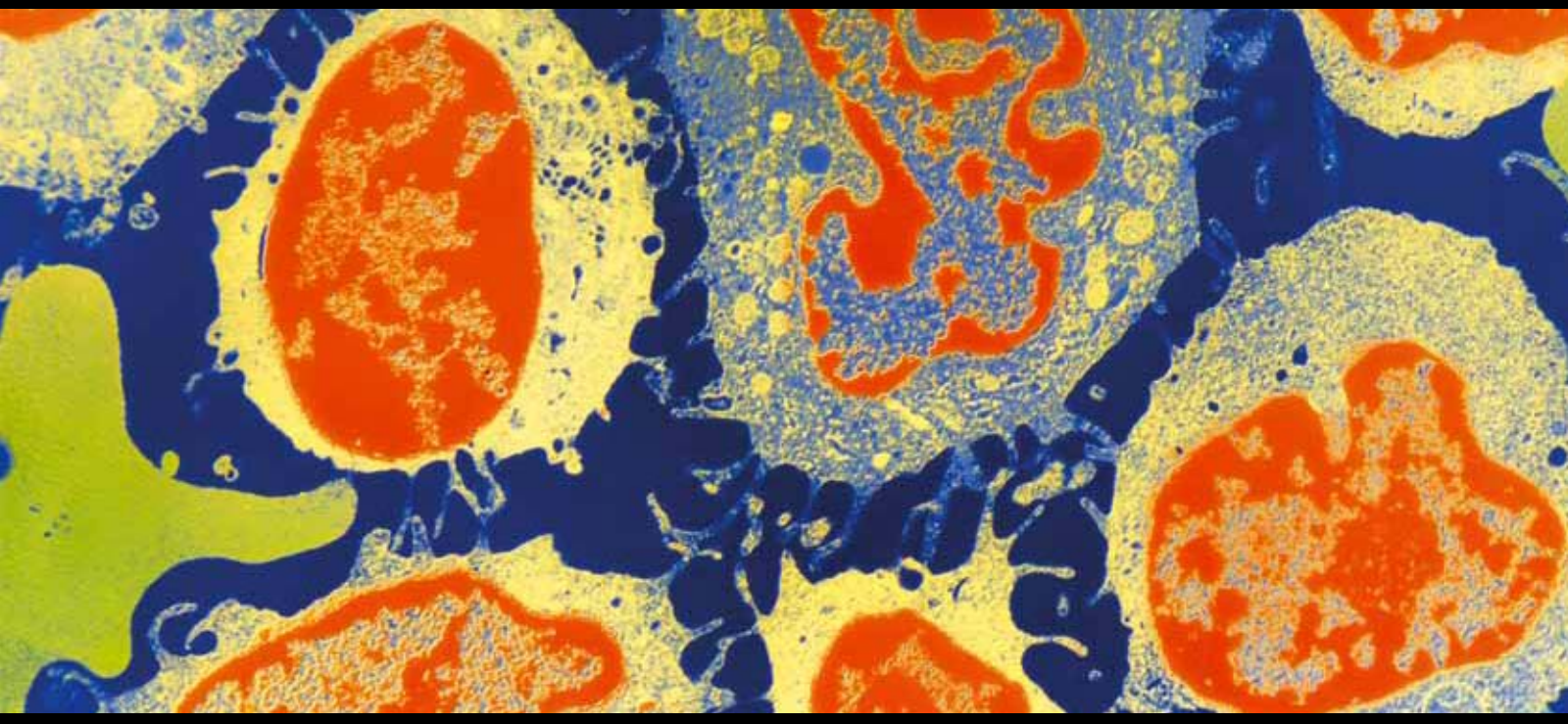
Journal of Oncology

Tumor Angiogenesis 2012

Guest Editors: Arkadiusz Z. Dudek, Kalpna Gupta,
Sundaram Ramakrishnan, and Debabrata Mukhopadhyay



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Editorial Board

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Editorial

Tumor Angiogenesis 2012

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It has been more than 40 years since the initial prospect emerged that tumor angiogenesis could be targeted for cancer therapy. This proposal was based on the very simple observation that tumors are capable of stimulating new vascularization. Later the concept of an angiogenic switch was proposed; where cancer cells evolve from a dormant state into angiogenic phenotype attracting blood vessels growth and invading neighboring and distant sites. Since Folkman's landmark observations [1] a more complex picture of angiogenesis has emerged replete with a complex tumor microenvironment and interactions with multiple cell types within the tumor. Numerous anti angiogenic agents were developed successfully for the treatment of renal cell carcinoma, lung cancer, breast cancer, brain tumors, and colorectal carcinoma, but resistance to these therapies soon emerged. This stimulated additional research that led to the better understanding of the very complex interaction of tumor angiogenesis and the tumor microenvironment responsible for the lack of lasting response to angiogenesis targeted therapy.

In this issue of Tumor Angiogenesis, several review papers discuss the current status of our understanding of and therapy for ovarian carcinoma, head, and neck cancer, melanoma, glioma, and leukemia (papers by A. Amini et al., C. Carla et al., U. Adamcic et al., K. Shirai et al., S. R. Choudhury et al., and A. Trujillo et al.)

The role of pericytes by E. Fakhrejahani and M. Toi T. Duong et al.'s contribution on the role of lymphangiogenesis enhance the spectrum of different cellular and structural components contributing to cancer progression and metastases.

The most novel contribution emerges from the role of external stimuli in the promotion of angiogenesis. Diet as a modifier of tumor angiogenesis by W. W. Li et al. and I. Urits et al., substantiates the role of external control of microenvironment by diet, which is complemented by the observations of K. Luk et al. on pericyte-endothelial interaction influenced by morphine generally used to treat severe pain in patients with cancer.

Insights into newer signaling pathways such as, PTEN (S. Rodriguez, and U. Huynh-Do), c-Kit (K. Rupertus et al.) and transcription factor FoxC2 (T. Kume) provide an understanding of yet to be conquered targets to efficiently treat cancer. Additionally, imaging techniques for tumor angiogenesis discussed by K. Tanaka et al. also offer novel approaches to understand tumor angiogenesis.

The interaction between tumor vasculature and the microenvironment is reviewed by E. Fakhrejahani, M. Toi, K. Rupertus et al., and S. Niland et al., and inflammation and cancer is examined by J. I. Arias et al.

These publications provide an up to date comprehensive state of research on tumor angiogenesis. We therefore hope that this issue will help the readers understand the underlying complex biology and challenges in developing successful antiangiogenic therapy.

Arkadiusz Z. Dudek
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Sundaram Ramakrishnan
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Review Article

Tumor Lymphangiogenesis as a Potential Therapeutic Target

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Metastasis the spread of cancer cells to distant organs, is the main cause of death for cancer patients. Metastasis is often mediated by lymphatic vessels that invade the primary tumor, and an early sign of metastasis is the presence of cancer cells in the regional lymph node (the first lymph node colonized by metastasizing cancer cells from a primary tumor). Understanding the interplay between tumorigenesis and lymphangiogenesis (the formation of lymphatic vessels associated with tumor growth) will provide us with new insights into mechanisms that modulate metastatic spread. In the long term, these insights will help to define new molecular targets that could be used to block lymphatic vessel-mediated metastasis and increase patient survival. Here, we review the molecular mechanisms of embryonic lymphangiogenesis and those that are recapitulated in tumor lymphangiogenesis, with a view to identifying potential targets for therapies designed to suppress tumor lymphangiogenesis and hence metastasis.

1. Introduction

The spread of cancer to secondary sites (metastasis) is the main cause of morbidity for patients with solid tumors. Understanding of the molecular and cellular mechanisms that underpin tumor metastasis is therefore an important goal in cancer biology. Over a century ago, Stephen Paget proposed a "seed and soil" theory in which tumor cells have their propensity to seed certain particularly favourable organs [1]. For instance, prostate cancer often metastasizes to bones, colon cancer to the liver, and melanoma to the brain. This theory reflects the need of cancer cells to find a suitable milieu with appropriate molecular and cellular characteristics to be able to survive. In 1928, James Ewing suggested an alternative metastatic theory, proposing that cancer cell metastasis depends solely on available anatomical and mechanical routes [2]. Although tumorigenic cells certainly need to access the lymphatic or blood vascular system to spread, in accordance with Ewing's theory, they also have different propensity to seed some organs in favour of the others. It is clear that both anatomical/mechanical and "seed and soil" theories partly explain the metastatic pattern.

Recent studies have revealed that the lymphatic vasculature is one of the major routes for tumor metastasis, raising the possibility that blocking tumor lymphangiogenesis might prevent the very initial stage of tumor spreading from the primary site. This review focuses on tumor lymphangiogenesis, its effect on cancer metastasis, and how targeting tumor lymphangiogenesis may provide a potential therapeutic strategy to treat cancer metastasis.

2. Lymphatic Vascular System and Its Function

The structural and functional features of the lymphatic vascular system make it particularly suited to a role as a major route of metastasis. The lymphatic system plays a vital role in maintaining tissue fluid homeostasis by draining protein-rich fluid from the interstitial space back to the general blood circulation. The lymphatic system is divided into the conducting vessel network and lymphoid tissue. The lymphatic vessel network consists of lymphatic capillaries, precollecting vessels, collecting vessels, and the thoracic duct. The initial lymphatic capillaries have a thin wall, built from a single layer of endothelial cells, and play a role in taking up the interstitial tissue fluid. Because cell-to-cell contact in these vessels is loose and specialized for fluid uptake, they are also well suited to invasion by cancer cells. Furthermore, the lymphatic capillaries are distributed throughout the body except for some avascular tissues such as the epidermis, cartilage, cornea, hair, nails, and some vascularized organs such as the brain and the retina (reviewed by [3]). Their broad distribution in the whole body therefore also provides ready

routes for cancer cell metastasis. The larger lymphatic vessels have a smooth muscle cell layer and basement membrane surrounding the inner lining of endothelial cells (reviewed by [4]). In addition, they also contain intraluminal valves, which allow unidirectional flow to the thoracic duct in order to reach the general blood circulation (reviewed by [3]).

Lymphoid tissue includes structurally well-organized lymph nodes and loosely organized lymphoid follicles. The lymph nodes are located at intervals along the lymphatic vascular tree and filter the lymph. The lymph node therefore represents a preferred site for lodgement of metastasizing cancer cells during tumorigenesis [5].

Another important function of the lymphatic system is immune trafficking and surveillance. Through the lymphatic network, immune cells from the peripheral tissues navigate to regional lymph nodes in order to stimulate the immune response [6]. This navigation is also critical in modulating inflammatory lymphangiogenesis. Interestingly, this mechanism can also be utilised by cancer cells to escape from the primary tumor site and metastasize to the regional lymph nodes (discussed later in part 4.2.3).

3. Embryonic Lymphatic Vessel Development

Since the lymphatic developmental program can be reactivated during tumor lymphangiogenesis, it is important to understand early lymphatic vessel development in the embryo and the key factors involved in this process. It has been suggested that lymphatic vessels in mammals arise in the embryo from the preexisting blood vasculature and more particularly from the cardinal vein (CV) [7, 8]. Lymphatic endothelial cell (LEC) precursors from the CV migrate outwards and form the lymph sac (LS), from which lymphatic vessels start to develop throughout the body, connecting to form either deep or superficial lymphatic vessels (Figure 1(a)). Recent molecular studies based on lymphatic phenotypes of mutant mice have revealed several factors that regulate these steps in the embryo (Table 1). In this section, we will discuss the factors involved in establishing the lymphatic vasculature in the embryo, as a prelude to discussion of factors that are also involved in adult pathological conditions, especially tumor lymphangiogenesis.

3.1. Lymphatic Endothelial Cell Specification and Expansion of the Lymphatic Network

3.1.1. Lymphatic Endothelial Cell Specification. During early lymphatic vascular development, lymphatic endothelial hyaluronan receptor-1 (LYVE-1) and vascular endothelial growth factor receptor-3 (VEGFR-3) are first expressed at sites where lymphangiogenesis will occur in the cardinal vein around 8.5 dpc [43]. Later, polarized expression of SOX18 is found in the dorsal-lateral side of the cardinal vein at 9.0 dpc [10]. SOX18 directly activates the transcription of *Prox1* gene, which encodes the homeodomain transcription factor PROX1 (prospero-related homeobox-1) [10]. In addition, another transcription factor, COUP-TFII, has been identified as being essential for modulation of PROX1 expression in the cardinal vein [44]. These SOX18$^+$/COUP-TFII$^+$/PROX1$^+$ lymphatic endothelial precursor cells then delaminate from the CV to form lymph sacs, the primary plexus of the lymphatic vasculature, around 11.5 dpc in mouse embryo [43, 45] (Figure 1(a)).

3.1.2. Expansion of the Lymphatic Vascular Network. A dorsolateral gradient of VEGF-C guides the developing lymphatic endothelial cells during this early phase [46]. Disruption of *Vegfc* in mice, *Xenopus* tadpoles, and zebrafish leads to a defect in migration of early lymphatic endothelial cells from the cardinal veins to form a lymphatic plexus [18–20]. VEGFR-3 is a specific receptor tyrosine kinase that binds to VEGF-C and VEGF-D and is highly expressed by blood endothelial cells (BECs) before the differentiation of lymphatic vasculature. However, its expression becomes restricted to lymphatic endothelial cells after 11.5 dpc [47]. VEGF-C/VEGFR-3 signalling induces proliferation, migration, and survival of endothelial cells [48], and transgenic overexpression of VEGF-C in the skin promotes lymphangiogenesis [49]. Maintenance of VEGF-C/VEGFR-3 signalling therefore is important in regulation of lymphatic vascular expansion.

A coreceptor for VEGF-C, neuropilin receptor-2 (Nrp-2), is also expressed only within the veins and lymphatics [29]. Both VEGF-C and VEGF-D bind to Nrp-2, and this ligand stimulation leads to internalization of Nrp-2 together with VEGFR-3 [50]. This finding suggests that both Nrp-2 and VEGFR-3 together increase the affinity of LECs toward VEGF-C gradients during lymphatic development.

After LEC specification and establishment of the lymphatic plexus, separation of the lymphatic vasculature from the blood vasculature is one of the most critical stages required to ensure proper function of the two vessel networks. Several key factors and different cell types that are involved in this process have been recently reviewed elsewhere, including tyrosine kinase SYK and its adaptor protein SLP-76, expressed by circulating endothelial progenitor cells, and podoplanin and C-type lectin receptor 2 (CLEC-2), expressed in platelets (see [51] for review) (Table 1).

3.2. Lymphatic Vessel Remodelling and Maturation. The next stages involved in the remodelling and maturation of the lymphatic network include the formation of lymphatic capillary network from the primary lymphatic plexus, and the assembly of collecting lymphatic vessels with recruitment of smooth muscle cells (SMCs) and formation of lymphatic valves [27, 52]. Angiopoietin-2 (Ang2), a growth factor binding to its receptor tyrosine kinase Tie2, has been found to be involved in lymphatic maturation. *Ang2* mutant mice display an abnormal lymphatic network due to defective recruitment of smooth muscle cells to the lymphatic collecting vasculature [23]. Further, overexpression of Ang1, Ang2, and Ang3/Ang4 in adult tissues promotes lymphatic sprouting *in vivo* [53–55].

The role of transmembrane growth factor ephrin-B2 in postnatal remodeling of lymphatic vasculature has also been explored using mice that express a mutated form of ephrin-B2 lacking the carboxy-terminal site for binding PDZ-domain-containing proteins. These mutant mice displayed

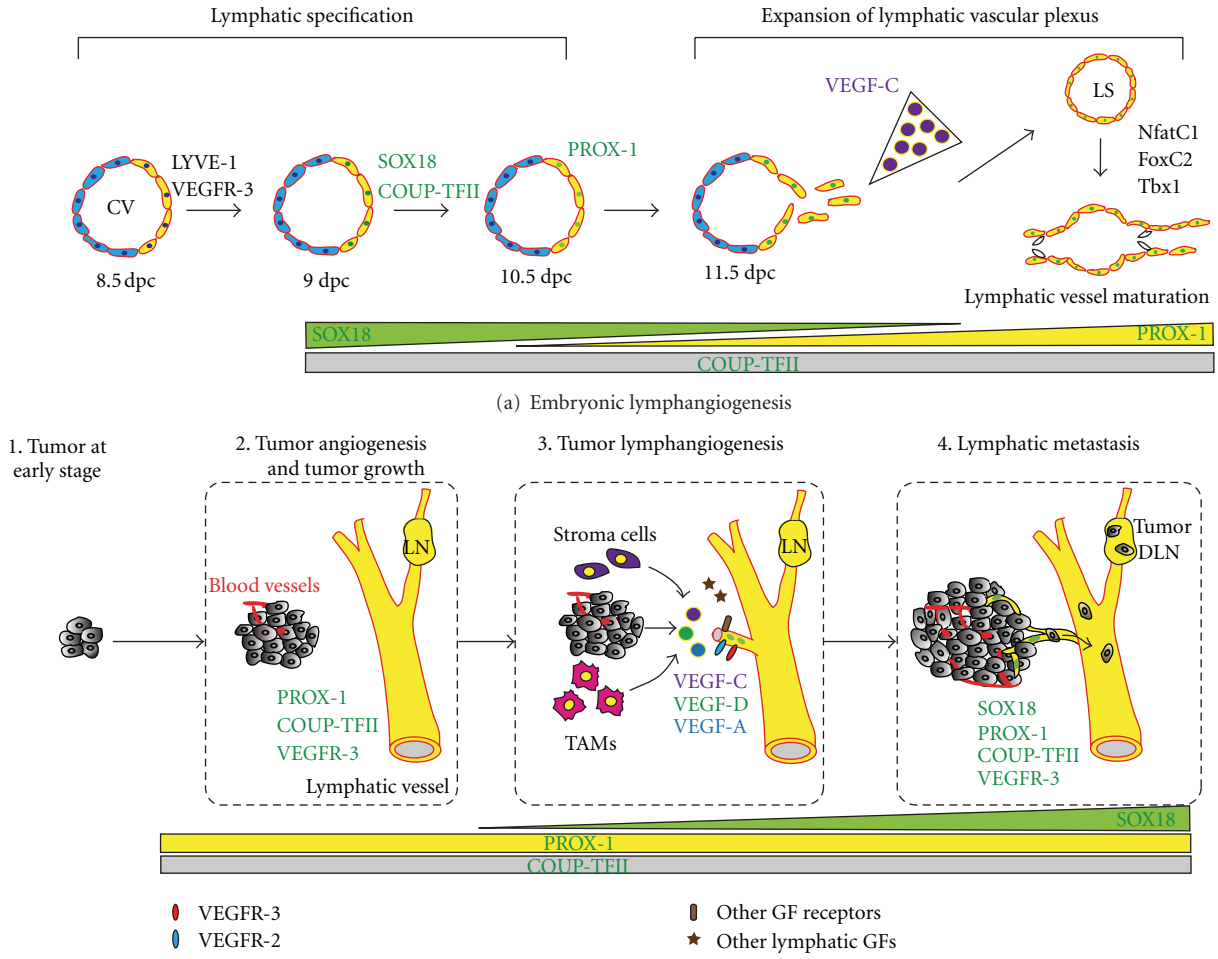

Figure 1: Embryonic lymphangiogenesis versus tumor lymphangiogenesis. (a) During early lymphatic vessel development, lymphatic endothelial precursor cells (SOX18$^+$/COUP-TFII$^+$/PROX-1$^+$) from the CV migrate outwards and form lymph sacs (LS), from which lymphatic vessels start to extend throughout the body. (b) In a tumor microenvironment, various lymphatic growth factors are secreted from tumor cells, inflammatory cells (e.g., TAMs), and stroma cells. These factors stimulate the formation of tumor neolymphatics, either in the peritumoral or intratumoral area, which facilitate the intravasation of cancer cells into lymphatic vessels. Interestingly, several key early factors in embryonic lymphangiogenesis also play critical roles during tumor lymphangiogenesis. In particular, SOX18 is not required for maintenance of adult lymphatics but appears to be reactivated and regulate the formation of tumor neolymphatics. CV, cardinal vein; LS, lymph sac; dpc, *days coitum*; GF, growth factor; LN, lymph node; DLN, draining lymph node; TAMs, tumor-associated macrophages.

major lymphatic defects, including disturbed postnatal remodeling of their primary lymphatic capillary plexus, hyperplasia, and lack of luminal valve formation, whereas the blood vasculature phenotype remained normal [30, 56, 57].

Taken together, it is clear that many factors are known to be involved in controlling the finely tuned stages of lymphatic vessel development in the embryo (summarized in Table 1). Understanding the early embryonic steps of lymphatic vessel formation will increase our knowledge of how a developmental program is reactivated in the adult under pathological condition, and the consequences of its dysregulation. In the following section, we will discuss the process of adult neo-lymphangiogenesis during pathological conditions, particularly in tumor metastasis.

4. Lymphangiogenesis and Tumor Metastasis

4.1. Tumor Microenvironment: Stroma versus Tumor Vasculature in Metastasis. Metastasis is a complex multistep process identified as the invasion-metastasis cascade, beginning with local invasion then intravasation of cancer cells into blood and lymphatic vessels, transit of cancer cells through these vascular trees, extravasation to the lymph node or distant organs, micrometastasis with small cancer nodules, and finally an invasion step in which micrometastasis becomes macrometastasis [58]. The ability of cancer cells to metastasize depends on many triggers such as the intrinsic properties of the tumor itself and the tumor microenvironment [59].

The tumor microenvironment consists of cancer cells, noncancer cells (e.g., endothelial cells (ECs), cancer-associated

Table 1: Lymphangiogenic factors in the early steps of embryonic development and in adult.

Key factors	Defects in lymphatic vascular system	
	Human syndrome	Mutant animals
Transcription factors		
SOX18	Dominant-negative mutations of SOX18 have been linked with hypotrichosis-lymphedema-telangiectasia syndrome (OMIM no. 607823) [9].	*Sox18*-null mice are devoid of lymphatic vessels and die *in utero* at 14.5 dpc from a generalized oedema [10].
COUP-TFII		Conditional inactivation of COUP-TFII during embryogenesis causes edema, haemorrhage, and blood-filled lymphatics [11].
PROX-1		(i) *Prox1*−/− is embryonic lethality at approximately 14.5 dpc due to lack of lymphatic vasculature [12]. (ii) Conditional loss of *Prox1* function in the adult has been shown to induce LECs to revert to a blood vascular phenotype [13].
TBX1	TBX1 mutation causes DiGeorge human syndrome, which is associated with multiple congenital anomalies.	Mouse embryos with conditional deletion of Tbx1 in endothelial cells display widespread lymphangiogenesis defects and have perinatal death [14].
NFATc-1		(i) NFATc1-deficient mice showed irregular patterning of the LEC sprouting from the jugular lymph sac [15]. (ii) NFATc-1 and FOXC2 are downstream of VEGFR-3, cooperate in regulating the differentiation of lymphatic capillaries and valves formation [16].
FOXC2	Mutation in transcription factor FOXC2 caused lymphedema-distichiasis (LD) in human (OMIM no. 153400).	*Foxc2*−/− mice have abnormal lymphatic vascular patterning, increased pericyte investment of lymphatic vessels, and loss of valves in the collecting vessels [17].
Growth factors		
VEGF-C		(i) The disruption of VEGF-C in mice, *Xenopus* tadpoles, and zebrafish leads to a defect in migration of early lymphatic endothelial cells away from cardinal veins to form lymphatic plexus [18–20]. (ii) *Vegfc*−/− mouse embryos completely lack lymphatic vasculature [18]. (iii) *Vegfc*−/−; *Vegfd*−/− double knockout fails to recapitulate the early embryonic lethality observed in *Vegfr3*−/− mice [21].
VEGF-D		VEGF-D deficiency mice displayed no lymphatic vessel dysfunction, suggesting that VEGF-D is dispensable and might not play a major role in lymphatic development [22].
Angiopoietin-2		*Ang2*-mutant mice display an abnormal lymphatic network due to defective recruitment of smooth muscle cells to the lymphatic collecting vasculature [23].
Adrenomedullin		*AM*-, *calcrl*-, *RAMP2*-null mice died midgestation with formation of interstitial lymphedema. Loss of AM signalling caused abnormal jugular lymphatic vessels due to reduced LEC proliferation [24].
Receptors/transmembrane proteins		
VEGFR-3	Heterozygous tyrosine kinase-inactivating missense point mutations of *VEGFR-3* gene have been identified as a major cause of the Milroy disease (OMIM no. 153100).	*Vegfr3* knockout mice display cardiovascular defects, severe blood vessel defects, and embryonic death [25].
Integrin $\alpha 9\beta 1$		(i) Integrin-$\alpha 9\beta 1$-deficient mice die after birth due to chylothorax, an accumulation of lymph in pleural cavity [26]. (ii) Integrin-$\alpha 9$-deficient mice further were described as having abnormal lymphatic valves and impaired fluid transport [27].

Table 1: Continued.

Key factors	Defects in lymphatic vascular system	
	Human syndrome	Mutant animals
LYVE-1		(i) Mice lacking this receptor have normal lymphatic vessels. (ii) LYVE-1 is expressed at the site where lymphangiogenesis will occur in the cardinal vein around 8.5 dpc [3].
Podoplanin		*Podoplanin*−/− mice died at birth and have lymphatic defects, associated with decreased lymphatic transport, lymphedema and dilation of lymphatic vessels [28].
Neuropilin-2		*Nrp2*−/− mice show absence or severe reduction of small lymphatic vessels and capillaries during development, while arteries, veins, and collecting lymphatics developed normally [29].
Ephrin-B2		Mice expressing a mutated form of Ephrin-B2 have major lymphatic defects, including disturbed postnatal lymphatic remodeling, hyperplasia, and lack of luminal valve formation, whereas the blood vasculature remained normal [30].
Clp24, Claudin-like protein of 24 kDa		(i) *Clp24* knockdown in *Danio rerio* and *Xenopus* laevis display defective lymphatic development. (ii) *Clp24*-/- mice have enlarged lymphatic vessels with abnormal patterning and smooth muscle cell recruitment [31].
Liprin β1		Knock-down liprin β1 in *Xenopus laevis* tadpoles using morpholino leads to edema, defective assembly of lymphatic vessels [32].
Synectin		Knockdown of synectin in zebrafish causes impaired formation of the thoracic duct and defective lymphangiogenic sprouting [33].
ALK1, activin receptor-like kinase 1		(i) ALK1 is a member of TGF-β type I family of receptors. (ii) Blockade of ALK1 signalling using ALK1Fc results in failed remodelling of lymphatic vascular in neonatal mice [34].
Others		
SYK and SLP-76		Loss of SYK or SLP-76 function results in embryonic hemorrhage, arteriovenous shunting, blood-lymphatic connections, and blood-filled lymphatics [35].
CCBE1	Mutation in CCBE1 associates with the Hennekam syndrome, a generalised lymphatic dysplasia in humans [36].	CCBE1 has been identified as essential factor for embryonic lymphangiogenesis and venous sprouting in zebrafish model [37].
Aspp1, apoptosis-stimulating protein of p53		*Aspp1*−/− mice have embryonic subcutaneous edema, delayed lymphatic vessel formation, defective lymphatic drainage function and mispatterned collecting lymphatic vessels [38].
Emilin-1		*Emilin1*−/− mice result in hyperplasia, enlargement, irregular pattern of lymphatic vessels with a reduction of anchoring filaments [39].
miR-31, microRNA-targeting PROX1		Gain of miR-31 function leads to impaired venous sprouting and lymphatic vascular development in *Xenopus* and zebrafish; miR-31 is identified as negative regulator of lymphatic development [40].
Rac1, Rho family GTPase		Deletion of endothelial *Rac1* in mice causes impaired lymphatic-blood vessel separation, identified by edema, haemorrhage, and embryonic lethality, whereas blood vessels remain normal [41].
Spred-1/2		Spred-1/2 -deficient embryos display subcutaneous haemorrhage, edema, dilated and blood-filled lymphatic vessels and die *in utero* [42].

fibroblasts (CAFs), mesenchymal stem cells (MSCs), tumor-associated macrophages (TAMs)), and noncellular components (extracellular matrix—ECM) [60]. Interaction between cancer cells and their adjacent microenvironment leads to a significant impact on the tumor progression and metastasis (see for review [60]). For instance, tumor chemoattractants including colony-stimulating factors (CSF-1) [61, 62], CC chemokines [63], and VEGF [64] stimulate the recruitment of the infiltrating cells (e.g., monocytes/macrophages) in the lymphatic and blood vessels towards the tumor. Further, several factors secreted by tumors, including interleukin-10, -4 (IL-10, -4), transforming growth factor-β (TGF-β), and CSF-1, can switch these TAMs into polarized type II or M2 macrophages [65]. Importantly, M2 macrophages have reduced T-cell activity, poor antigen-presenting capacity and concomitantly release several protumorigenic factors (TGF-β, IL-10), proangiogenic factors (VEGF, IL-1β), prolymphangiogenic factors (VEGF-C, VEGF-D), and extracellular matrix proteases (matrix metalloproteinases—MMPs) [65, 66]. During tumorigenesis, connective tissue growth factor (CTGF) is also highly expressed [67], which may lead to continued activation of the TGF-β signalling pathway [68]. Further, TGF-β secreted by tumor cells or host inflammatory cells might induce fibroblasts in the tumor microenvironment to become activated fibroblasts (myofibroblasts), which express high levels of α-smooth muscle actin [69, 70]. These activated fibroblasts in turn produce MMPs, which cleave E-cadherin and therefore further induce epithelial to mesenchymal transition (EMT) [71]. Cancer cells undergoing EMT have increased invasive ability because of their loose cell-to-cell contact and acquired mesenchymal properties [72]. In addition, TGF-β secreted by tumors also can induce activin receptor-like kinase 1 (ALK1 receptor) expressed by ECs, leading to endothelial cell proliferation, migration, remodelling [73], and eventually triggering tumor angiogenesis or lymphangiogenesis.

All of the events and complex interactions in the tumor microenvironment alter the nature of tumor stroma cells, which in turn significantly affects tumor progression and metastasis. However, since cell migration through connective tissue is relatively difficult and slow, cancer cells are able to spread more quickly and efficiently via blood or lymphatic vessels [60].

Here, we will focus on one aspect of the tumor microenvironment by examining tumor lymphangiogenesis and its impact on tumor metastasis. Lymphatic vessels, which have high permeability and a lack of tight junction structure compared to blood vessels, are particularly accessible for tumor cell invasion. Clinical studies on breast, cervical, head and neck, and ovarian cancer have revealed that, in most patients, an early sign of cancer spread is metastatic cells located in the regional draining lymph node [74] (reviewed by [5]). Clinical studies have also shown that the process of metastasis occurs in an orderly pattern, starting from the primary site, spreading through the lymphatic channel, and then to regional sentinel lymph nodes before disseminating systemically to distant organs (Figure 1(b)). Studies of micrometastasis in the sentinel lymph node have shown that 80% of metastasis follows this pattern, whereas 20% showed systemic metastasis bypassing the lymphatic system [75]. The lymphatic vasculature is thus one of the major routes for tumor metastasis and therefore is considered a potential target for blocking the spread of cancer.

4.2. Tumor Lymphangiogenesis: Cellular and Molecular Mechanisms

4.2.1. Growth Factors Involved in Tumor Lymphangiogenesis. During the 1990s, the first lymphangiogenic factor, VEGF-C, was identified [76]. Overexpression of VEGF-C by tumor cells can induce lymphangiogenesis and increase metastasis to the regional lymph node in a mouse model of breast and pancreatic cancer [77–80]. As mentioned above, TAM has also been identified as a stroma cell critically responsible for production of lymphatic growth factors, VEGF-C, and -D [66, 81] (Figure 1(b)). In addition, VEGF-C overexpression induced enlargement of tumor-associated lymphatic vessels that can increase lymph flow and facilitate intravasation of cancer cells into the lymphatics [77] (Figure 2(b)). VEGF-C has been further shown to induce intercellular gaps that facilitate entry of tumor cells into the lumen of the vessels [82]. More than 65 studies have shown that VEGF-C expression correlates with lymph node metastasis and poor prognosis in a range of human tumors [14, 83–89]. In patients with melanoma, mRNA levels of VEGF-C also correlate with stage of tumor progression [90].

Another structurally related lymphatic growth factor is VEGF-D, which also can bind to VEGFR-3 and activate lymphangiogenesis [91]. VEGF-C and -D share a central VEGF homology domain (VHD), containing receptor-binding sites, flanked by N- and C-terminal propeptides, which can be proteolytically cleaved to produce mature forms with higher affinity to receptors [92, 93]. These mature forms of VEGF-C and -D also can bind to VEGFR-2 and therefore can also promote angiogenesis [94–96]. *Vegf-d*-deficient mice display a lack of lymphatic vascular phenotype, suggesting that VEGF-D might not play a major role in embryonic lymphatic vessel development [22, 97]. However, VEGF-D has been shown to play a role in stimulation of tumor neo-lymphangiogenesis, as the expression of VEGF-D in tumor cells induced tumor lymphangiogenesis and lymph node metastasis in several tumor mouse models [98, 99]. In addition, *vegf-d*-null mice displayed a reduction in peritumoral lymphangiogenesis and lymph node metastasis in an orthotopic pancreatic tumor model [100]. Analysis of VEGF-C and -D expression level in excised patient tumor tissues revealed that levels of these growth factors are associated with poor outcome and lymph node metastasis [101–103].

Another VEGF family member, VEGF-A, initially identified as a key positive regulator of angiogenesis, primarily binds to VEGFR-1 and VEGFR-2 [104]. VEGF-A has no known function during embryonic lymphangiogenesis. However, VEGF-A has been shown to induce tumor lymphangiogenesis and tumor metastasis to regional and distant lymph nodes [105], and VEGF-A overexpressing tumors have high numbers of macrophages [106]. Further, Cursiefen et al. (2004) have shown an indirect lymphangiogenic role for VEGF-A via recruitment of bone marrow-derived

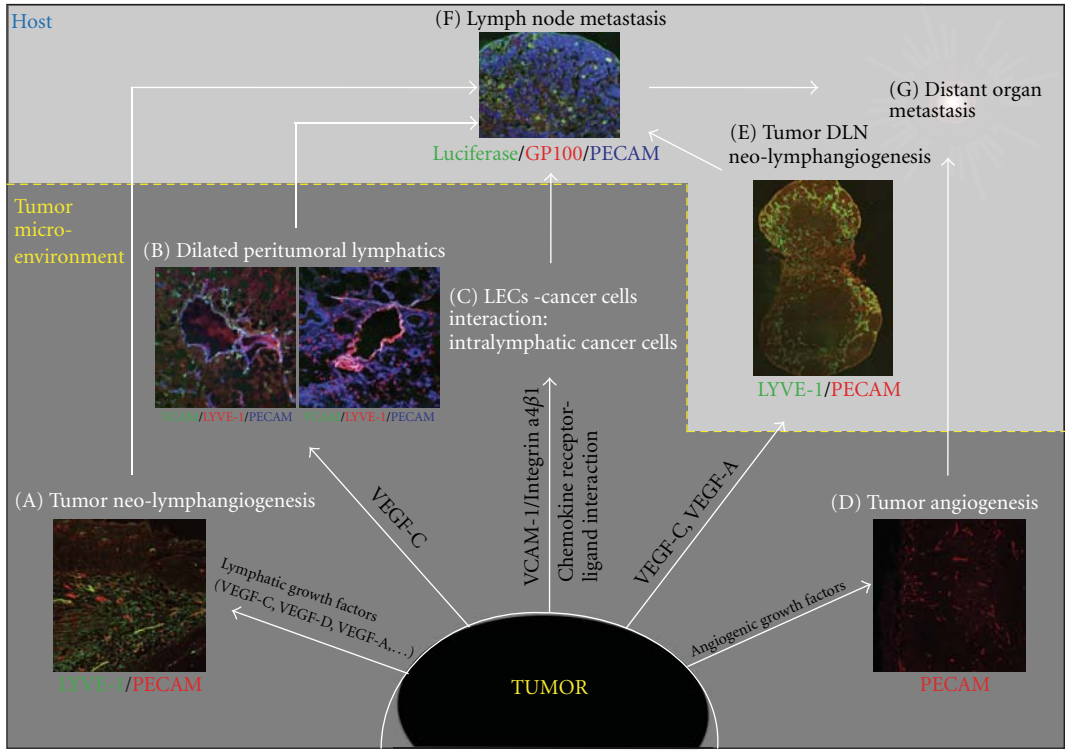

Figure 2: Biology of tumor lymphangiogenesis and metastasis. (A), (B) Stimulation of tumor neo-lymphangiogenesis and enlargement of tumor lymphatics can facilitate intravasation of cancer cells into the lymphatics. (C) The interaction between tumor cells and LECs via tumor cell receptors (e.g., Integrin $\alpha 4\beta 1$) and endothelial cell adhesion molecules (e.g., VCAM-1) or via chemokine receptor ligand interaction (e.g., CCR7/CCL21) can facilitate the invasion of cancer cells into lymphatic vessels (intralymphatic cancer cells). (E) Notably, lymphangiogenesis also occurs at the tumor draining lymph node (DLN) before metastasis of cancer cells to this site, probably to generate a favourable environment for in-coming metastatic cancer cells at this site. (F) Intralymphatic cancer cells then metastasize to the tumor DLN. (D), (G) Additionally, tumor angiogenesis also contributes to distant organ metastasis. The tumor microenvironment has a critical impact on tumor progression and metastasis. LECs, lymphatic endothelial cells; DLN, draining lymph node.

macrophages (BDMs) by using a mouse model of inflammatory-induced corneal neovascularization. These BDMs in turn secrete angiogenic and lymphangiogenic factors that can stimulate both blood and lymphatic out-growth [107].

Several other factors have been recently identified as inducers of lymphangiogenesis, including hepatocyte growth factor, Angiopoietins 1 and 2 (Ang-1, -2), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor-BB (PDGF-BB), growth hormone (GH), adrenomedullin (AM), insulin-like growth factors 1 and 2, and endothelin-1 (ET-1) (the involvement of these factors in tumor lymphangiogenesis is summarized in Table 2). Some of these factors have already been identified from embryonic lymphatic vessel development studies, whereas others were discovered in tumor lymphangiogenesis studies. Further studies aimed at indentifying lymphangiogenic growth factors will help to provide more potential molecule targets in inhibiting tumor neo-lymphangiogenesis and metastasis.

4.2.2. Peritumoral and Intratumoral Lymphatics. The relative distance of the tumor to the lymphatic bed can also affect the ability of cancer cells to metastasize. It is well established that peritumoral lymphatics are predominantly responsible for the uptake of cancer cells during metastasis [128]. In fact, lymph node metastasis associated with melanoma can be predicted more accurately by quantitation of peritumoral lymphatic vessels than by quantitation of intratumoral vessels [129]. Also in a clinical study on a cohort of 123 patients with gastric cancer, peritumoral lymphatics were shown to exhibit higher density when compared to intratumoral lymphatics, and importantly these peritumoral lymphatics also play a role in gastric cancer progression [130].

In contrast, the role of intratumoral lymphatic vessels has remained unclear and controversial. Intratumoral lymphatics have been thought to be nonfunctional and are typically collapsed due to the high pressure found in intratumoural environment [128]. Nevertheless, in a mouse model of tumor overexpression of VEGF-C/VEGF-D, proliferation of intratumoral lymphatics was shown to correlate with lymph node metastasis [79, 98, 99, 131]. However, it still remains to be clarified whether function of intratumoral lymphatics is critical for tumor invasion and distant organ metastasis.

4.2.3. Interaction between Tumor Cells and Lymphatic Vasculature. Interactions between tumor cell surface receptors and endothelial cell adhesion molecules are thought to contribute to tumor cell arrest and extravasation during blood vessel-mediated metastasis. It has been shown that

Table 2: Tumor lymphangiogenic growth factors and their receptors.

Lymphangiogenic factors	Receptors	Mechanism of action/association with cancer	References
VEGF-C	VEGFR-2, VEGFR-3	(i) Overexpression of VEGF-C by tumor induces tumor lymphangiogenesis, dilated lymphatics and increases metastasis to lymph node. (ii) Proteolytic VEGF-C also binds to VEGFR-2 and therefore can also induce tumor angiogenesis	[77–80, 94]
VEGF-D	VEGFR-2, VEGFR-3	(i) VEGF-D plays a role in stimulation of tumor lymphangiogenesis and lymph node metastasis. (ii) Proteolytic VEGF-D also binds to VEGFR-2 and can induce tumor angiogenesis.	[98–100, 108]
VEGF-A	VEGFR-2	(i) VEGF-A induces tumor lymphangiogenesis and tumor metastasis to regional lymph node.	[105]
FGF-2	FGFR-3 [109]	(i) Induces both angiogenesis and lymphangiogenesis through the control of VEGF-C and VEGF-D expression. (ii) Increased expression of FGF-2 is associated with lymphatic metastasis.	[110, 111]
Hepatocyte growth factor (HGF)	c-met	(i) Overexpression of HGF in mice/intradermal delivered HGF induces lymphatic vessel hyperplasia. (ii) HGF stimulate the outgrowth of peritumoral lymphatics, via activation of VEGFR-3. (iii) HGF contribute to lymphatic metastasis when overexpressed in tumor.	[112, 113]
Insulin-like growth factor-1, 2	Insulin-like growth factor receptor	(i) IGF-1, -2 induce lymphangiogenesis in a mouse cornea assay. (ii) IGF-IR is involved in angiogenesis and lymphangiogenesis through modulation of VEGF ligand expression in gastric cancer cell line MKN45.	[106, 114]
Ephrin-B2	Eph. receptor tyrosine kinase	(i) PDZ interaction site in Ephrin-B2 is required for the remodelling of lymphatic vasculature. (ii) Tumor angiogenesis was inhibited in *Ephrin-B*-mutant mice in an orthotopic glioma tumor model.	[30, 115]
Angiopoietin-1, -2 (Ang-1, -2)	Tie-2	(i) Overexpression of Ang-1 in adult mouse tissues leads to lymphatic sprouting and hyperplasia. (ii) Ang-1 is moderately expressed by tumor cells; Ang-2 is expressed by activated endothelial cells and upregulated during tumorigenesis. (iii) Ang-2 levels are associated with disease progression in melanoma patients.	[55, 116, 117]
PDGF-BB	PDGFR-α and -β	Expression of PDGF-BB in murine fibrosarcoma cells induced intratumoral lymphangiogenesis and promote lymphatic metastasis	[118]
Growth hormone (GH)	Growth hormone receptor	(i) GH promotes lymphangiogenesis in the granulation tissue of full-thickness skin wounds. (ii) Ectopic GH expression has been found in breast cancer and pancreatic cancer tissue.	[119–121]
Adrenomedulin (AM)	Calcrl associated with RAMP2 (*)	AM is a multifunctional regulatory peptide that is overexpressed in cancer cells and help them to develop to malignant growth.	[122]
Endothelin-1 (ET-1)	Endotheline B receptor (ET_BR)	(i) ET-1/ET_BR expression is correlated with lymphatic invasion in human breast cancers. (ii) ET-1/ET_BR enhances VEGF-A/C and VEGFR-3 expression and induces formation of lymphatic vessels. (iii) ET-1 is one of significantly upregulated genes in LEC isolated from metastatic LN.	[123–125]
Neutrin-4		(i) Netrin-4 is expressed in human breast tumor lymphatic and blood vessels. (ii) In mouse model of netrin-4 overexpressing breast cancer, lymph node metastasis and lung metastasis were significantly increased. (iii) Netrin-4 stimulates lymphatic permeability via activation of small GTPase and Src family kinase/FAK and downregulating tight junction protein.	[126]
Fibronectin	Integrin $\alpha4\beta1$	High expression of integrin $\alpha4\beta1$ is detected on tumor lymphatic endothelium.	[127]

* Calcrl: calcitonin receptor-like receptor; RAMP2: receptor activity-modifying protein.

the interaction of melanoma cell integrin $\alpha 4\beta 1$ (very late antigen-4, VLA-4) with VCAM-1 is critical for tumor cell arrest [132, 133]. Therefore, the expression of VCAM-1 on tumor lymphatics could lead to increased interaction with cancer cells and further facilitate metastasis. In addition, organ-specific increases in VCAM-1 expression correspond with reported clinical patterns of melanoma metastasis [134, 135].

During inflammatory response, lymphatic vessels play a critical role in the migration of dendritic cells to the draining lymph node to initiate the adaptive immune response [6]. The inflammatory cells interact with the lymphatic endothelium to find their way to the next lymphatic vessels and transmigrate into the vascular lumen [6]. Recent studies have revealed that this interaction occurs through the specific expression of ligands and their receptors. Lymphatic endothelium actively secretes the chemokine (C-C motif) ligand 21 (CCL21), which binds to the C-C chemokine receptor type 7 (CCR7) expressed on dendritic cells, thus creating a chemoattracting gradient for dendritic cells that migrate toward the lymphatic vasculature [136, 137]. Interestingly, tumor cells also can use this physiological chemokine receptor/ligand interaction to metastasize to the regional lymph node [138, 139]. In fact, CCR7 is expressed in some malignant melanoma cell lines [140], and it has been shown in melanoma mouse model that lymphatics can attract cancer cells through secretion of endogenous chemokine [141, 142]. Human breast cancer cells express the chemokine receptors CXCR4 and CCR7 [140]. Further, their respective ligands CXCL12 and CCL21 are highly expressed in the target organs of breast cancer metastasis that can partly explain the metastatic pattern in breast cancer patients [140]. In addition, fibroblasts, which constitute the majority of stromal cells in the tumor microenvironment of breast carcinoma, play an important role in establishment of the CXCL12-CXCR4 axis. In fact, CAFs elevate CXCL12 secretion, which in turn can stimulate proliferation and migration of CXCR4-expressing cancer cells in the tumor microenvironment [143, 144].

Understanding the interaction between tumor cells and LECs can help to identify an alternative way to block the intravasation of cancer cells into lymphatics. Inhibiting key factors involved in this process would provide novel potential therapeutic solution.

4.2.4. Intralymphatic Cancer Cells. Lymphatic invasion at either the primary tumor site or distant metastatic organs is characterised by the existence of cancer cells inside the lumen of the lymphatic vasculature (intralymphatic cancer cells or tumor emboli). The frequency of lymphatic invasion has been investigated in melanoma and gastric and breast cancer [145–147]. Importantly, it has been shown that lymphatic invasion occurs more frequently than blood vessel invasion (16% versus 3% in melanoma) [145–147]. 75% of melanoma patients that present intratumoral or peritumoral lymphatic invasion also exhibit sentinel lymph node metastasis [145]. Lymphatic invasion is therefore one of the most important adverse prognostic indicators for cancer recurrence rate and sentinel lymph node metastasis [145, 146, 148, 149].

Intralymphatic cancer cells have been also detected in distant organs. In a study using a mouse model of lymphangitic carcinomatosis, an extremely aggressive form of lung metastasis, cells expressing VEGF-C were specifically identified inside the peribronchial lymphatic vessels [150]. This observation suggests that conditioning of the intralymphatic vessel milieu with particular factors may have growth-promoting activity, which in turn facilitates tumor survival and promotes metastasis [150]. Since cancer cells remain essentially intra-lymphatic and do not invade the alveolar region, lungs still remain functional until a very advanced stage of the disease. This model of lung cancer metastasis recapitulates the human cancer situation in which patients with pulmonary lymphangitic carcinomatosis typically do not experience symptoms until a very late stage of disease when cancer cells start to extravasate from the lymphatics to invade the alveolar region of the lung [150].

4.2.5. Neo-Lymphangiogenesis in the Tumor Draining Lymph Node. It has been shown that the primary tumor has the ability to induce neo-lymphangiogenesis in the lymph node itself, so as to establish a "platform" from which cancer cells can disseminate [105, 129, 151, 152] (Figure 2(e)). VEGF-A-overexpressing primary tumors induce lymphangiogenesis at the sentinel lymph node even before cancer cells metastasize to this site [105]. Further, in mouse models of skin carcinogenesis in which VEGF-C was overexpressed in skin, lymphangiogenesis occurred at both the primary tumor site and the tumor draining lymph nodes [151]. VEGF-A and VEGF-C secreted from the primary tumor site can be drained to the regional lymph node where lymphangiogenesis is stimulated prior to the invasion of metastatic cancer cells. Once cancer cells metastasize to the regional lymph node, lymphangiogenesis is further enhanced [105, 151]. This observation indicates that lymphangiogenesis in the premetastatic lymph node creates a favourable environment, a premetastatic niche that might support the survival of in-coming metastatic cancer cells [83]. Tumor-induced neo-lymphangiogenesis in the regional lymph node triggers an increase of lymph flow. This upregulation in flow is a permissive factor that can actively enhance metastatic rate via the lymphatics [153]. Importantly, lymph node lymphangiogenesis is also detected in cancer patients suffering from melanoma and breast cancer [154, 155], two cancer types known for their high rate of metastasis.

Additionally, neo-lymphangiogenesis in a distant organ has also been investigated in a mouse model of breast cancer cells that overexpress VEGF-C [150]. The induction of lymphangiogenesis by VEGF-C at a secondary tumor site in the lung was shown to facilitate the expansion of already disseminated cancer cells throughout the lung tissue [150].

4.3. Cellular Origin of Tumor Lymphatic Endothelial Cells

4.3.1. Neolymphatic Vessels Arise Mainly from the Preexisting Vasculature. Identifying the cellular origin of tumor LECs can help to identify targets for anti-lymphangiogenic drugs in tumors. Growth of lymphatic vessels from preexisting vessels (neo-lymphangiogenesis) is regionally induced during

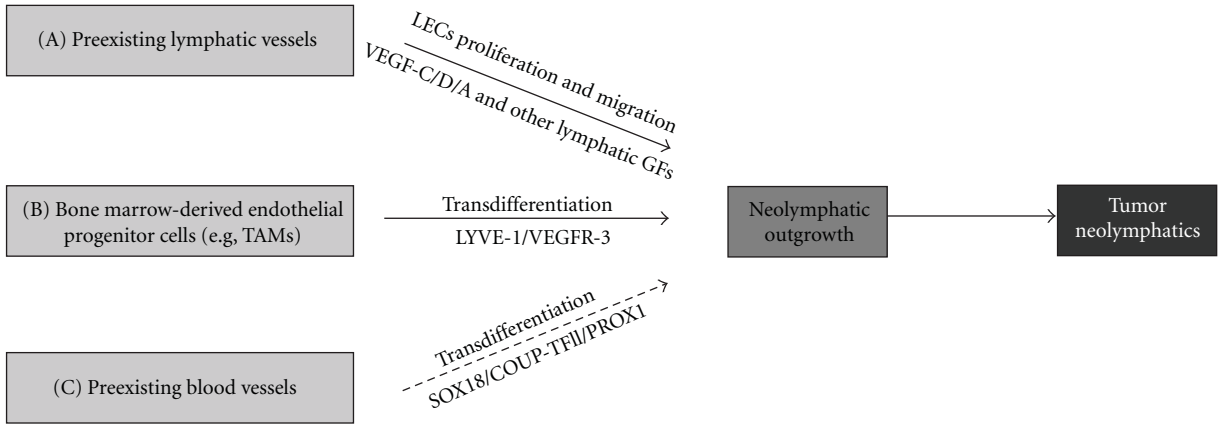

Figure 3: Potential cellular origins of tumor lymphatic endothelial cells. (A) Neolymphatics mainly arise from preexisting vasculature by proliferation and migration of LECs. (B) Bone marrow-derived endothelial progenitor cells (e.g., tumor-associated macrophages—TAMs) can also transdifferentiate into LECs, which further incorporate into the pre-existing lymphatic vasculature. (C) BECs can transdifferentiate into LECs under stimulation of reexpressed lymphatic transcription factors and lymphatic growth factor receptors. This mechanism has not been shown in *in vivo* (dashed line arrow).

tumorigenesis (Figure 3(a)). There is a strong body of evidence in the literature suggesting that neolymphatics mainly arise from preexisting lymphatic vessels, whereas bone marrow-derived endothelial progenitor cells did not significantly contribute to the formation of tumor lymphatic vessels in mouse models of melanoma and lung cancer [3, 156, 157]. This tumor-induced lymphangiogenesis is controlled by the stimulation of various lymphatic growth factors secreted by tumor cells, stroma cells, and inflammatory cells in the tumor microenvironment.

4.3.2. Transdifferentiation from Nonendothelial Cell Types. Several independent studies have demonstrated significant contribution of bone marrow-derived cells (BMDCs) to the formation of new blood vessels during tumor angiogenesis [158, 159]. BMDCs, including endothelial progenitor cells, are recruited to angiogenic sites to support the formation of new vessels [158–160]. Endothelial progenitor cells have been shown to play a critical role in regulating the angiogenic switch that eventually affects metastatic progression from micrometastasis to macrometastasis in mouse models of pulmonary metastasis [161].

Since there is a biological association between angiogenesis and lymphangiogenesis, it is important to identify whether BMDCs also play critical role during pathological lymphangiogenesis. It has not yet been established whether expansion of lymphatic vasculature during pathological conditions is critically driven by incorporation of endothelial progenitor cells. Endothelial progenitor cells are present in the newly formed lymphatic vessels in a corneal lymphangiogenesis mouse model and also in peritumoral lymphatic vessels of a fibrosarcoma [162]. Remarkably, depletion of bone marrow cells suppressed lymphangiogenesis in inflamed corneas that were implanted with fibroblast growth factor-2 (FGF-2) [162]. In another model of mouse inflammation after corneal transplant, Maruyama et al. (2005) showed that $CD11b^+$ macrophages infiltrate the corneal stroma and transdifferentiate into lymphatic endothelial cells that integrate into existing lymphatic vessels [163]. Study of de novo lymphangiogenesis in human kidney transplants provided further evidence for the participation of recipient-derived lymphatic progenitor cells [164]. Specifically, myeloid cells present in murine inflamed corneas were found to express specific lymphatic marker VEGFR-3, and these specific cells also integrate into lymphatic vasculature during inflammation [165].

In a further study using bone marrow transplantation and genetic lineage-tracing, Zumsteg et al. (2009) demonstrated that cells derived from the myeloid lineage can contribute to tumor lymphangiogenesis by transdifferentiating to LECs and incorporating into tumor-associated lymphatics in a transgenic mouse model of pancreatic β-cell carcinogenesis and mouse model of transplanted prostate cancer [166] (Figure 3(b)).

Plasticity of macrophages has been demonstrated by the finding that these cells can transform from naïve monocytes into VEGF-C-producing cells. Additionally, TAMs have been shown to also express the lymphatic marker VEGFR-3 [66]. However, the contribution of BMDCs to tumor lymphangiogenesis is rather still controversial. In a study using Lewis lung carcinoma and B16-F1 melanoma cells in syngenic mice, no integration of BMDCs into newly formed lymphatic vessels was detected [156]. Therefore, more studies need to be performed to validate the transdifferentiation pathway of TAMs into LECs during tumorigenesis.

Recently, it has been reported that bone marrow-derived mesenchymal stem cells (MSCs) may also be able to differentiate into endothelial cells (ECs) under certain conditions [167, 168]. MSCs can form networks in a tube formation assay *in vitro* and also highly express endosialin, a tumor endothelial marker present in the microvascular and stroma of human tumors [167]. Under hypoxic culture conditions, human MSCs can differentiate into endothelial cells and show a significant increase in endothelial specific markers

such as CD34, VWF, FLK1, FLT1, TIE2 [168]. Importantly, MSCs infiltrate tumors in high number and have been shown to enhance breast cancer cell metastasis [169]. These studies implicate important roles of MSCs during tumorigenesis, one of which is that MSCs may differentiate into ECs˜and therefore contribute to tumor angiogenesis and lymphangiogenesis. Conversely, ECs treated with bone morphogenetic protein (BMP4) or TGF-β2 can be reverted to a multipotent cell with MSCs phenotype [170]. This indicates that ECs and MSCs are able to interchange their phenotype. This transdifferentiation may be conditioned by the tumor microenvironment and further contributes to tumor progression.

4.3.3. Transdifferentiation from Blood Endothelial Cells-Endothelial Cell Plasticity. During embryonic lymphangiogenesis, lymphatic endothelial precursor cells arise from venous endothelial cells in cardinal vein. Notably, this specific population of venous endothelial cells expresses several key transcription factors, including SOX18, COUP-TFII, and PROX-1 that regulate the differentiation of venous endothelial cells into LECs [10, 12, 44]. Therefore, under pathological conditions in the adult, reactivation of a specific combination of transcription factors may modulate the plasticity of endothelial cells by turning on the molecular program required for transition from a BEC phenotype to a LEC fate (Figure 3(c)).

In support of this concept, the transcription factor COUP-TFII that is essential for inducing PROX1 expression in venous endothelial cells and triggering the lymphatic differentiation program [44] has been also shown to be required for adult lymphangiogenesis in an animal model of cancer [11] (Table 3). Similarly, although transcription factor SOX18 is not required for the maintenance of the LEC phenotype in adult during physiological condition, it is re-expressed on tumor blood vessels [172] and neolymphatics (unpublished data) suggesting a potential role in tumor-induced lymphangiogenesis. Potentially, the re-expression of SOX18 in BECs may trigger PROX-1 transactivation and induce the acquisition of a LEC phenotype. Moreover, blood vessels have been reported to express lymphatic marker VEGFR-3 in some tumors and chronic wounds [173–175]. The expression of VEGFR-3 on BECs not only can contribute to angiogenenic activation via the VEGF pathway but also can induce the LEC phenotype, suggesting that its expression may be indicative of phenotypic transition between blood and lymphatic vessels.

Although there is no direct evidence so far supporting the concept of transdifferentiation from BECs, it is plausible to consider that embryonic lymphatic vascular development is recapitulated in a tumor setting. Further, experimental depletion of the venous endothelium or the macrophage population in a tumor model will yield a definitive answer to the question of key cellular differentiation mechanisms. Indentifying these differentiation programs can lead to more therapeutic options in targeting critical differentiation pathways that trigger lymphangiogenic switch during tumorigenesis.

5. Lymphatic Vasculature as a Potential Therapeutic Target

5.1. Limitations of Antiangiogenic Therapy. Although it has been well established in preclinical and clinical studies that antiangiogenic therapies have antitumoral effects and survival benefits, it also has emerged that tumor cells can eventually elicit multiple mechanisms of resistance that allow them to adapt to a new milieu. Angiogenic inhibitors (such as VEGFR2-specific antibody and sunitinib—an oral, small-molecule, multitargeted receptor tyrosine kinase inhibitor) targeting the VEGF pathway have been shown to display antitumor effects in mouse models of pancreatic neuroendocrine carcinoma and glioblastoma but concomitantly induced tumor progression to greater malignancy with adaptive "evasive resistance" [183]. This mechanism is followed by increased invasion and distant metastasis. Notably, while both angiogenic inhibitors induced liver metastasis, sunitinib did not enhance lymph node metastasis [183]. The preferred explanation for this effect is that sunitinib potently blocks not only VEGFR-2 and platelet-derived growth factor receptors (PDGFRs) but also specific lymphatic receptor VEGFR-3 [184, 185]. The inhibition of VEGFR-3 in this context can block tumor lymphangiogenesis and lymph node metastasis. This raises the prospect that a potential therapeutic strategy could address both blood and lymphatic vessels to maximize antitumor and antimetastasis effects. Further, glioblastoma patients involved in antiangiogenic therapies, including VEGF ligand-trapping antibody and bevacizumab (a humanized monoclonal antibody that binds to VEGF-A), showed a proinvasive adaptive response where multifocal recurrence of tumors developed during the course of the therapy [186–188].

The critical challenge is to manage metastatic disease after the primary tumor has been surgically removed or has been inhibited by antiangiogenic agents. This raises the question of how anti-lymphangiogenic therapeutics might help in blocking both lymph node and distant organ metastasis. Hence, the use of antiangiogenic agents could be considered alongside anti-lymphangiogenic therapeutic approaches with the aim of improving current therapy.

5.2. Targeting the VEGF Family. A number of independent studies have now shown that inhibiting tumor-induced neolymphangiogenesis can dramatically reduce the metastatic spread of cancer in mouse models [83, 189, 190] (see [191] for review). Recently, several therapeutic strategies that target outgrowth of lymphatics via the VEGFR-3/VEGF-C/VEGF-D axis have been developed, based on preclinical animal models (Table 4) or on clinical trials using VEGFR tyrosine kinase inhibitors (Table 5). It is important to note that VEGF-C/VEGFR-3 signalling is not required for the maintenance of lymphatic vasculature in the adult, as prolonged inhibition of the VEGFR-3 pathway using soluble VEGFR-3 decoy receptor does not affect preexisting lymphatic vessels in the adult [192].

In preclinical studies, the therapeutic effects of targeting VEGF pathways have been evaluated using antibodies to neutralize lymphatic growth factors/receptor, or a soluble form

Table 3: Tumor lymphangiogenic transcription factors.

Transcription factors	Target genes	Association with cancer	References
PROX-1	Genes involved in proteolysis, lymphatic differentiation, cell adhesion, and migration	(i) Prox1 is strongly expressed by human Kaposi's sarcoma (a neoplasm of KSHV-infected vascular endothelium). (*) (ii) Highly expressed Prox1 induces lymphatic reprogramming, more aggressive tumor growing, and local invasion.	[176, 177]
SOX18	Prox-1, VCAM-1, Claudin-5	(i) SOX18 plays a critical role in initial steps of tumor angiogenesis and subsequent induction of tumor growth. (ii) SOX18 has also been found to express on tumor neolymphatics, suggest its potential role in regulation of tumor lymphangiogenesis.	[172, 178, 179] (unpublished data)
COUP-TFII	(i) Nrp2, coreceptor for VEGF-C (ii) Suppress VEGFR-1 expression in ECs	(i) Essential factor for tumor-induced neo-lymphangiogenesis in spontaneous mouse breast cancer model (ii) Control pancreatic islet tumor angiogenesis by regulating VEGF/VEGFR-2 signalling	[11, 180]
FOXC2	Integrin β3 subunit, Dll4, Hey2, CXCR4	(i) FOXC2 might regulate tumor angiogenesis by target genes including integrin β3, CXCR4, and Delta-like 4 (Dll4). (ii) High FOXC2 expression (mRNA level) group showed a higher incidence of advanced tumor stage, lymph node metastasis, and lymphatic invasion in esophageal cancer patients.	[181, 182]

* KSHV: Kaposi's sarcoma-associated herpesvirus (the involvement of lymphatic transcription factors—NFATc1 and Tbx1— in cancer metastasis has not been reported recently).

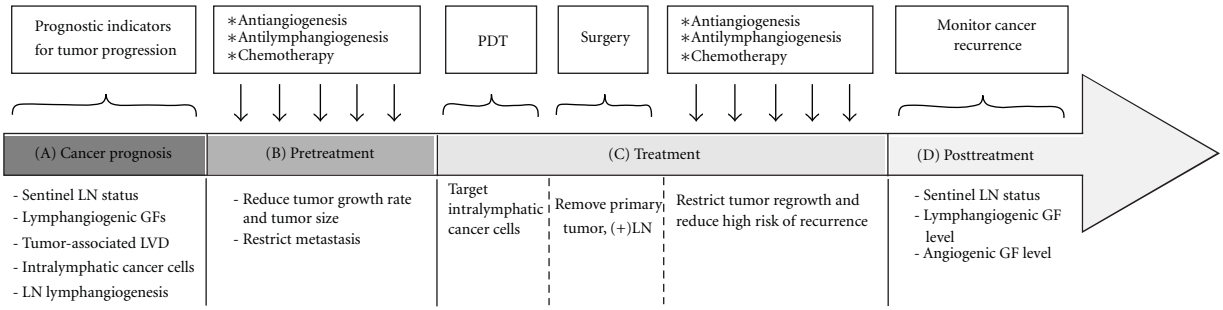

Figure 4: Schematic for potential clinical strategies in treatment of metastatic disease. (A) Tumor progression can be evaluated based on several prognostic indicators including tumor lymphangiogenesis and sentinal LNs status. These steps will guide the therapeutic decision to adopt anti-lymphangiogenic strategies if the tumor appears to be lymphangiogenesis-dependent and/or to have lymph node metastasis. (B) Antiangiogenesis, anti-lymphangiogenesis, and chemotherapy can be applied to reduce tumor growth and restrict metastasis before surgery. For advanced disease or nonresectable tumors, there will be no surgery [171]. (C) Photodynamic therapy (PDT) also can be performed before removal of the primary tumor, to eradicate in-transit tumor cells and prevent tumor relapse. Anti-lymphangiogenic, antiangiogenic, and chemotherapy can also be applied later, to prevent tumor regrowth and metastasis. (D) Cancer recurrence can be monitored by checking sentinel LN status, lymphangiogenic and angiogenic growth factor levels. PDT, photodynamic therapy; LN, lymph node; LVD, lymphatic vessel density; GF, growth factor (adapted from [171]).

of VEGFR-3 to trap VEGF-C/D. Neutralization of VEGF-D with a specific antibody or genetic ablation of VEGF-D appears to suppress tumor metastasis in mice [100, 204, 205]. Further, anti-VEGF-R3-blocking antibody or VEGF-C/-D trap strategy (a soluble VEGF-R3 immunoglobulin G Fc-domain fusion protein) has been shown to reduce the rate of lymph node metastasis in mouse models by 60–70% [3, 129, 157, 192, 197, 206, 207] (see [51] for review).

Further, VEGF-C has been known to also bind to Nrp-2 coreceptor and play a role in regulation of small lymphatic vessel and capillary remodelling [29, 208]. An antibody against the Nrp-2 coreceptor that blocks VEGF-C binding has been shown to reduce tumor lymphangiogenesis and metastasis to regional lymph nodes and distant organs [189]. Targeting Nrp-2 therefore has been considered as a potential way to block tumor spread *via* inhibition of neo-lymphangiogenesis.

Another indirect approach is to target the pathway controlling VEGF-C/-D proteolysis. Proteolysis improves the affinity of VEGF-C and -D for both VEGFR-2 and VEGFR-3, which can further increase the induction of these growth factors during tumor angiogenesis and lymphangiogenesis

Table 4: Preclinical studies of anti-lymphangiogenic effect on tumor metastasis.

Drugs	Experiment/cancer model	Effect	References
siRNA VEGF-C	Mouse mammary tumor model (C166-siVEGFC)	Reduction in tumor lymphangiogenesis, lymph node metastasis, and spontaneous lung metastasis	[193]
Human monoclonal antibody VC (anti-VEGF-C)	*In vitro* binding affinity of antibody was tested	(i) Bind with high specificity and affinity to full processed mature form of human VEGF-C (ii) Inhibit the binding of VEGF-C to VEGFR-2 and VEGFR-3	[194]
VEGFR31-Ig	Mouse model of a highly metastatic human hepatocellular carcinoma (HCCLM3)	(i) Simultaneously bind VEGF-A, VEGF-C. (ii) Block both tumor angiogenesis and lymphangiogenesis, effectively inhibit primary tumor growth, metastasis to lung and lymph node	[195]
VD1 monoclonal Ab (anti-VEGF-D)	Immunodeficient mice, 293EBNA express VEGF-D (mAbs raised to VDH of hVEGF-D (antagonists))	Reduce the LN metastasis from 61% to 0%	[98]
Monoclonal Ab to VEGFR-3	Regeneration of adult lymphatic vessels	Block the regeneration of lymphatic vessels in adult	[196]
VEGFR-3 monoclonal antibody	Mouse model of MDA-MB-435/GFP human breast cancer transfected with human VEGF-C cDNA	Suppress tumor lymphangiogenesis and restrict metastatic spread to lymph nodes and distant organs	[197]
Soluble VEGFR-3 (VEGFR-3-Ig)	Highly metastasis human lung cancer cells (LNM35) stably expressing VEGFR-3-Ig or recombinant adenovirus expressing VEGFR-3-Ig were injected to LNM35 tumor-bearing mice	(i) Inhibition of intra- and peritumoral lymphangiogenesis (ii) Metastasis to LN was inhibited (iii) Metastasis to lung occurred in all mice group	[198]
Soluble VEGFR-3 (VEGFR-3-Rg)	Immunocompetent rat model induced with highly metastatic MT-450 cancer cells expressing VEGFR-3 soluble	(i) Reduction in the number of peritumor lymphatic vessels (ii) Suppression of metastasis formation both in regional LNs and lungs	[199]
Soluble VEGFR-3 decoy receptor (sVEGFR3-Fc)	Mouse model of human melanoma, human prostate injected with recombinant adeno-associated viral vector sVEGFR3-Fc (rAAV-sVEGFR3-Fc) Treatment before tumor implantation	(i) Melanoma: inhibit LN metastasis, but have less effect on lung metastasis (ii) Prostate: inhibit LN and lung metastasis (iii) Inhibition of tumor-associated lymphangiogenesis	[192]
Soluble VEGFR-3	Mouse model of prostate cancer (PC-3): subcutaneously or surgical orthotopic implantation	Reduction in intratumoral lymphatics, but metastasis to LN was not significantly affected	[200]
Ki23057	Mouse model of gastric cancer induced by orthotopic inoculation of OCUM-2MLN cells	(i) Ki23057 is a tyrosine kinase inhibitor, block autophosphorylation of VEGFR-3 (ii) Reduced significantly lymphatic invasion and lymphangiogenesis (iii) Reduced size of orthotopic tumors and number of metastatic LN	[201]
Anti-neutropilin-2	Mouse model of breast adenocarcinoma (66C14) and rodent glioblastoma (C6)	Reduction in tumor lymphangiogenesis, metastasis to sentinel lymph nodes and distant organs	[189]
Celecoxib (COX-2 inhibitor)	Mouse model of highly metastasis human lung adeocarcinoma	Suppression of the lymphangiogenesis and lymph node metastasis through downregulation of VEGF-C expression.	[202]
Antagonists of integrin $\alpha 4\beta 1$	Mouse model of Lewis lung carcinoma and B16 melanoma cancer	Significant suppression of lymphangiogenesis and metastasis	[203]

Table 5: Clinical trials of tyrosine kinase inhibitors on the VEGF pathway.

Drugs	Clinical trials	Target	References
PTK787/ZK 222584 (chloroanilino-pyridylmethyl phthalazine succinate)	Phase III for colorectal cancer Phase I, II for advanced hepatocellular carcinoma patients (in combination with intravenous doxorubicin)	Target VEGFR-3, -2, -1, PDGFR-beta (need new strategies for trials to specifically monitor effects on metastasis)	[210, 211]
CEP-7055 (N, N-dimethyl glycine ester)	Phase I as an oral-administered therapy for various malignancies	Target VEGFR-3, -2, -1	[212]
BAY 43-9006 (Bi-aryl urea)	Phase III for renal cell carcinoma Phase II for multiple tumor types (e.g., prostate, ovarian, pancreatic, breast, and lung cancers. . .)	Target VEGFR-3, -2 tyrosine kinase, PDGFR-beta, FGFR-1	[213]
JNJ-26483327	Phase I for patients with advanced solid tumors	Multitargeted tyrosine kinase inhibitor, inhibiting kinase of (EGFR)-1, -2, -4; VEGFR-3, Src family (Lyn, Fyn, Yes)	[214]
SU-014813	Phase I for patients with advance solid tumors	Oral multitargeted tyrosine kinase inhibitor	[215]

(modified from the table in paper "Focus on lymphangiogenesis in tumor metastasis"- Cancer cell, Achen et al., 2005 [206]).

[94, 108]. Recently, a novel enediynyl peptide inhibitor has been developed to block the furin-mediated processing of pro-VEGF-C to mature VEGF-C [209]; further studies using animal models need to be performed to clarify the *in vivo* effect and mechanism of this inhibition.

To date, several VEGF-receptor tyrosine-kinase inhibitors have entered phase I, II, or III clinical trials for cancer treatment, including BAY 43-9006, CEP-7055, PTK787/ZK 222584, JNJ-26483327, and SU-014813 (Table 5). These VEGF-receptor tyrosine-kinase inhibitors are well tolerated, display low toxicity and positive results such as an increase in response rate, progression-free survival, and overall survival, and have been observed in advanced colorectal, renal cell, breast, and non-small-cell lung cancer. This response is observed when treatment of VEGF-receptor tyrosine-kinase inhibitors is used alone or in combination with chemotherapy [216]. Although these multiple VEGF-receptor tyrosine-kinase inhibitors also affect tumor lymphangiogenesis via the VEGFR-3 pathway, most clinical investigations have focused on antiangiogenic and antitumor growth effects, and only a handful of reports describe antilymphangiogenic effect or anti-metastatic outcomes. More clinical evaluations on these tumor lymphatic aspects are required to develop a more efficient therapeutic approach against tumor growth and metastasis.

5.3. New Targets for Anti-Lymphangiogenesis . Over the past two decades, many key factors have been identified as important regulators for tumor lymphangiogenesis (Tables 2 and 3), but the major focus in anti-lymphangiogenic therapy has been targeting through VEGF-C and -D, and their membrane receptor VEGF-R3 and coreceptor (Nrp-2) [157, 206, 217]. Blocking only a single pathway related to the VEGF/VEGFR axis may not always be effective to prevent cancer metastasis. For instance, the lack of effect of PTK/ZK (a broad spectrum inhibitor of VEGF signalling) on tumor lymphangiogenesis and lymphatic metastasis in a mouse model of pancreatic β-cell carcinomas overexpressing VEGF-C or VEGF-D reveals the involvement of other pathways [218]. Adenoviral delivery of soluble VEGFR-3 also did not inhibit tumor lymphangiogenesis in these mice. This result suggests that the level of VEGF-C/D expression might be critical for drug effects and that there might be other important pathways involved in tumor lymphangiogenesis.

Various endogenous inhibitors of angiogenesis have been identified so far including matrix-derived group (e.g., collagen fragments, endostatin, tumstatin, ...), and non-matrix-derived group (e.g., interferons, angiostatin, ...) [219]; however, little is known about endogenous inhibitors of lymphangiogenesis. Using a mouse cornea model in which lymphangiogenesis is induced by factors including VEGF-A, FGF-2, and PDGF-BB, Vasohibin1 has been shown to have broad-spectrum anti-lymphangiogenic activity [220]. Vasohibin1 also appears to inhibit tumor lymphangiogenesis and regional lymph node metastasis in a mouse model of human lung cancer [220]. There is a need to identify novel endogenous lymphangiogenic inhibitors to broaden the therapeutic options in anticancer metastatic treatment.

Conspicuously, the current knowledge of the transcriptional control of pathological lymphangiogenesis has been disregarded, limiting the range of potential novel therapeutic targets. Recent studies have revealed the role of transcription factors in controlling neolymphatic formation during tumorigenesis. For instance, COUP-TFII has been shown to play a critical role in tumor lymphangiogenesis in a mouse model [11]. In addition, SOX18, a transcription factor regulating early vasculogenesis [221, 222] and lymphangiogenesis in the embryo [10], has been also identified to play a critical role in the initial steps of tumor angiogenesis and subsequent induction of the tumor growth. *Sox18*-mutant mice show greatly reduced tumor diameter compared to wild type [172]. The reexpression of SOX18 on tumor neo-lymphatics (unpublished data) suggests there might be an additional role of SOX18 in controlling tumor lymphangiogenesis. Considering that FOXC2 also plays a critical role during embryonic blood and lymphatic vessel development [17, 223], there is also evidence for the involvement of this transcription factor during tumor growth and angiogenesis. For instance, in aggressive basal-like breast cancers, FOXC2 is also highly expressed and contributes to cancer invasion and metastasis [224]. The tumoral endothelium in human and mouse express FOXC2, and *Foxc2* +/− heterozygous mutant

mice display reduced tumor growth due to a decrease in neoangiogenic activity [225]. The growing body of evidence supporting a critical of role of transcription factors as modulators of tumor-induced lymphangiogenesis provides new potential avenues in the design of novel therapeutic strategies. Engineering new ways to target transcription factors pharmacologically therefore represents an essential step towards further complementing therapeutic inhibition of the VEGF-VEGF-R axis.

Recent studies have broadened our knowledge about the molecular pathways that regulate tumor lymphatic formation and lymphatic spread. These include not only the group of lymphatic growth factors (Table 2) but also several transcriptional regulators (Table 3). In considering therapeutic application, targeting transcriptional factors may encounter the difficulty in delivery, as drugs need to be delivered to nucleus to be able to block the transcriptional factor targets. Nevertheless, further preclinical studies targeting both growth factors and transcription factors with an efficient delivery system may potentially inhibit tumor lymphangiogenesis and therefore metastasis. Additionally, further studies on the role of other groups of transcription factors that control tumor angiogenesis and tumor lymphangiogenesis will generate new therapeutic options for inhibiting the metastasis of solid tumors.

6. Summary and Conclusions

In preclinical studies using animal models, a variety of approaches have been investigated mainly targeting pro-lymphangiogenic signalling related to the VEGF axis, including neutralization using monoclonal antibodies, soluble receptors, chemical inhibitors, and shRNA. The next challenge is to establish translational studies to address metastasis *via* a more integrative approach that inhibit multiple pathways (related to both lymphatic growth factors and transcription factors) modulating tumor lymphangiogenesis. An anti-lymphangiogenic approach could be used together with antiangiogenic therapy and conventional chemotherapy, leading to a more efficient way to prevent cancer recurrence [171]. Recently, in a mouse model of gastric cancer, the combination of treatment with antiangiogenic agent (bevacizumab) and genetic blockade of IGF-1 (IGF-1R dominant negative) efficiently reduced tumor growth and importantly resulted in the complete regression of 43% of tumors by inhibiting both angiogenesis and lymphangiogenesis [114]. In humans, the benefit of treatments that combine sunitinib (an anti-angiogenic agent) with docetaxel (an anti-mitotic chemotherapeutic) has been evaluated in phase 1/2 clinical trials of prostate cancer patients. This combination was moderately well tolerated and showed a promising increase of progression-free survival [226].

Based on the most recent preclinical research, photodynamic ablation of in-transit metastatic cancer cells could also be applied to efficiently prevent the recurrence of cancer metastasis (Figure 4). This method relies on liposomes, which ensure lymphatic specific delivery of verteporfin, a drug that can be activated by a 689 nm laser light. The cytotoxic activity of light-activated verteporfin is thus restricted to lymphatic vessels and cancer cells within the vessel. This preclinical study on a mouse model has shown that the recurrent metastasis was reduced to 37.5% compared to untreated animals after laser treatment [227].

To advance the prospect of anti-lymphangiogenic therapy, the next step would be to initiate trials on cancer types in which lymphangiogenesis has been clearly identified as a risk factor. Moreover, there are still several issues that need to be clarified which relate to the efficiency of anti-lymphangiogenic therapy in blocking metastasis. Firstly, inhibition of lymphangiogenesis does not seem to affect preexisting vessels [196], which are still potential routes for cancer cell dissemination. Secondly, there are some possible side effects of targeting tumor-associated lymphatic vessels [228]. Inhibition of lymphangiogenesis might interfere with physiological process such as wound healing and tissue regeneration [173]. Finally, lymphedema is a complication in 20%–30% of breast cancer patients after surgery to remove the tumor-metastasized lymph node [229, 230]. Therefore, preclinical studies using animal models have been performed in an attempt to restore lymphatic vessel function in secondary lymphedema, including VEGF-C, VEGF-D gene transfer using adenovirus or naked plasmids and recombinant VEGF-C protein [3, 231].

In conclusion, the study of embryonic lymphatic vessel development has revealed key factors that play a central role in controlling tumor-induced lymphangiogenesis. However only the VEGF/VEGF-R axis has been thoroughly investigated and exploited with a view to restricting tumor growth and metastasis, and so far the outcomes in terms of patient survival have been limited. Therefore, it is important to continue efforts to indentify factors and molecular mechanisms in order to fully comprehend how tumor neo-lymphangiogenesis is regulated and participates in tumor metastasis. These discoveries will lead to identification of potential new molecular targets and design of novel therapeutic avenues of metastatic disease. In addition, further preclinical studies focusing on delivery systems, side effects, drug resistance, and combination of anti-angiogenic and anti-lymphangiogenic therapies may eventually improve the efficacy of current treatments.

Abbreviations

Ang: Angiopoietin
BEC: Blood endothelial cell
BMDCs: Bone marrow-derived cells
CAFs: Cancer-associated fibroblasts
CV: Cardinal vein
dpc: days post coitum
ECs: Endothelial cells
LEC: Lymphatic endothelial cell
LN: Lymph node
LS: Lymph sac
MSCs: Mesenchymal stem cells
TAMs: Tumor-associated macrophages
VEGF: Vascular endothelial cell growth factor
VEGFR: Vascular endothelial cell growth factor receptor.

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Research Article

Influence of Morphine on Pericyte-Endothelial Interaction: Implications for Antiangiogenic Therapy

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Morphine stimulates tumor angiogenesis and cancer progression in mice. We examined if morphine influences endothelial-pericyte interaction via platelet-derived growth factor-BB (PDGF-BB) and PDGF receptor-β (PDGFR-β). Clinically relevant doses of morphine stimulated PDGF-BB secretion from human umbilical vein endothelial cells and activated PDGFR-β and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) phosphorylation in human pericytes. These *in vitro* effects of morphine were translated into promotion of tumor angiogenesis in a transgenic mice model of breast cancer when treated with clinically used dose of morphine. Increased vessel-associated immunoreactivity of desmin and PDGFR-β was observed on pericytes in tumors of morphine-treated mice. These data suggest that morphine potentiates endothelial-pericyte interaction via PDGF-BB/PDGFR-β signaling and promotes tumor angiogenesis, pericyte recruitment, and coverage of tumor vessels. We speculate that morphine may impair the effectiveness of antiangiogenic therapy by influencing vascular pericyte coverage.

1. Introduction

Angiogenesis, sprouting of new blood vessels from the existing vessels, is critical for cancer progression and metastases [1]. Endothelial cells, the building blocks of blood vessels, and endothelial cell-specific cytokine vascular endothelial growth factor (VEGF) and its receptors have therefore been a target of anticancer therapies [2–4]. Several VEGF and endothelial cell specific therapies are either in clinical use or clinical trials. However, drug resistance and ineffectiveness are a major challenge limiting the success of these promising new drugs. Recent studies suggest that vasculature is not merely an endothelial structure, rather it is closely associated with mural cells including pericytes and vascular smooth muscle cells (VSMCs) [5–7]. The exact role of pericyte coverage of tumor vasculature is not clear, but paradoxical roles are proposed that favor inhibition of angiogenesis on one hand and a barrier to antiangiogenic therapy on the other [4, 8, 9].

Pericyte coverage of the vascular endothelium is controlled by several cytokines including the platelet-derived growth factors (PDGFs) and VEGF [10, 11]. PDGF-BB secreted by endothelial cells acts as an attractant to recruit PDGFR-β-expressing pericytes and pericyte progenitor cells to the endothelium [12]. In turn, pericytes provide guidance to endothelial sprouts, scaffolding for the vasculature to grow, and stabilize the vessel wall [5, 13, 14]. Thus, endogenous and exogenous molecules that influence pericyte-endothelial interaction may influence tumor angiogenesis and interfere with therapies directed towards them. For example, morphine stimulates the expression of PDGF-BB in human brain microvascular endothelial cells (HBMECs) [15] and coactivates PDGFR-β signaling in the mouse retinal microvascular endothelial (mREC) and mesangial cells (specialized mural cells) in the kidney [16, 17].

Morphine used in clinically relevant doses promotes angiogenesis *in vitro* and *in vivo* and increases vascular permeability [18–20]. This proangiogenic activity of morphine is translated into promotion of breast and lung cancer in mice [18, 19, 21]. Additionally, morphine promotes breast and lung cancer cell proliferation and migration. Opioid

receptors (ORs) particularly mu opioid receptor (MOR) mediate the analgesic effect of morphine and are highly expressed in human lung cancer [21–23]. Morphine and its congeners are used to treat pain due to cancer, particularly in the advanced stages of malignancy when most of the therapies are ineffective. It is likely that morphine influences endothelial-pericyte interaction and may further contribute to ineffectiveness of targeted therapies.

Therefore, we examined morphine-induced endothelial and pericyte-specific activity mediated by PDGF-BB/PDGFR-β signaling. We used primary human umbilical vein endothelial cells (HUVECs) and human placenta-derived pericytes for *in vitro* studies and a transgenic mouse model of breast cancer, which mimics the evolutionary spectrum of human disease. We found that morphine stimulates PDGF-BB secretion by HUVEC and phosphorylation of PDGFR-β, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and signal transducer and activator of transcription 3 (STAT3) in pericytes. Complementary to these *in vitro* observations, morphine in clinically used doses increased desmin- and PDGFRβ-positive cells in the tumor vasculature of mice, suggestive of increased proliferation and/or recruitment of vessel-associated pericytes.

2. Materials and Methods

2.1. Tumor Model and Drug Treatments. Female transgenic mice carrying a rat C3(1) simian virus 40 large tumor antigen (C3TAG) fusion gene that develop highly invasive breast tumors were used. Female C3TAG mice show the evolutionary spectrum of human infiltrating ductal carcinoma [24]. These mice develop ductal epithelial atypia at 8 weeks, progression to intraepithelial neoplasia at 12 weeks (resembling human ductal carcinoma in situ), and invasive carcinoma and grossly palpable tumors at 16 weeks. Tumors predominantly metastasize hematogenously to the lungs, and also to the liver, adrenals, and heart. By 6 months of age, all the mice die because of universal development of multifocal mammary adenocarcinomas. We have used this model in previous studies to target tumor angiogenesis using blood outgrowth endothelial cells expressing sFlt1 [25]. Three-month-old C3TAG mice were subcutaneously injected with morphine sulfate (Baxter Esilerderle Healthcare, Cherry Hill, NJ) at 0.5 mg/Kg/d for 2 wks and the dose escalated every two weeks to 0.75 mg/Kg/d, 1.0 mg/Kg/d, 1.25 mg/Kg/d, and 1.5 mg/Kg/d, or with PBS, for a period of seven weeks. All reagents were from Sigma-Aldrich, St. Louis, MO, unless specified.

2.2. HUVEC Culture. Human umbilical vascular endothelial cells (HUVECs) were isolated from umbilical cords and cultured as previously described [26]. Complete HUVEC culture medium (CHCM) consisted of medium 199 (Life Technologies, Inc., Gaithersburg, MD), 40 μg/mL heparin sodium salt, 15% fetal bovine serum, 2.4% 200 mM L-glutamine, 100 units/mL penicillin, 100 units/mL streptomycin, 100 units/mL fungizone, 0.04% freshly thawed ENDO-GRO, and 0.1% sodium pyruvate. Primary HUVECs between passages one and three were used for all experiments. HUVECs were cultured in serum and growth factor-free medium (SFM) to examine the effect of morphine on PDGF-BB expression and for signaling studies. SFM consisted of medium MCDB 131 (Life Technologies, Inc., Gaithersburg, MD), dibutyryl cAMP, heparin, L-glutamine, Pen/Strep/Fungizone, and hydrocortisone as described [18].

2.3. Pericyte Culture. Human pericytes from placenta were purchased from PromoCell (PromoCell, Heidelberg, Germany) and cultivated per the manufacturer's instructions in pericyte growth medium. Pericytes were serum and growth factor starved in pericyte serum and growth factor-free medium (PSFM). PSFM consisted of medium 199 (Life Technologies, Inc., Gaithersburg, MD), 0.5% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, 100 units/mL fungizone, and 2 mM L-Glutamine.

2.4. ELISA. HUVEC were serum and growth factor starved in SFM overnight followed by incubation with different concentrations of morphine indicated in the figures for an additional 48 hrs. The supernatant from HUVEC was analyzed for PDGF-BB using an ELISA Kit (RayBiotech, Norcross, GA). Absorbance was read at 450 nm using an ELISA reader (Synergy HT, Winooski, VT). The concentration of PDGF-BB in the supernatant was calculated using the standard curve prepared in parallel with each experiment. SFM cultured without HUVEC in parallel to the experiment was used as a blank/negative control.

2.5. Western Blot Analysis. Pericytes were serum starved overnight as described and stimulated with 0.1 μM morphine or 20 ng/mL of PDGF-BB. Cell lysates were prepared as described by us earlier using a cocktail of protease inhibitors [18]. Protein lysates containing 100 μg of protein were separated on a 3–15% gradient SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane (Immobilon; Millipore, Bedford, MA). Protein bands were detected using 1 : 250 phospho-PDGFR-β (Upstate, Lake Placid, NY), 1 : 500 PDGFR-β, 1 : 1000 phospho-STAT3, 1 : 1000 STAT3, 1 : 1000 phospho-MAPK/ERK, and 1 : 1000 MAPK/ERK (all from Cell Signaling Technology, Danvers, MA). Alkaline phosphate-conjugated secondary antibodies (1 : 5000, Santa Cruz Biotechnology, Santa Cruz, CA) and ECF system (Amersham Bioscience, Buckinghamshire, UK) were used to detect chemiluminescent signals on a Storm 860 Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Protein bands were quantitated by densitometric analysis using ImageJ Software (National Institutes of Health, Bethesda, MD).

2.6. Immunofluorescent Staining. Tumors were frozen in liquid nitrogen, embedded in optimal cutting temperature compound (OCT), and cut into 6 μM cryosections. Sections were fixed in 4% paraformaldehyde and immunostained using the following primary antibodies at the indicated dilutions: 1 : 100 rabbit anti-PDGFR-β (Upstate, Lake Placid,

NY); 1 : 100 mouse antismooth muscle actin (α-SMA; Sigma, St. Louis, MO); 1 : 50 goat antidesmin (Santa Cruz); 1 : 50 rat anti-CD31-FITC (BD Pharmingen, San Diego, CA). Species-specific secondary antibodies conjugated with Cy3 or TRITC were used at the following dilutions: 1 : 400 donkey anti-rabbit IgG-Cy3 (Jackson Laboratories, West Grove, PA) and 1 : 50 donkey anti-goat IgG-TRITC (Santa Cruz). In addition, isotype-matched IgG was used as control (Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescent images were visualized and obtained using an Olympus IX70 epifluorescent microscope with an attached Olympus DP70 digital camera (Olympus America Inc., Center Valley, PA).

2.7. Quantitation of Immunoreactive Pixels and Tumor Angiogenesis. Pericyte markers, desmin, α-SMA, and PDGFR-β, were examined in relation to endothelial cell marker CD31. Superimposed images were analyzed with Adobe Photoshop to calculate the ratio of desmin, α-SMA, or PDGFR-β relative to CD31-positive cells. Ratios were based on the fluorescent intensity of the proteins. For morphometric analysis of tumor angiogenesis, CD31-positive images were binarized and skeletonized using Adobe Photoshop and the Image Processing Tool-Kit Plug-in Functions for Adobe Photoshop (Reindeer Games, Asheville, NC), and total lengths, ends, and nodes of vessels were quantified as described by us earlier [18].

2.8. Statistical Analysis. All data are expressed as mean ± SEM. All statistical analyses were performed using Prism software (GraphPad Prism Inc., San Diego, CA). Significance was determined using unpaired, Student's t-tests. $P < 0.05$ was considered significant.

3. Results

3.1. Morphine Stimulates PDGF-BB Secretion by HUVEC. Morphine induces PDGF-BB expression in HBMECs [15], but this needs to be secreted to have a paracrine effect on pericytes. We examined if PDGF-B was secreted into the culture supernatants by HUVEC stimulated with morphine for 48 h. Morphine at the doses of 0.1 and 1 μM stimulated about a 2-fold increase in the secretion of PDGF-BB in the culture medium as compared to PBS (Figure 1). However, 1 mM concentration of morphine did not have a significant effect on PDGF-BB secretion as compared to PBS. Phase contrast microscopy and Trypan blue staining of HUVEC incubated with 0.1 and 1 μM morphine showed that more than 99% cells were alive and appeared normal (data not shown). In contrast, more than 99% of HUVECs were dead after incubation with 1 mM morphine. Morphine concentrations at 0.1 and 1 μM are consistent with the observed plasma/serum concentration of diverse patient population treated with morphine, which ranges between 2 nM and 3.5 mM [27, 28]. Earlier studies from our laboratory demonstrated that 1 mM morphine was cytotoxic to human dermal microvascular endothelial cells (HDMECs) [18]. Thus, clinically relevant concentration of morphine stimulates PDGF-BB secretion from endothelial cells, a key step in endothelial-pericyte interaction.

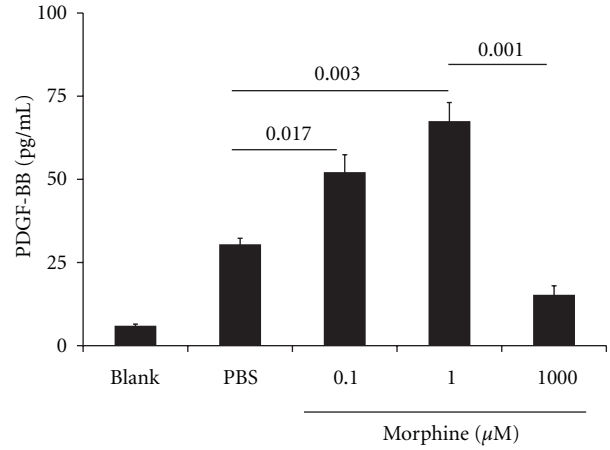

Figure 1: Morphine stimulates PDGF-BB release from endothelial cells. PDGF-BB was analyzed in the cell culture supernatants of HUVEC incubated with different concentrations of morphine or PBS in serum- and growth factor-free medium for 48 h at 37°C. In parallel, serum- and growth factor-free medium was incubated in flasks without cells to serve as blank. A dose-dependent increase in PDGF-BB is seen between 0.1 and 1 μM morphine, whereas no statistically significant increase occurred with 1 mM morphine, as compared to PBS. Each bar is the mean ± SEM of three separate experiments from 3 different cultures of HUVEC.

3.2. Morphine Activates PDGFR-β, MAPK/ERK, and Stat3 Signaling in Pericytes. Proliferation, recruitment, and endothelial interaction of pericytes with endothelium are dependent upon PDGFR-β signaling. We observed that 0.1 μM morphine as well as 20 ng/mL PDGF-BB stimulates sustained activation of PDGFR-β phosphorylation on pericytes from 5 min to 60 min of incubation (Figures 2(a) and 2(b)). Interestingly, both morphine and PDGF-BB significantly stimulated MAPK/ERK phosphorylation in a time-dependent manner, which returned to baseline after 60 min of incubation. On the other hand, both morphine and PDGF-BB stimulated the phosphorylation of STAT3, but it was not statistically significant. Therefore, both morphine and PDGF-BB stimulate PDGFR-β and MAPK/ERK signaling in pericytes.

3.3. Morphine Promotes Angiogenesis in Tumors of C3TAG Mice. Three-month-old C3TAG mice bearing multiple breast tumors, which grow spontaneously, were treated with clinically relevant escalating dose of morphine, for seven weeks. Multiple tumors in different sizes were dissected out, but only tumors about 1 cm × 0.5 cm were analyzed for tumor angiogenesis (Figures 3(a)–3(d)). Morphometric analysis was performed on tumor sections stained with anti-CD31-FITC. Different parameters of angiogenesis analyzed included vessel density (a), total length of vessels (b), number of vessels (ends, (c)), and branching (nodes, (d)). A significant increase was observed in each of these parameters in morphine-treated mice as compared to PBS-treated mice. Excessive branching (nodes) and increased number of vessels is a typical feature of disorganized growth of vasculature in

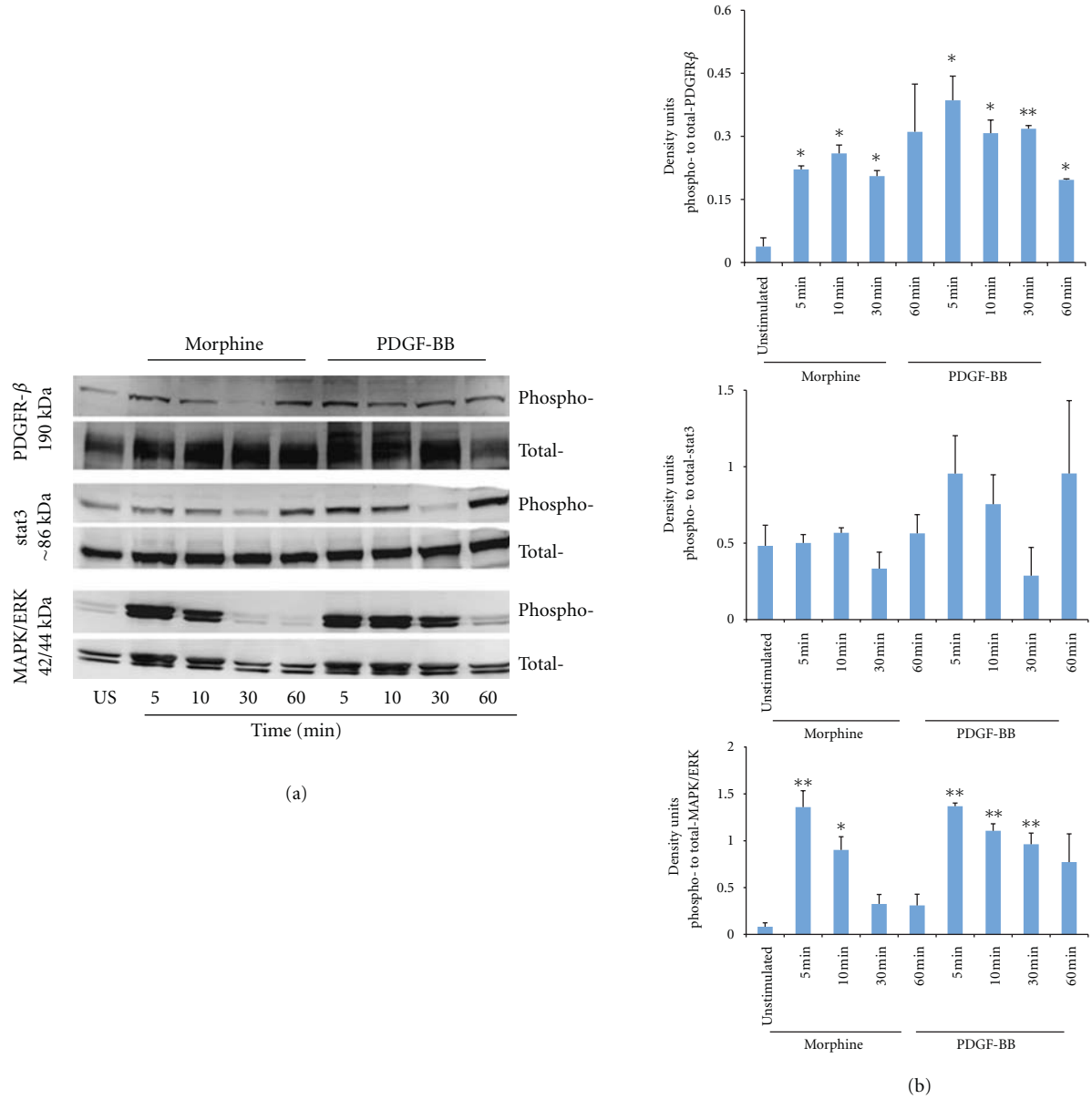

Figure 2: Morphine coactivates PDGFR-β phosphorylation and MAPK/ERK signaling in human pericytes. Human placenta-derived pericytes were incubated with 0.1 μM morphine or 20 ng/mL PDGF-BB for indicated time or with PBS, followed by cell lysis using protease inhibitors. Lysates containing 100 μg protein were resolved on 3–15% SDS gel and Western blotted as described in the methods. (a) Representative image of five different Western blots for phospho- and total-PDGFR-β, MAPK/ERK, and STAT3 is shown. (b) Densitometric analysis of protein bands is represented as a ratio of phospho- to total-protein for each protein. Each bar is the mean ± SEM of five different blots. $^{*}P < 0.01$, $^{**}P < 0.001$, for each time point compared to unstimulated in each graph.

tumors. Morphine, therefore, further augments tumor angiogenesis.

3.4. Morphine Treatment Results in Increased Desmin Immunoreactivity but Does Not Influence α-SMA Immunoreactivity in Tumors. Tumors from C3TAG mice treated with morphine as described above were costained with anti-CD31-FITC (green vasculature) and desmin (red) or with anti-CD31-FITC (green) and α-SMA (red). Immunofluorescent images show a significant increase in desmin immunoreactivity in morphine treated as compared to PBS-treated mouse tumors (Figure 4(a), top row). Most of the desmin staining colocalized with CD31-postive endothelium in a random fashion and showed a significant increase with morphine treatment as compared to PBS (Figure 4(b)). In some areas, desmin staining is independent of CD31 staining (magenta arrow in (a)). Some strongly desmin-positive (red) cells also appeared on the vessel sprouts and tip cells (yellow arrow and enlarged region shown separately in Figure 4(c)) indicative of supporting the formation and guidance of new vessels. In contrast, α-SMA immunoreactivity was strong in both morphine- and PBS-treated tumors without any significant

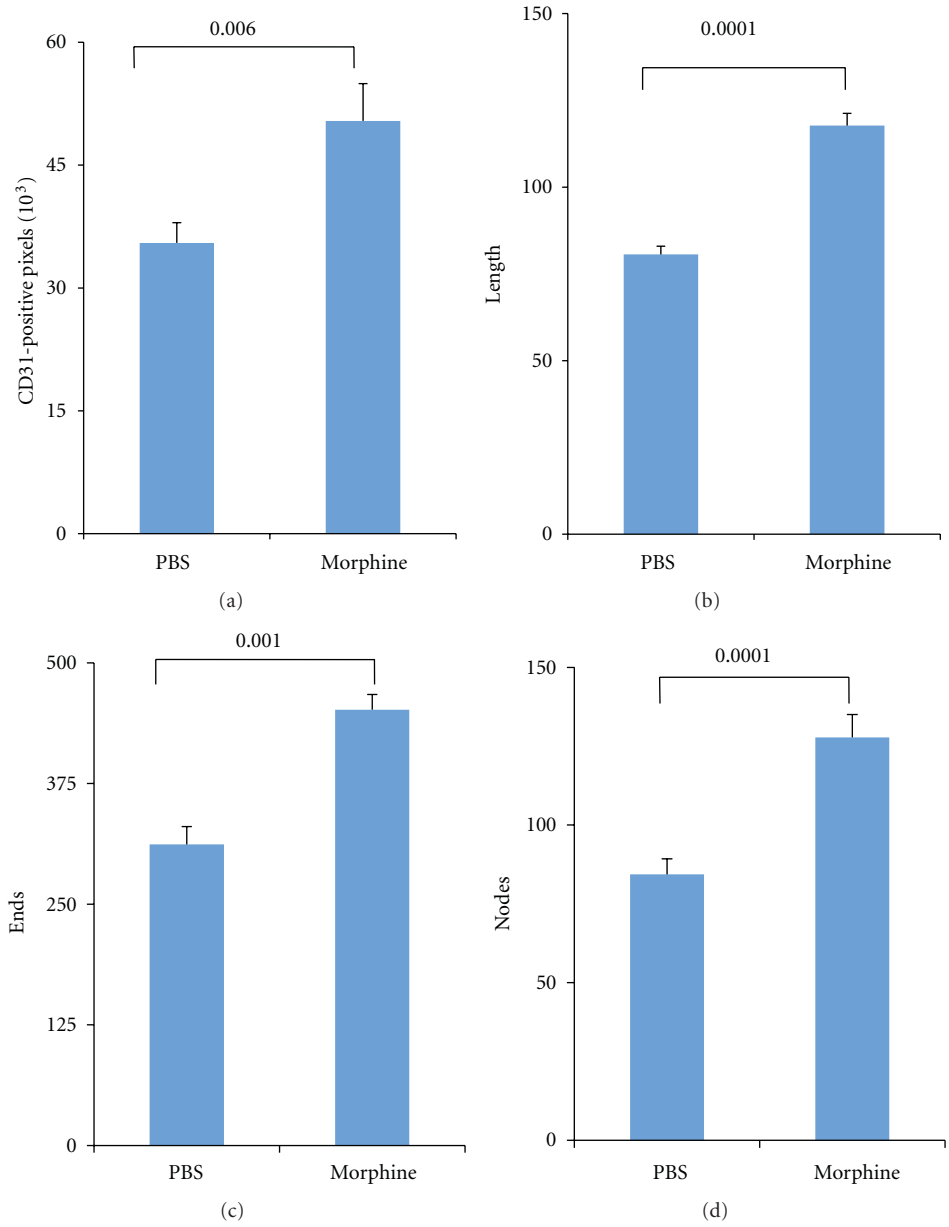

Figure 3: Morphine stimulates tumor angiogenesis. C3TAG mice at 3 months of age were subcutaneously injected with morphine at 0.5 mg/Kg/d for 2 wks, and the dose was escalated every two weeks to 0.75 mg/Kg/d, 1.0 mg/Kg/d, 1.25 mg/Kg/d, and 1.5 mg/Kg/d, or with PBS, for a period of seven weeks. Sections of tumors larger than 1 cm × 0.5 cm were immunostained with anti-CD31-FITC, followed by morphometric analysis to quantify different parameters specific to tumor angiogenesis. (a) CD31-positive pixels indicate total pixels for CD31 immunostaining per image. (b) Length suggests the total length of vessels per image. (c) Ends denote the number of vessels per image. (d) Nodes suggest the number of branch points in an image. Each bar represents mean ± SEM of sections from five different tumors obtained from 5 different mice per treatment.

difference between the two treatment groups (Figures 4(a) and 4(b)). Notably, all a-SMA immunoreactivity colocalized with vascular endothelium.

3.5. Increased Expression of Vascular PDGFR-β Immunoreactivity in Tumors of Mice Treated with Morphine. Tumor sections of mice treated with morphine show strong costaining of PDGFR-β in association with endothelium, which appears orange due to the overlaying of red and green images of PDGFR-β and CD31, respectively (Figure 5(a)). Quantitatively also vessel-associated PDGFR-β immunoreactivity was significantly higher in morphine as compared to PBS-treated mouse tumors (Figure 5(b)). Most of the PDGFR-β immunoreactivity colocalized uniformly with vessels and in the area surrounding the vasculature (red arrows) in morphine treated mice. In contrast, in PBS-treated mice, most of the PDGFR-β immunoreactivity colocalized with nonvascular cells, likely with tumor cells. Of note, specific

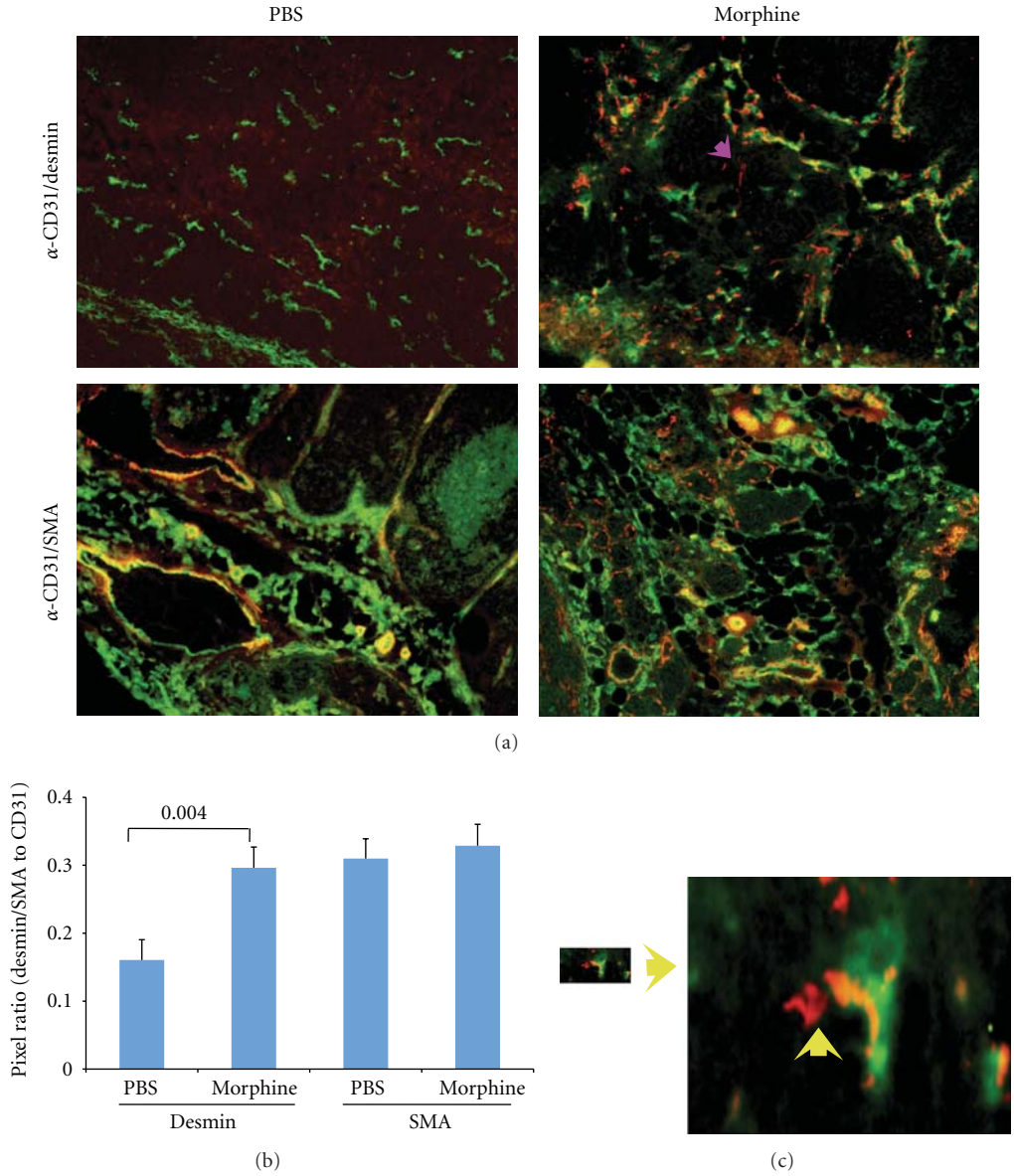

Figure 4: Morphine stimulates vessel-associated Desmin expression in mouse tumors. C3TAG mice were treated with morphine or PBS as described in Figure 3 and the methods. (a) Upper row shows tumor sections stained with anti-CD31 for vasculature (green) and desmin (red). Morphine-treated tumors show strong desmin (red) staining associated with tumor endothelium (green) as well as independent of vasculature (magenta arrow). Orange staining suggests an overlap between red and green staining for desmin and vasculature, respectively. Lower row shows strong costaining of α-smooth muscle actin (SMA, red) with CD31 (green), in both PBS and morphine-treated mice tumors. Magnification $\times 150$. Each image represents 5 different tumors from 5 different mice per treatment. (b) Ratios of desmin to CD31- and α-SMA to CD31-immunoreactive pixels are shown. A significant difference is observed in desmin/CD31 ratio between morphine and PBS treatment but not in α-SMA/CD31 ratio. Each bar represents mean $\pm$ SEM of immunoreactive pixels from five tumors (3 different sections of each tumor) obtained from 5 different mice per treatment. (c) Enlargement of area shown with yellow arrow in (a) for CD31/desmin staining in morphine-treated mice. It shows colocalization of desmin staining in the sprouting endothelial cell and in the tip cells.

colocalization of PDGFR-β was seen on vascular sprouts (orange arrows) and at vessel branch points, in PBS group. Thus, it appears that in the tumors of these mice, PDGFR-β is associated with vessel branching and sprouts, whereas morphine treatment increases endothelium-associated pericyte density and pericyte coverage of vasculature.

4. Discussion

Clinically used doses of morphine/opioids act on endothelium and tumor cells resulting in tumor progression *in vitro* and *in vivo* experimental studies [18, 21, 22, 29–31]. Significantly higher expression of MOR on human lung cancer

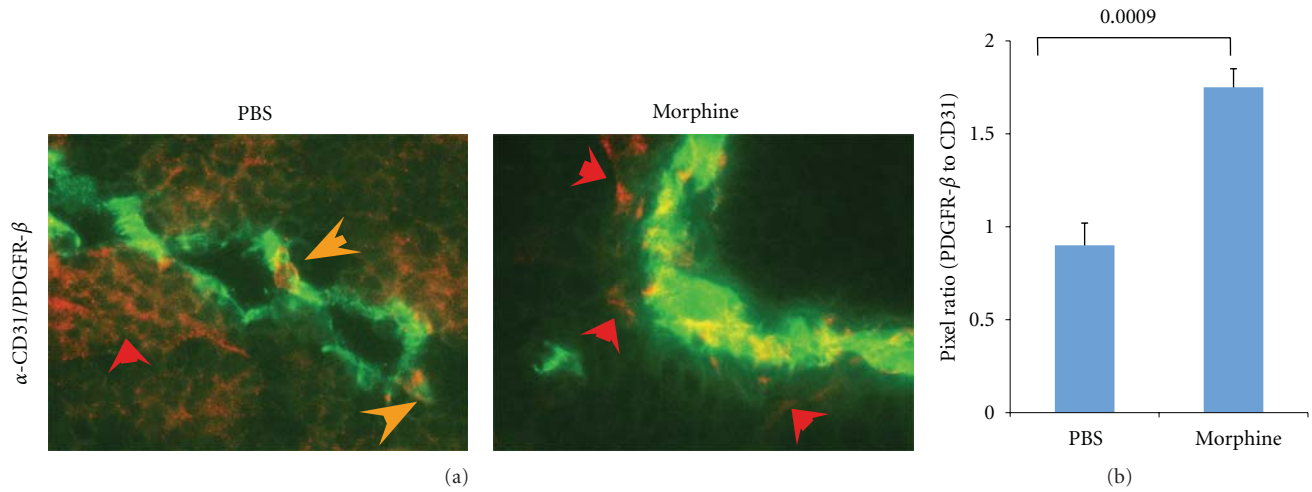

Figure 5: Morphine stimulates vessel-associated PDGFR-β expression in mouse tumors. C3TAG mice were treated with morphine or PBS as described in Figure 3 and the methods. (a) Tumor sections were immunostained with anti-CD31 (green) and anti-PDGFR-β (red). Vasculature from morphine-treated mouse tumors shows strong costaining for PDGFR-β, whereas PDGFR-β staining is predominantly observed in nonvascular compartments of PBS-treated mouse tumors. Vessel-associated PDGFR-β is observed only near the branch points of vasculature in PBS-treated group (orange arrows). Each image represents 5 different tumors from 5 different mice per treatment. Magnification $\times$900. (b) Ratios of PDGFR-β to CD31, immunoreactive pixels are shown. A significant difference is observed in PDGFR-β/CD31 ratio between morphine and PBS treatment. Each bar represents mean $\pm$ SEM of immunoreactive pixels from five tumors (3 different sections of each tumor) obtained from 5 different mice per treatment.

tissue as compared to nonmalignant tissue in the same organ complements this activity of morphine [21–23]. Therefore, this study was undertaken to examine if pericytes were directly or indirectly influenced by clinically relevant doses of morphine. We observed that morphine stimulated PDGF-BB secretion by endothelial cells, a critical mediator of endothelial-pericyte crosstalk, thus indirectly influencing pericyte activity. Morphine also activated PDGFR-β signaling and MAPK/ERK phosphorylation on human pericytes. These activities of morphine on endothelial cells and pericytes correlate with an increase in angiogenesis, vessel associated-desmin and -PDDGFR-β expressing pericytes in transgenic mice with breast cancer. Our observations on morphine-induced vascular-pericyte interaction may have implications upon the effectiveness of antiangiogenic therapy.

PDGF-BB plays a central role in the recruitment and growth of pericytes and in endothelial-pericyte interaction in a paracrine manner [5, 13]. In this relationship, endothelial cells secrete PDGF-BB, which acts upon the pericytes and progenitor cells and recruits them to the endothelium. Increased secretion of PDGF-BB by HUVEC when stimulated with 0.1 and 1 μM morphine demonstrates that morphine plays a salutary role in HUVEC-pericyte interaction. However, 1 mM morphine did not have an effect on PDGF-BB secretion by HUVEC. It is noteworthy that in patients treated with a range of morphine doses for diverse conditions including cancer, morphine concentration ranged between 2 nM and 3.5 μM in the plasma/serum [27, 28], which are in the range of 0.1 and 1 μM concentration showing stimulation of PDGF-BB secretion by HUVEC. The 1 mM concentration of morphine is highly unlikely to be present in the plasma of patients, because an extremely high dose of morphine will be required to achieve this plasma concentration, which in turn may have severe side effects and therefore not used clinically. We demonstrated earlier that 1 mM morphine was cytotoxic to HDMEC [18], and in the present study, we found that HUVECs incubated with 1 mM morphine for 48 h were not alive. Our observation of PDGF-BB secretion by HUVEC in this study is further supported by an increase in expression of PDGF-BB in HBMEC by 10^{-7} M morphine, but not by 10^{-5} M morphine [15]. Together, these data suggest that clinically relevant dose of morphine has a stimulatory effect on PDGF-BB production by endothelial cells, which can act in an autocrine and paracrine manner via PDGFR-β to promote angiogenesis and pericyte growth and recruitment.

PDGF-BB is known to activate PDGFR-β and several downstream signaling pathways that promote cell proliferation, survival, and differentiation, including MAPK/ERK and STAT3 [14, 32, 33]. We found that morphine coactivates VEGFR2 and PDGFR-β in mouse retinal microvascular endothelial cells (mRECs) which immunoprecipitated with MOR [16]. Our earlier studies also showed morphine-induced MAPK/ERK, Stat3, and Akt phosphorylation in mREC and HDMEC [16, 18]. In HBMECs, also morphine activated MAPK/ERK and PKB/Akt phosphorylation [15]. More recently, we observed that morphine coactivates PDGFR-β signaling in kidney mesangial cells *in vitro* and *in vivo* [17]. MOR silencing on kidney mesangial cells led to a significant decrease in morphine-induced phosphorylation of PDGFR-β, MAPK/ERK, Stat3, and PKB/Akt, suggestive of MOR-PDGFR-β crosstalk. This is highly significant considering that MOR agonist drugs including morphine are used to treat pain in cancer and that PDGFR-β signaling is involved in pericyte growth and recruitment. Our observations herein that morphine activates PDGFR-β and MAPK/ERK phosphorylation in pericytes to the same extent as that induced

by PDGF-BB suggest that morphine may increase pericyte recruitment to the endothelium and also increase tumor angiogenesis.

Consistent with morphine-induced secretion of PDGF-BB by HUVEC and activation of PDGFR-β and MAPK/ERK signaling in pericytes, we observed increased angiogenesis in the tumors of C3TAG mice treated with morphine using clinically relevant doses. Morphine-induced tumor angiogenesis is in agreement with morphine-induced angiogenesis *in vitro* and *in vivo* and promotion of breast and lung tumors in mice [18–22]. Morphine-induced angiogenesis was replete with excessive vessel branching and a significantly larger number of vessels, typical of tumor angiogenesis. These data demonstrate that morphine promotes angiogenesis in a breast cancer model, which recapitulates the evolutionary spectrum of human breast cancer. Together, the promotion of angiogenesis and PDGF-BB/PDGFR-β induced endothelial-pericyte interaction promoted by morphine may influence anti-angiogenic therapy.

Increased tumor angiogenesis in morphine-treated mice was accompanied by increased vessel-associated desmin-expressing pericytes, but not α-SMA-expressing pericytes. It is believed that α-SMA is not expressed on pericytes associated with normal capillaries, which express desmin, while vSMCs on arterioles and pericytes on venules express desmin as well as α-SMA [34, 35]. On the other hand, α-SMA is suggested to be a marker of pericytes [36]. Irrespective of the treatment, all tumor sections showed a strong expression of α-SMA on tumor vessels. It is therefore likely that α-SMA is strongly expressed on certain type of vessels in this tumor model, which appear to be similar to arterioles and venules and are not influenced by morphine. Notably, strong desmin immunoreactivity in tumors of morphine-treated mice colocalized with endothelial sprouts and tip cells and in close proximity to endothelial cells. This is an indication of increased pericyte differentiation and recruitment induced by morphine in the vicinity of endothelium. Similarly, cells expressing PDGFR-β increasingly colocalized with vasculature in morphine group and in close vicinity to endothelium, further demonstrating increased pericyte recruitment and vascular coverage. Indeed increased PDGFR-b signaling leads to increased pericyte coverage of the vasculature [14]. Interestingly, in PBS-treated mice, vessel-associated PDGFR-β-expressing pericytes were few, and were sparsely located at vessel branch points and on the vascular sprouts. Appreciably high PDGFR-β expression was observed on nonendothelial cells, perhaps on tumor cells, in PBS-treated mice. Increased microvessel density and thicker PDGFR-β-expressing pericyte coverage were associated with highly metastatic human KM12SM colon cancer cecal tumors in nude mice as compared to low metastatic KM12C cell tumors [37]. Therefore, increased vessel-associated pericyte coverage in tumors of morphine-treated mice in this study complements increased metastases observed by us in subcutaneous SCK breast tumors in *A/J* mice [19]. It is likely that morphine influences tumor angiogenesis, progression, and metastases by stimulating endothelial-pericyte interaction and increased pericyte recruitment and coverage of vasculature, thus increasing resistance to antiangiogenic therapy by limiting the accessibility of drugs to the endothelium on one hand and promoting angiogenesis on the other.

While using anti-angiogenic therapy, contribution of opioids (if coadministered) to the therapeutic outcomes requires consideration. To date, there are no clinical data on the effect of morphine on cancer progression and metastases. However, OR antagonist naltrexone inhibited ovarian cancer progression in mice [38] and improved the outcome of cisplatin therapy in ovarian cancer [39]. Inhibition of advanced nonmetastatic and metastatic pancreatic cancer was also reported in patients receiving low-dose naltrexone with an antioxidant therapy with α-lipoic acid [40]. Morphine-induced PDGF-BB expression in HBMEC was inhibited by naltrexone [15], suggestive of an OR-mediated mechanism. Furthermore, a peripherally only acting MOR antagonist, methylnaltrexone, inhibited opioid-induced angiogenesis [20]. Therefore, coadministration of peripherally acting MOR antagonists that do not compromise morphine analgesia may improve the outcome of anti-angiogenic therapy.

In conclusion, we show that morphine stimulates PDGF-BB secretion by endothelial cells and activates PDGFR-β and MAPK/ERK signaling in pericytes, thus mediating endothelial-pericyte interaction. This cellular activity of morphine correlates with increased angiogenesis replete with pericyte recruitment and coverage of tumor vasculature. Thus, morphine treatment may influence the effectiveness of anti-angiogenic drugs.

Conflict of Interests

The authors declared that there is no conflict of interests.

Authors' Contribution

K. Luk and S. Boatman, performed experiments, quantitative analysis, preparation of figures and wrote the paper. K. N. Johnson performed Western analysis; O. A. Dudek and N. Ristau performed immunostaining; D. Vang assisted with quantitative and statistical analysis; J. Nguyen assisted with cell culture; K. Gupta designed and supervised the entire study and edited the paper. K. Luk and S. Boatman have equally contributed to this work.

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Research Article

Vascular Disrupting Agent Arsenic Trioxide Enhances Thermoradiotherapy of Solid Tumors

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Our previous studies demonstrated arsenic trioxide- (ATO-) induced selective tumor vascular disruption and augmentation of thermal or radiotherapy effect against solid tumors. These results suggested that a trimodality approach of radiation, ATO, and local hyperthermia may have potent therapeutic efficacy against solid tumors. Here, we report the antitumor effect of hypofractionated radiation followed by ATO administration and local 42.5°C hyperthermia and the effects of cisplatin and thermoradiotherapy. We found that the therapeutic efficacy of ATO-based thermoradiotherapy was equal or greater than that of cisplatin-based thermoradiotherapy, and marked evidence of *in vivo* apoptosis and tumor necrosis were observed in ATO-treated tumors. We conclude that ATO-based thermoradiotherapy is a powerful means to control tumor growth by using vascular disruption to augment the effects of thermal and radiation therapy.

1. Introduction

As_2O_3 (ATO) has now been widely studied and used successfully as a therapeutic agent for acute promyelocytic leukemia, as it causes differentiation and apoptosis of leukemic cells [1–6]. This FDA-approved compound has also demonstrated significant potential as a solid tumor anti-vascular and angiogenesis inhibiting agent in preclinical studies. We were among the first groups to discover the potent and selective tumor vascular disruption caused by this agent [7]. Subsequently we have demonstrated significant thermosensitization of tumors and an increase in tumor response to radiation therapy induced by ATO treatment [8–10]. Although there have been numerous reports in the literature, relatively little is clear about the cellular and physiological mechanisms of ATO effect on solid tumors *in vivo*, especially in regard to antivascular effects of the drug and how to use these effects to design effective clinical regimens [7, 11–14].

From our earlier findings, we concluded that selective tumor vascular shutdown caused by ATO induced boldly demarcated necrotic regions. We also observed an increase in mean or median tumor oxygenation values for up to a week after ATO therapy [9, 10, 15]. These studies, and the low toxicity caused by ATO in our experience, suggested that a regimen consisting of radiation, ATO, and local hyperthermia may be more effective than the already clinically used cisplatin-based combination therapy.

Phase III clinical studies using thermoradiotherapy with cisplatin have begun [16]. This clinical trial was expanded to a multicenter, international trial against cervical cancer due to the encouraging initial results. More than 15 different Phase I, II, or III studies to compare the effects of cisplatin/radiation and heat/cisplatin/radiation have been performed in the past 20 years [17]. However, use of ATO, a vascular disrupting agent with a different mechanism of action than conventional cisplatin therapy and which may be effective in patients resistant or insensitive to platinum-based

regimens, could be a better candidate for the treatment of solid tumors. The possible advantages of a tumor and stromal- (i.e., vascular-) targeted multimodality approach to improved cancer treatment are discussed in the context of using ATO or similar vascular disrupting agents to sensitize the tumor to thermoradiotherapy.

2. Materials and Methods

2.1. Cell Line

FSaII Tumor. This fibrosarcoma of C3H mice (Jackson Laboratories) was originally obtained from Dr. Herman Suit (Massachusetts General Hospital). Stock cells are stored in liquid nitrogen, and new cultures are established every 2-3 months. FSaII tumor cells grow well in RPMI-1640 medium supplemented with 10% bovine calf serum.

2.2. Tumor Induction. FSaII tumor cells in exponential growth phase were harvested using 0.25% trypsin in Hepes-buffered medium, washed, and counted. A subcutaneous injection of 2×10^5 cells in 0.05 mL serum-free medium was made in the hind thigh of female C3H mice.

2.3. Arsenic Trioxide. An i.p. injection of 4–8 mg/kg arsenic trioxide (ATO or Trisenox^{TM}, Cephalon Oncology, Inc., Frazer, Pa, USA) was performed by using a clinical grade 1 mg/mL stock solution for each mouse, and imaging or treatment was performed at specific times after this injection. Control mice were injected with an equal volume of phosphate-buffered saline, pH 7.4.

2.4. Cisplatin. Doses of 2 or 6 mg/kg cisplatin (Sigma-Aldrich, St. Louis, Mo, USA) were administered i.p. in saline at the frequency indicated for each of the studies comparing to ATO treatment.

2.5. Window Chamber Tumor Growth and Intravital Microscopy. Skin-fold chambers made of anodized aluminum frames were surgically implanted into a fold of dorsal skin in female nu/nu mice. Briefly, the dorsal skin was sandwiched between two identical anodized round aluminum frames. The 19 mm $\times$ 22 mm chamber was held fixed on the mouse by three screws between the frames. The skin was also attached to the chamber with 4-O silk. The skin on both sides of the viewing region was removed, exposing the dermis containing the microvasculature. Excess fascia on the dermis was removed to assist clear visualization of the microvasculature. Windows milled from quartz glass microslides (Chase Scientific Glass, Rockwood, Tenn, USA) were used to cover the vascular area. The distance between the windows was maintained at 450 μm by a spacing gasket on the inside of the frames, leaving room for seeded tumor to grow. 1-2 $\times$ 10^6 tumor cells were added in 30 uL of matrigel just before placement of the glass windows. Treatments and imaging were performed over the course of tumor growth and treatment as described [18].

2.6. FLIVO Reagent. FLIVO (FAM-VAD-FMK, 50 μg per vial, Immunochemistry Technologies, LLC, Bloomington, Minn, USA) was dissolved in 50 μL of DMSO and diluted by the addition of 200 μL of sterile PBS, pH 7.4. At 30 min after an i.v. injection of 0.1 mL of FLIVO cell permeant probe via the lateral tail vein, fluorescent images were captured at 20X using a Hamamatsu C2400 camera (Hamamatsu, Japan) and Broadway Imaging Software (Data Translation, Marlboro, Mass, USA) on an Eclipse TE200 bench-top microscope (Nikon, Japan).

2.7. Histological Analysis. Tumor-bearing mice were sacrificed, and the tumor was removed at the specified time point after treatment. The tissue was fixed in 10% neutral buffered formalin, and, after processing and embedding, tissue sections at 5 μM were prepared and stained with hematoxylin and eosin. An Olympus BX40 microscope was used to image multiple fields at 20x magnification which were then fit together using Adobe Photoshop.

2.8. X-Irradiation. Tumors were locally irradiated with 5 Gy per fraction by a Philips 250 Kv X-ray machine at a dose rate of 1.4 Gy/min. The body was shielded with lead with only the tumor and foot exposed to the X-ray beam.

2.9. Hyperthermia. Tumors were heated by immersing the tumor-bearing legs of anesthetized mice into preheated water for 60 min as described previously [8]. The water temperature and the temperature of tissues were routinely measured with needle-type (29 gauge) thermocouples. The thermoprofiles in tumors and normal tissues during water-bath heating have been thoroughly studied in our group [8]. The temperature in the tumors during water bath heating is consistently 0.3–0.5°C below the water temperature. All temperatures quoted refer to the water temperature during hyperthermia treatment.

2.10. Assessment of Tumor Growth. Tumor volume was measured with a caliper (Scienceware) and calculated according to the equation: $(a^2 \times b)/2$, where "a" is the width and "b" the length of the tumor.

3. Results

As shown in Figure 1(a) cisplatin at 2 mg/kg i.p. and Figure 1(b) ATO at 8 mg/kg i.p. given every 4 days for a total of three treatments were able to significantly improve the antitumor effect of radiation combined with hyperthermia against FSaII tumors. The single modalities were only slightly effective, and the dual-modality combinations further improved the tumor growth delay, but the trimodality treatments were clearly the most effective in delaying tumor growth, especially in the case of ATO where an approximate 2-fold increase in growth delay compared to any other treatment occurred ($P < 0.001$ by day 8 of therapy onward). Cisplatin-based thermoradiotherapy was only significantly different from the dual-modality treatments by day 16 after start of treatment ($P = 0.03$).

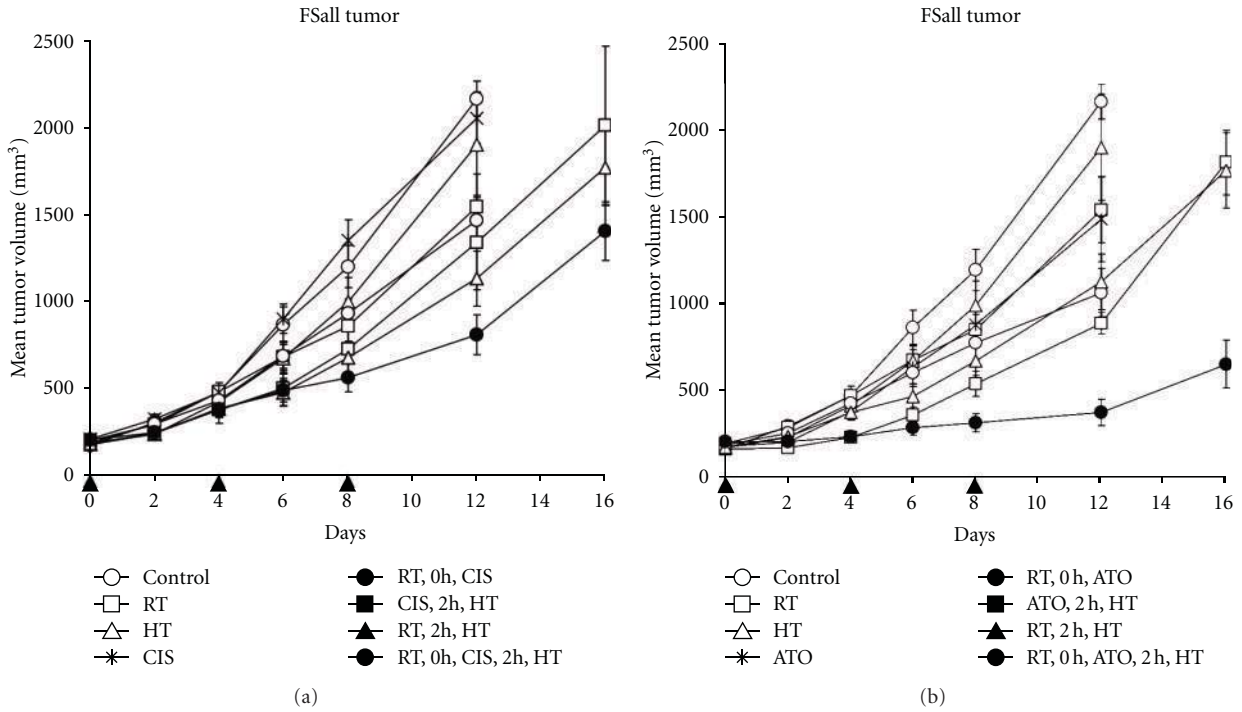

Figure 1: Comparison of arsenic trioxide and cisplatin enhanced thermoradiotherapy of FSaII fibrosarcoma tumors applied every 4 days as indicated by the arrows on the x-axis. Cisplatin was given at 2 mg/kg, while ATO was given at 8 mg/kg. Radiation fractions were 5 Gy, and hyperthermia was applied at 42.5°C (60 min). In the dual- or trimodality treatment groups, radiation was given first, followed by ATO or cisplatin injection within 30 min and hyperthermia was applied beginning 2 h after the completion of radiation. N = 5–7 mice per group. Errors bars represent 1 SEM.

In order to more definitively understand the potential of ATO as a part of a trimodality regimen, we did a subsequent study using an equimolar amount of cisplatin or ATO (4 mg/kg ATO MW: 198 versus 6 mg/kg cisplatin MW: 300, resp.). In this study, ATO was again found to be more effective than cisplatin in augmenting the effects of radiation and hyperthermia on tumor inhibition ($P = 0.02$ versus cisplatin-based therapy by day 10 after start of treatment). As shown in Figure 2, ATO maximally increased tumor growth delay by a factor of 2.5-fold as compared to control tumor growth, while cisplatin increased the tumor growth delay response by a factor of about 2-fold on average compared to control tumors in this group of animals.

A typical composite image of the entire tumor gross section from an untreated tumor and from tumors treated with either cisplatin-based or ATO-based trimodality therapy is shown in Figure 2 (inset). The effects of repeated radiation, arsenic trioxide, and heat treatments are clearly evident in the marked rings of live and dead regions of tissue in the ATO-treated mouse, while the cisplatin-based therapy indicates there were more widespread regions of viable tumor remaining after treatment and the control tumor shows homogenous staining of live cells with very little architectural variation. The images shown are the typical results obtained from groups of 3 control or trimodality-treated tumors that were prepared for histology and studied.

We subsequently investigated the *in vivo* cellular and physiological mechanisms by which ATO selectively damages solid tumor and increases the effect of hyperthermia and radiation. We employed the dorsal skin-fold window chamber (DSFC) technique for intravital imaging of tumor tissue [18–21] to visualize real-time changes in tumor physiology and vascularity. As shown in Figure 3, ATO causes substantial and noticeable vascular damage in relatively large areas of the tumor by 2 h after i.p. injection of tumor bearing mice. A fluorescent pan-caspase probe (FLICA or FLIVO Poly-(pan) caspase detection) was used for *in vivo* assessment of cell death and viability, as we previously reported [21]. Figure 4 shows changes in the amount of apoptosis occurring in FSaII tumors grown in the window chamber in control tumors, 2 h after a single dose of 8 mg/kg ATO i.p., 2 h after 5 Gy followed by 30 min of 42.5°C heating or 2 h after 5 Gy and 8 mg/kg ATO i.p. followed by 42.5°C heating, as detected by the polycaspase inhibitor. There appeared to be increased apoptotic activity in the representative regions of the tumor in the mouse treated with ATO alone as well as the mouse that received trimodality treatment compared to control tumor or tumor treated with heat and radiation. An intensity plot was made for each image (Figure 4), and the overall mean pixel intensity was calculated for each group. Upon statistical analysis of the mean intensity values, the trimodality mean pixel intensity was found to be significantly increased compared to control, ATO alone, or heat combined with radiation exposure ($P < 0.03$). ATO treatment alone appeared to visually cause more apoptosis than that in tumor

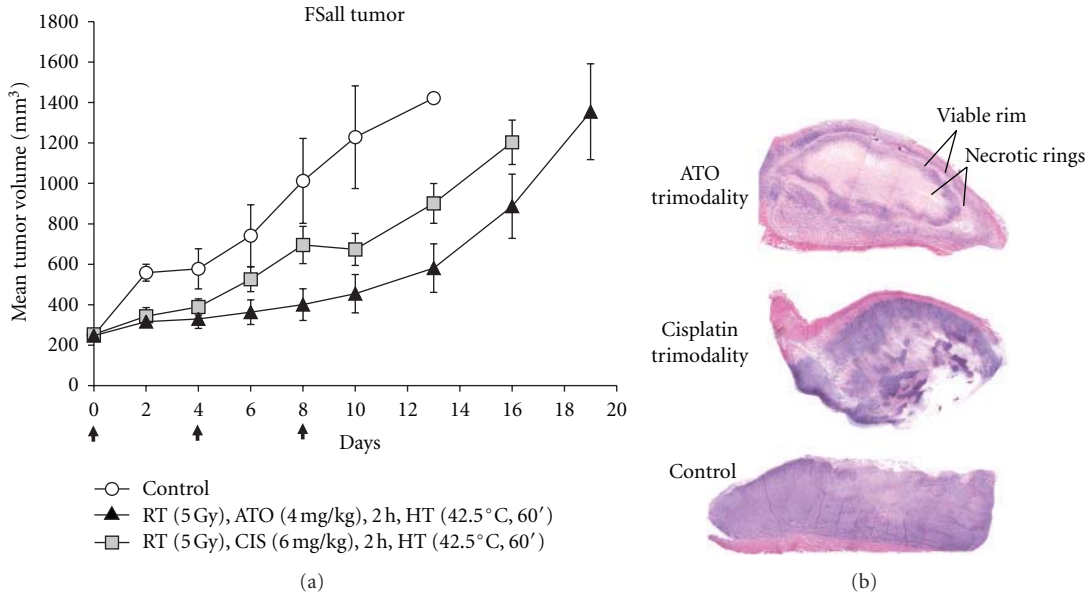

Figure 2: Equimolar comparison of arsenic trioxide and cisplatin as part of a recurring trimodality regimen every 4 days against FSaII fibrosarcoma tumors. As in the previous figure, in the dual- or trimodality treatment groups, radiation was given first, followed by ATO or cisplatin injection within 30 min and hyperthermia was applied beginning 2 h after the completion of radiation. $N = 6$–8 mice per group. Error bars represent 1 SEM. INSET: histological evidence of antitumor effects of repeated trimodality therapy applied every 4 days, 3 times in total in FSaII tumors (tumors taken from treatment groups on the last day of measurement) compared to control tumor.

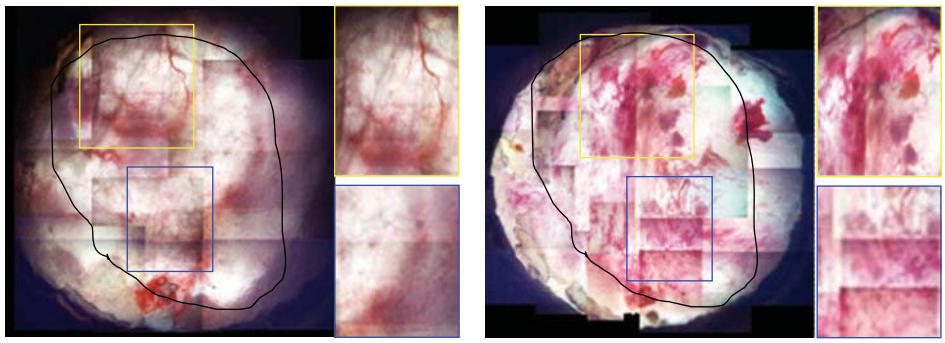

Figure 3: Representative window chamber imaging of 8 mg/kg i.p. arsenic trioxide-induced vascular damage in FSaII fibrosarcoma grown in nude mice. FSaII tumor was implanted and 7 days later the tumor was imaged before and 2 h after an i.p. injection of 8 mg/kg ATO. The tumor is outlined in black and the boxes have been placed in the areas where typical marked evidence of vascular damage occurred.

treated with heat combined with radiation or in control tumor, but this was not found to be statistically significant.

4. Discussion

The results of the current study suggest that fractionated radiation in concert with arsenic trioxide and clinically achievable thermal doses is a viable option against tumors that may not be adequately treated by mono- or dual-therapy regimens or may be insensitive to conventional cisplatin-based chemotherapy. Our results agree with a number of other intriguing studies employing a variety of trimodality approaches, some with other antivascular or antiangiogenic agents [22–30]. In view of these results and the clinical benefit obtained with trimodality approaches involving more traditional therapeutics [16, 17, 26, 31, 32], continued development and implementation of this treatment strategy appears to be promising.

Many previously studied triple-combination regimens do not include hyperthermia, but instead pair an antiangiogenic or other biological targeting agent with more standard chemotherapy and radiation [25, 33]. These type of studies are intriguing, but it should be noted that they are quite different from studies employing thermal therapy since the

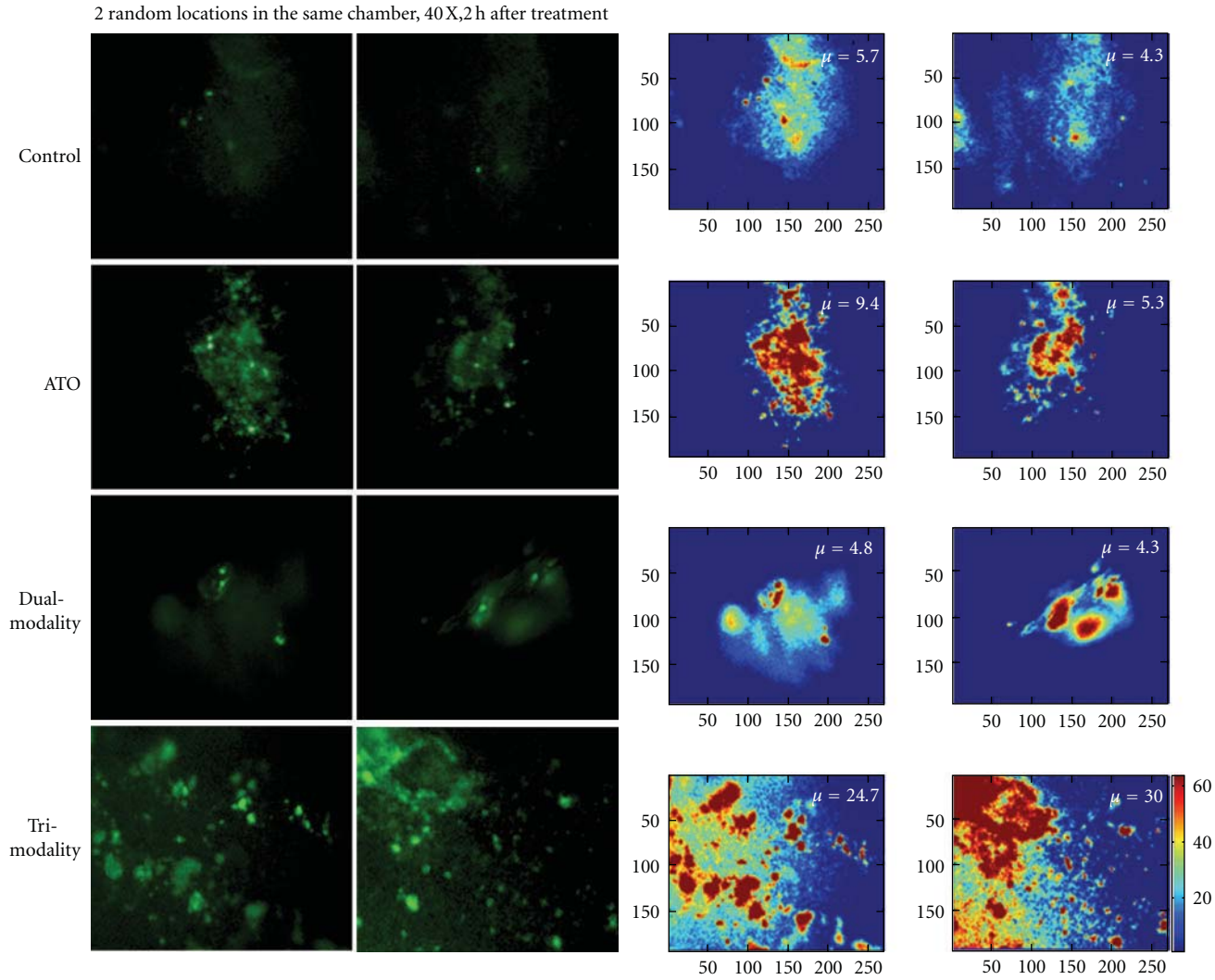

Figure 4: ATO, heat, and radiation induce apoptotic activity by 2 h after combined treatments in tumor of treated mouse. *In vivo* imaging of polycaspase inhibitor in FSaII tumor grown in the window chamber and treated with 8 mg/kg arsenic trioxide, thermoradiotherapy (dual-modality, 5 Gy followed by 42.5°C (30 min) 2 h later), or arsenic trioxide combined with thermoradiotherapy (trimodality; 5 Gy, 8 mg/kg ATO, 2 h, 42.5°C (30 min). The right insets are intensity plots of the images on the left, created in Matlab. μ: mean pixel intensity for all pixels in each image. The color bar represents the relative intensities of individual pixels.

mechanism of action and rationale for sequencing will likely be very different for each strategy. Work with the vascular-targeting agent combretastatin has been highly encouraging and supports the intelligent use of agents that target the tumor vasculature in heat-based multimodality regimens [29, 30, 34, 35]. However, the authors of these preclinical and clinical studies all highlight the critical need for well-controlled clinical trials where the sequencing of the agents is of utmost importance. These studies and our rationally designed study based on known synergism of ATO, radiation, and hyperthermia strongly suggest that careful attention to how treatments are combined will maximize the benefits for patients.

Our earlier work had demonstrated enhancement of radiation-induced growth delay when ATO was given before or after radiation. However, since we knew that tumor blood flow was rapidly reduced or abolished in certain regions of the tumor upon ATO administration, it appeared logical to avoid induction of hypoxia before radiation by administering ATO after each radiation fraction. The application of heat to the tumor was elected to be after the radiation and the ATO injection for two reasons. First, although there may be a benefit in increasing the tumor oxygenation and thus the tumor radiosensitivity when heat is applied prior to radiation, we had observed a more significant thermosensitization when heat was applied after ATO injection at the point of greatest blood flow shutdown. Secondly, it is well known that radiation damage repair is inhibited by heating after radiation exposure. Therefore, the combination of ATO-induced tumor and endothelial thermosensitivity and potential inhibition of DNA damage repair were expected to cause the greatest improvement in

radiation-induced tumor growth delay. Indeed, the tumor growth was suspended in some animals during the period of therapy, suggesting that continued therapy may even obtain tumor cures using this multimodality approach. It is possible that other sequences and/or frequencies of treatment may be even more potent in these models. The important result is that arsenic trioxide, as a novel vascular disrupting agent, can be effectively added to a thermoradiotherapy regimen that may have certain advantages and synergy compared to regimens employing traditional chemotherapy.

We further studied the effects of ATO alone or in combination with heat and radiation therapy on tumor blood flow and apoptosis *in vivo* by employing intravital microscopy and histological analysis. A current focus of our group is to delineate the kinetics of vascular cell apoptosis and tumor cell apoptosis after these treatments. Ultimately, apoptosis detection *in vivo* could be used as a surrogate marker of treatment response in clinical situations. Overall, we have clearly demonstrated here, and previously, that ATO can be a powerful radiation- or thermosensitizing agent [8, 9]. Others have also recently found significant effects of ATO therapy on the tumor control obtained by thermal ablation or fractionated radiation therapy [11, 36]. Newer agents based on trivalent arsenicals that may selectively target tumor endothelium have reached clinical trials, and; thus, there is reason to believe that acceptable clinical doses of arsenic-based therapy would be achievable in the clinic and/or better delivery strategies will be available and contribute to positive therapeutic outcomes [13, 37, 38].

Equal or better antitumor activity of arsenic trioxide-based thermoradiotherapy was observed in our study compared with a cisplatin-based regimen. The study with cisplatin at equimolar ratio to ATO (Figure 2) was particularly enlightening in that it allows us to hypothesize that ATO has the potential to improve upon currently employed trimodality therapy against cervical cancer. Out of the several antivascular strategies currently being tested either preclinically or in clinical trials, it seems likely that at least one of these agents will become a realistic option to the clinician wanting to maximize the effects of a thermoradiotherapy treatment plan.

5. Conclusions

Clinical trial development of an arsenical-based trimodality strategy may be warranted, especially in view of the wealth of clinical experience already accumulated with arsenic against leukemia, lymphoma, and other solid tumors. The continued success of several radiation-, chemotherapy- and hyperthermia-based trials suggest that there is much to be gained with agents that selectively target and disrupt solid tumor vasculature and angiogenic capability with different, yet complementary, mechanisms of action such as ATO, thermal therapy, and radiation therapy.

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Research Article

Dietary Restriction Promotes Vessel Maturation in a Mouse Astrocytoma

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Mature vasculature contains an endothelial cell lining with a surrounding sheath of pericytes/vascular smooth muscle cells (VSMCs). Tumor vessels are immature and lack a pericyte sheath. Colocalization of vascular endothelial growth factor receptor 2 (VEGFR-2) and platelet-derived growth factor receptor beta (PDGF-Rβ) reduces pericyte ensheathment of tumor vessels. We found that a 30% dietary restriction (DR) enhanced vessel maturation in the mouse CT-2A astrocytoma. DR reduced microvessel density and VEGF expression in the astrocytoma, while increasing recruitment of pericytes, positive for alpha-smooth muscle actin (α-SMA). Moreover, DR reduced colocalization of VEGF-R2 and PDGF-Rβ, but did not reduce total PDGF-Rβ expression. These findings suggest that DR promoted vessel normalization by preventing VEGF-induced inhibition of the PDGF signaling axis in pericytes. DR appears to shift the tumor vasculature from a leaky immature state to a more mature state. We suggest that vessel normalization could improve delivery of therapeutic drugs to brain tumors.

1. Introduction

Tumor vascularization, vital to neoplastic progression, provides nutrients and oxygen to the tumor [1–3]. Proliferation of vessel-forming endothelial cells is a limiting factor for tumor growth [4–7]. Accordingly, targeting tumor vessel proliferation decreases blood flow and nutrient availability, thus slowing tumor growth [8]. Tumors induce the proliferative vascular response of host blood vessels by influencing the local balance of angiogenic regulators, a rate-limiting step termed the angiogenic switch [9, 10]. The uncontrolled production of angiogenic stimulators and the absence of inhibitors favor vessel growth [10–12].

Normal tissue vasculature contains an endothelial lining with a surrounding sheath of pericytes/vascular smooth muscle cells (VSMCs) [13]. In contrast to healthy vessels, tumor vessels are immature, often mal-shaped, irregular, and have a tortuous structure with a leaky endothelial cell lining [13, 14]. The process of blood vessel maturation involves ensheathment of neovascular sprouts by α-smooth-muscle-actin- (α-SMA-) positive pericytes [15]. Pericytes contact endothelial cells and play an active role in endothelial cell function and blood flow regulation [15–17]. Mature vessels contain a variety of contractile proteins including α-SMA, which is often used as a pericyte marker [15, 18, 19].

The instability of tumor blood vessels is associated with the absence of a smooth muscle cell sheath [11]. Abnormalities in tumor vessel shape and structure not only impair drug delivery, but also can facilitate metastatic spread [20, 21]. While it may seem that an increase in blood vessel quantity would provide sufficient oxygen to tumors, the abnormal vessels deliver less oxygen leading to a hypoxic tumor environment [13]. This will further stimulate tumor growth and aberrant angiogenesis [22, 23]. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) signaling drives angiogenesis and recruitment of perivascular cells to surround the newly formed blood vessels [24]. VEGF stimulates endothelial cell migration, proliferation, survival, permeability, and lumen formation and has become a prime target of antiangiogenic therapy [13]. Blockage of VEGF signaling induces vessel normalization and inhibition of new vessel growth (16). In addition to the pruning of immature blood vessels, inhibition of VEGF expression also increases pericyte cell coverage and vessel maturation [25, 26].

Platelet-derived growth factor (PDGF) coordinates pericyte coverage of vascular sprouts through PDGF-Rβ on vascular smooth muscle cells [27]. Greenberg et al. showed that, in addition to stimulating endothelial cell proliferation, VEGF also inhibits neovascularization via its capacity to disrupt vascular smooth muscle cell function [24]. Specifically, VEGF prevents pericyte coverage of nascent vascular sprouts leading to vessel destabilization. VEGF activation of VEGF-R2 suppresses PDGF-Rβ signaling in VSMCs through the assembly of a complex consisting of the two receptors. Inhibition of VEGF-R2 prevents the formation of this receptor complex and restores tissue angiogenesis. Moreover, genetic deletion of tumor cell VEGF also disrupts the receptor complex and consequently increases tumor vessel maturation. These findings are important as they reveal a dichotomous role for VEGF signaling as a promoter of endothelial cell function and as an inhibitor of VSMCs and vessel maturation [24, 26, 28, 29].

VEGF expression is greater in tumor cells than in normal cells [30–33]. Reduced VEGF expression reduces angiogenesis while increasing vessel maturation [24]. Mukherjee et al. demonstrated that a 30% dietary restriction (DR) inhibits angiogenesis and reduces prostate tumor growth [34]. We showed that DR in mice reduces microvessel density in experimental mouse and human brain tumors [35, 36]. Powolny et al. demonstrated that DR attenuates tumor growth and reduces vascular density. They also found that a 40% DR significantly reduced VEGF gene and protein expression in rat prostate tumors [37]. These studies show that DR is a potentially viable nontoxic therapeutic approach for managing malignant brain tumor growth, for reducing tumor angiogenesis, and for increasing long-term survival in mice bearing orthotopically implanted tumors [35–38].

DR is produced by restricting the total caloric content administered to subjects. However, a distinction from starvation is that DR does not cause anorexia or malnutrition [34, 39–42]. It is important to note that the total reduction of calories, rather than the macronutritional content of the food, proves most important to producing the effects of reducing tumor growth and in limiting angiogenesis [34, 39]. Although prior studies showed that dietary restriction is antiangiogenic when initiated early in tumor development, no prior studies have identified the mechanisms by which dietary restriction is effective in correcting vasculature.

In this paper, we show that DR enhances vessel maturation and stabilization in the highly vascularized CT-2A mouse astrocytoma. In addition to reducing VEGF expression, we also found that DR decreased colocalization of VEGF-R2 with PDGF-Rβ. Our findings suggest that DR imparts its antiangiogenic and vessel maturating effects on the CT-2A tumor via the reduction of VEGF expression promoting VSMC ensheathment of vascular sprouts.

2. Materials and Methods

2.1. Mice. Mice of the C57BL/6J strain were obtained from the Jackson laboratory (Bar Harbor, ME, USA). The mice were propagated in the animal care facility of the Biology Department of Boston College, using animal husbandry conditions described previously [43]. Male mice (8–10 weeks of age) were used for the studies and were provided with food either ad libitum (AL) or under restricted conditions (as described below). Water was provided ad libitum to all mice. The animal room was maintained at 22°C, and cotton nesting pads were provided for additional warmth. All animal experiments were carried out with ethical committee approval in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Care Committee.

2.2. Brain Tumor Model. The syngeneic CT-2A experimental mouse brain tumor was generated in our laboratory after implantation of 20-methylcholanthrene into the cerebral cortex of a C57BL/6 mouse according to the procedure of Zimmerman [45, 46]. Histologically, the CT-2A brain tumor is broadly classified as a poorly differentiated highly malignant anaplastic astrocytoma [46]. The tumor grows orthotopically as a soft, noncohesive, and highly vascularised mass.

2.3. Intracerebral Tumor Implantation. The CT-2A tumor was implanted into the cerebral cortex of C57BL/6J mice using a trocar as we previously described [47, 48]. Briefly, mice were anaesthetized with pentobarbital (Vet Labs, Inc) intraperitoneally and their heads were shaved and swabbed with 70% ethyl alcohol under sterile conditions. Small CT-2A tumor pieces (1 mm^3) from a C57BL/6J donor mouse were implanted into the right cerebral hemisphere of anaesthetized recipient mice as we recently described [48]. All of the mice recovered from the surgical procedure and were returned to their cages when fully active. Initiation of tumors from intact tumor pieces is preferable to initiation from cultured cells since the pieces already contain an established microenvironment that facilitates tumor growth.

2.4. Dietary Restriction (DR). The mice were separated prior to the beginning of the experiment and randomly assigned to either control group that was fed AL or to an experimental group that was fed a total dietary restriction (DR) of 30%. Each mouse was housed singly in a plastic shoe box cage with a filter top and was given a cotton nesting pad for warmth. DR was initiated 2 days following tumor implantation and was continued for 11 days following implantation. Total DR maintains a constant ratio of nutrients to energy; that is, the average daily food intake (grams) for the AL fed mice was determined every other day and the DR-fed mice were given 70% of that quantity on a daily basis. All mice received PROLAB chow (Agaway Inc.), which contains a balance of mouse nutritional ingredients and, according to the manufacturer's specification, delivers 4.4 Kcal/g gross energy. Body weights of all mice were recorded every other day.

2.5. Tumor Growth and Histology. Intracerebral tumor growth was analyzed directly by measuring total tumor wet weight. Tumors were dissected from normal-appearing brain

tissue, were frozen, and then were weighed. Tumor samples for histology were fixed in 10% neutral buffered formalin (Sigma) and embedded in paraffin. They were sectioned at 5 μm, stained with haematoxylin and eosin, and examined by light microscopy.

2.6. Measurement of Plasma Glucose and Lipids. Mice were anesthetized with isoflurane (Halocarbon Laboratories, River Edge, NJ, USA) and euthanized by exsanguination, involving collection of blood from the heart in heparinized tubes. The blood was centrifuged at 6,000 g for 10 min, the plasma was collected, and aliquots were stored at −80°C until analysis. Plasma glucose concentration was measured in a spectrophotometer using the Stanbio Enzymatic Glucose Procedure (Stanbio). High-performance thin-layer chromatography was used to evaluate plasma lipids according to our standard procedures [49].

2.7. Western Blot Analysis. Frozen CT-2A tumor and contralateral normal brain tissues were homogenized in lysis buffer, on ice. Lysates were transferred to Eppendorf tubes, mixed on a rocker for 1 h at 4°C, and then centrifuged for 20 min. Supernatants were collected, and protein concentrations were estimated using the Bio-Rad detergent-compatible protein assay. Approximately 25 μg of total protein from each tissue sample were denatured with SDS-PAGE sample buffer and resolved with SDS-PAGE on 4% to 12% Bis-Tris gels (Invitrogen). Proteins were transferred to a polyvinylidene difluoride immobilon TM-P membrane (Millipore) overnight at 4°C and blocked in 5% nonfat powdered milk in TBS with tween 20 (pH 7.6) for 1 h at room temperature. Membranes were probed with primary antibodies overnight at 4°C with gentle shaking. The blots were then incubated with the appropriate secondary antibody for 1 h at room temperature, and bands were visualized with chemiluminescence. Each membrane was stripped and reprobed for β-actin as an internal loading control, and the ratio of the indicated protein to β-actin was analyzed by scanning densitometry.

2.8. Antibodies and Reagents. Antibodies were obtained against α-SMA (Sigma), β-Actin (Cell Signaling), Factor VIII (Dako), PDGF-Rβ (Santa Cruz), VEGF-R2 (Santa Cruz), and VEGF (Santa Cruz).

2.9. Confocal Microscopy. For the immunohistochemical studies, the tissue sections were deparaffinized, rehydrated, and washed. The tissue sections were then heat treated (95°C) in antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for 30 min. Tissue sections were blocked in goat serum (1 : 10 in PBS) for 1 h at room temperature, treated with primary antibody, followed by treatment with secondary antibody. Corresponding tissue sections without primary antibody served as negative controls. For confocal microscopy, digital images were obtained on a Leica DMI6000 inverted scope equipped with the Leica TCSSP5 confocal system, using HCX PL APO 409/1.25 NA oil and HCX PL APO 639/1.4 NA oil objective lenses. Leica confocal software was used to acquire images.

For α-SMA and Factor VIII immunoflourscent staining, sections were incubated with a cocktail of α-SMA and Factor VIII primary antibodies (1 : 100) in blocking buffer for 1 h at room temperature, followed by a cocktail of alexafluor 585 and 488, conjugated anti-mouse and anti-rabbit, respectively, secondary antibody (1 : 200) for 45 min at room temperature.

For colocalization of VEGF-R2 and PDGF-Rβ, sections were incubated with a cocktail of VEGF-R2 and PDGF-R2 primary antibodies (1 : 100) in blocking buffer overnight at 4°C, followed by a cocktail of alexafluor 585 and 488, conjugated anti-mouse and anti-rabbit, respectively, secondary antibody at 1 : 200 dilution for 45 min. All other conditions were as stated.

2.10. Immunohistochemistry. Tissue sections were processed similarly as for confocal microscopy. Sections were treated with VEGF, PDGF-Rβ, and Factor VIII primary antibodies overnight at 4°C followed by treatment with a biotinylated anti-rat secondary antibody at 1 : 100 dilution (Vector laboratories, Inc). The sections were then treated with avidin biotin complex followed by 3,3-diaminobenzidine as substrate for staining according to the manufacturer's protocol (Vectastain Elite ABC kit, Vector laboratories, Inc.). The sections were counterstained with haematoxylin and mounted. Corresponding tissue sections without primary antibody served as negative controls. The Zeiss Axioplan 2 light microscope was used to capture bright-field images.

3. Results

3.1. Dietary Restriction Reduces Body Weight, Blood Glucose, and Intracerebral Tumor Growth. The DR group exhibited an average body weight reduction of 22 ± 1% and an average tumor reduction of 76 ± 4%. Average tumor wet weight was significantly lower in the DR-fed mice (51 ± 7 mg) than in the AL-fed mice (209 ± 40 mg); $P < 0.01$, Student's t-test. Blood glucose levels (mmol/L) in the AL and DR mice were 8.6 ± 0.6 and 4.4 ± 1.0, respectively ($P < 0.05$, determined by two-tailed t-test). The blood glucose levels were reduced in the DR mice as we previously showed [36, 50]. No significant differences were detected between the AL and DR mice for the distribution of major lipids including cholesteryl esters, cholesterol, triglycerides, or phosphatidylcholine (data not shown). Mouse activity level increased under dietary energy restriction. This is a well-documented phenomenon that occurs in all mice when placed under calorie restriction and is due to increased foraging [51]. It is difficult to determine if increased physical activity causes psychological stress. It is well documented that general health and fitness improves significantly in mice when they are underfed with adequate nutrition [51, 52]. It is important to note that all tumors implanted grew in both the DR and AL groups, indicating that dietary restriction did not inhibit tumor take.

This study confirms previous observations that DR inhibits CT-2A tumor growth [36].

3.2. Dietary Restriction Reduces Microvessel Density and Hemorrhaging in the CT-2A Astrocytoma. H&E staining was used to evaluate the influence of DR on hemorrhagic blood vessels in CT-2A (Figure 1(a)). Light pink staining indicates normal brain tissue. Bright pink staining found within the CT-2A tumor tissue indicates hemorrhagic vasculature. The number of hemorrhagic vessels was noticeably less in tumors of DR-fed mice than in tumors of AL-fed mice. Factor VIII immunohistochemistry was used to evaluate the influence of DR on the density of vascular endothelial cells. The number of Factor-VIII-stained endothelial cells was noticeably less in sections of tumors from DR-fed mice than from AL-fed mice, indicating a reduction of microvessel density (Figures 1(b) and 1(c)).

3.3. Dietary Restriction Increases Maturation of Blood Vessels in the CT-2A Astrocytoma. Confocal microscopy was used to determine the influence of DR on localization of α-SMA and Factor VIII in blood vessels of CT-2A (Figure 2). α-SMA (red) was used as a marker for vascular smooth muscle cell (VSMC)/perictye coverage of blood vessels, and Factor VIII (green) was used as a marker for endothelial cells. Localization of VSMCs (red) with the endothelial cell lining (green) was greater in tumor vessels of DR-fed mice than in tumor vessels of AL-fed mice. These findings suggest that DR enhances VSMC/perictye coverage of CT-2A tumor vessels.

3.4. Dietary Restriction Increases α-SMA Expression and Reduces Factor VIII Expression in the CT-2A Astrocytoma. Western blot analysis was done to examine the effects of DR on the relative expression of α-SMA and Factor VIII in tumor blood vessels. Factor VIII expression was significantly lower while α-SMA expression was significantly higher in the CT-2A tumor when grown in DR-fed mice than when grown in AL-fed mice (Figure 3). The ratio of α-SMA to Factor VIII was significantly greater in the tumors of the DR mice as compared to the AL mice (Figure 3). An increase of this ratio in DR mice as compared to the AL group indicates a reduction of endothelial cell proliferation and a simultaneous increase of pericyte/VSMC vessel coverage.

3.5. Dietary Restriction Reduces VEGF Expression in the CT-2A Astrocytoma. Immunohistochemistry was used to determine the influence of dietary restriction on local VEGF expression in CT-2A. The brown VEGF staining intensity and quantity was noticeably less in tumors of DR-fed mice than in tumors of AL-fed mice (Figure 4). These findings are consistent with previous findings in plasma indicating that DR reduces VEGF expression [35, 36].

3.6. Dietary Restriction Reduces PDGF-Rβ and VEGF-R2 Association in the CT-2A Astrocytoma. Confocal microscopy showed that the amount of yellow staining, indicative of colocalization of PDGF-Rβ (red) and VEGF-R2 (green), was less in DR-fed mice compared to tumors of AL-fed mice (Figure 5(a)). Western blot analysis showed that PDGF-Rβ expression was similar in tumors of DR-fed and AL-fed mice (Figure 5(b)).

4. Discussion

We found for the first time that DR could enhance tumor blood vessel maturation in a malignant mouse astrocytoma. DR not only curtailed angiogenesis, but also increased vessel pericyte ensheathment in the highly vascularized CT-2A astrocytoma. DR reduced endothelial cell proliferation, as indicated by a reduction in staining for Factor VIII, a marker for endothelial cells. We also observed an increase of pericyte and vascular smooth muscle cell coverage of the endothelial cell lining in blood vessels, as indicated by an increase of α-SMA, a marker for VSMCs and VSMC-like pericytes. This was apparent from the increased ratio of α-SMA relative to Factor VIII. Our findings agree with the previously documented antiangiogenic effects of DR [34–36, 53]. Immunostaining of tumor sections with VEGF antibody showed an overall reduction of VEGF expression in DR-treated tumors. We suggest that the observed reduction in VEGF leads to the antiangiogenic and vessel maturating effects, via the VEGF-VEGF-R2 signaling axis.

The VEGF-VEGF-R2 signaling axis is a primary pathway in endothelial cell proliferation [54]. Moreover, Greenberg et al. implicated VEGF in a dichotomous role. Apart from acting as a promoter of endothelial cell function, VEGF also acts as a negative regulator of VSMCs and consequently vessel maturation [24]. We observed that DR reduced colocalization of VEGF-R2 and PDGF-Rβ in the CT-2A tumor. We also found that DR had no significant influence on PDGF-Rβ expression. These findings suggest that DR blocks the association of VEGF-R2 and PDGF-Rβ by reducing VEGF, thus preventing inhibition of the PDGF signaling axis due to colocolization of the two receptors [24]. Further studies will be needed to evaluate these signaling pathways.

An immature and leaky vasculature is a hallmark of solid tumors [55–57]. The leakiness of the tumor vasculature leads to elevated interstitial fluid pressure within the tumor. Tong et al. demonstrated that increased hydrostatic pressure within tumors hinders drug penetration across tumor vessels [58]. They also showed that penetration of large molecules into tumors is better through vessels with uniform pericyte coverage than through vessels with irregular pericyte coverage. We suggest that DR may improve drug delivery to the tumor via a similar mechanism. Denny et al. found that restriction of a ketogenic diet improved delivery of a small drug molecule into the mouse brain. They found that brain N-butyldeoxynojirmycin (NB-DNJ) content was 3.5-fold greater in the restricted ketogenic diet + NB-DNJ mice than in the NB-DNJ group alone suggesting that DR enhances delivery of the drug to the brain. This could allow for a lower dosing to achieve therapeutic effect [59]. Further research on the effects of dietary restriction on the brain vasculature is necessary to elucidate the mechanism by which diet and DR enhance drug delivery to the brain.

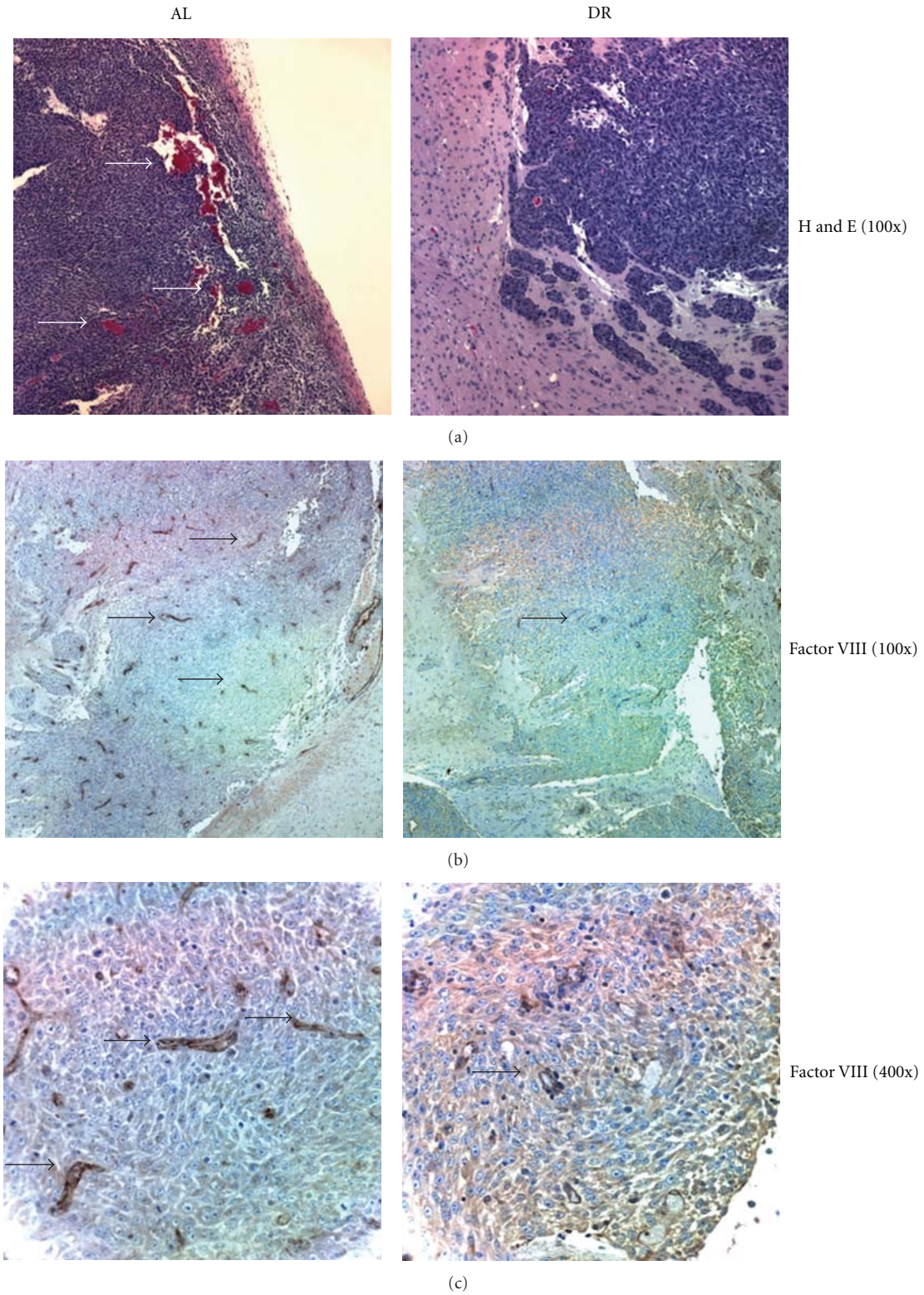

Figure 1: Dietary restriction reduces microvessel density and hemorrhaging in the CT-2A astrocytoma. (a) Vessel morphology. Arrows indicate hemorrhagenic regions. (b) Microvessel density. (c) Higher magnification. Arrows indicate positive Factor VIII vessel staining. Each stained section was representative of the entire tumor. All images were produced from digital photography.

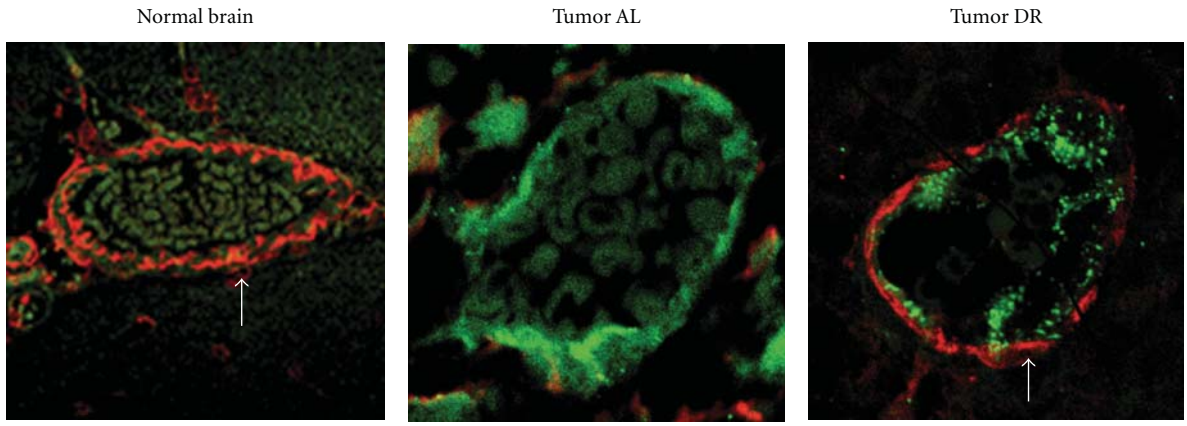

Figure 2: Influence of dietary restriction on blood vessel maturation in the CT-2A astrocytoma. Confocal analysis of normal brain and CT-2A tumor tissue double stained for α-SMA in vascular smooth muscle cells (red) and Factor VIII in vascular endothelial cells (green). Results show that α-SMA is greater in the vessels of the DR-fed tumor than in the vessels of the AL-fed tumor (indicated by white arrow). A blood vessel of a normal brain is shown for comparison. All other conditions were as described in Section 2.

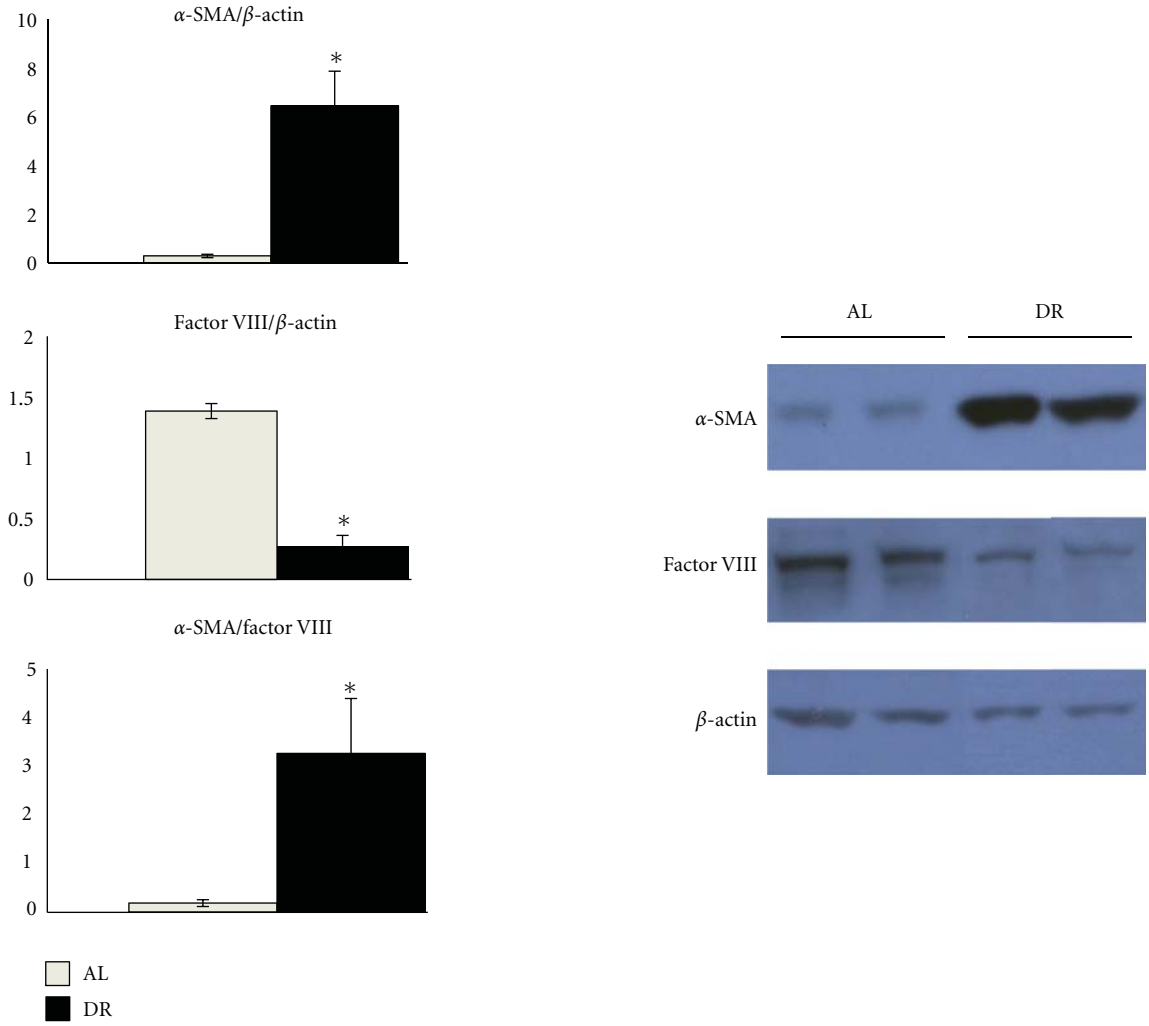

Figure 3: Dietary restriction increases α-SMA and reduces Factor VIII expression in the CT-2A astrocytoma. The histograms show the average relative expression of the indicated protein normalized to β-actin based on Western blot analysis. Equal amounts of protein were loaded into each lane of the Western blot (25 μg). Other conditions were as described in Section 2. Values are expressed as normalized means of three to four independent tissue samples per group ± SEM. The value is significantly different in the tumors of DR-fed mice than in the tumors of AL-fed mice: $^*P < 0.05$, Student's t-test. Two representative samples are shown for each tissue type.

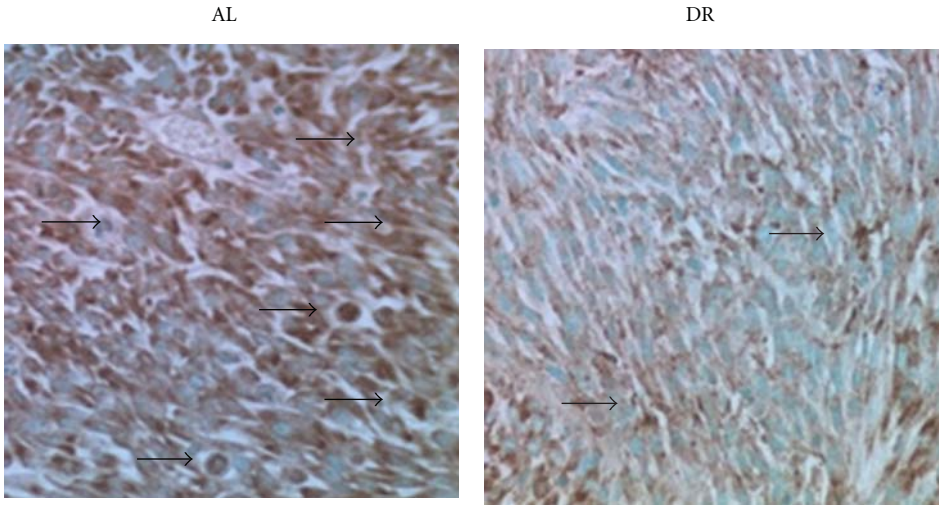

Figure 4: Dietary restriction reduces VEGF expression in the CT-2A astrocytoma. VEGF immunostained sections (400x). Results show that VEGF expression (brown stain) is less in the tumors of DR-fed mice than in the tumors of AL-fed mice. Black arrows indicate positive VEGF staining. Each stained section is representative of the entire tumor. All other conditions are as described in Section 2.

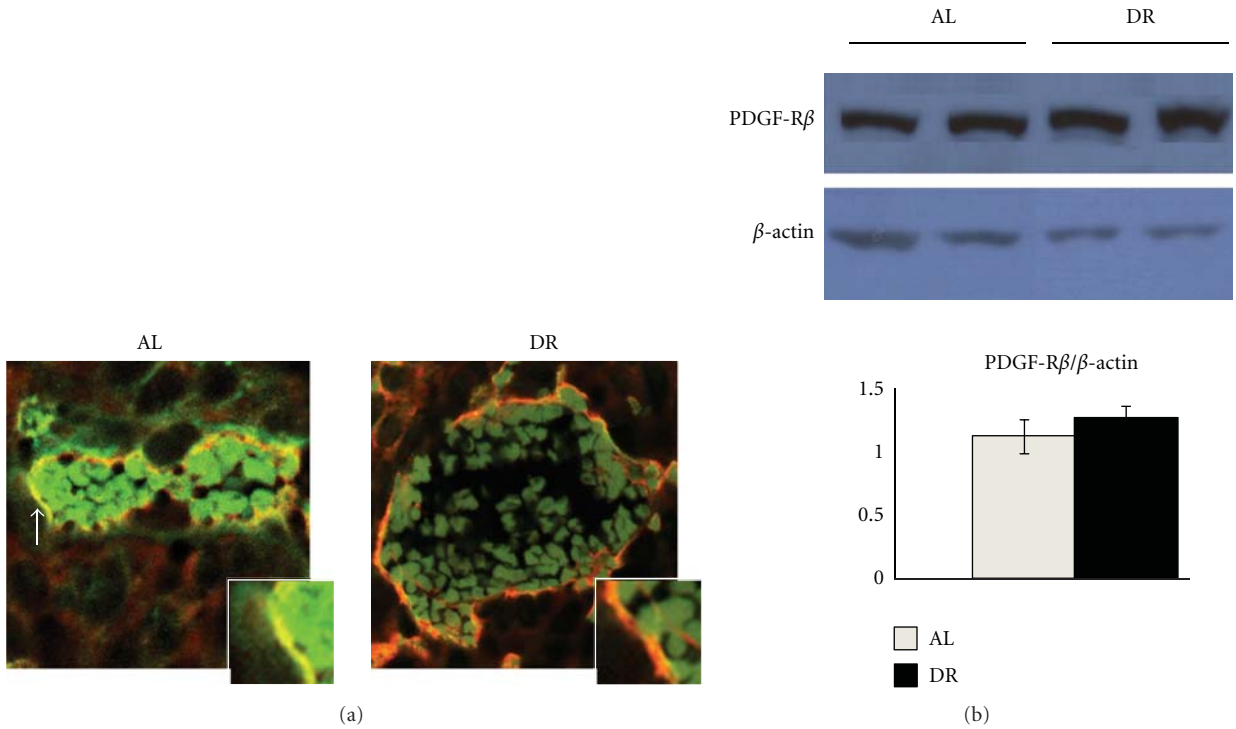

Figure 5: Dietary restriction reduces PDGF-Rβ and VEGF-R2 association in the CT-2A astrocytoma. (a) Confocal analysis of CT-2A tumor tissue double stained for VEGF-R2 (green) and PDGF-Rβ (red). Results show that the colocolization (yellow) of VEGF-R2 and PDGF-Rβ is less in the tumors of DR-fed mice than in the tumors of AL-fed mice. White arrow indicates colocolization. All other conditions are as described in Section 2. (b) The histogram shows that the average relative expression of PDGF-Rβ normalized to β-actin based on Western blot analysis is similar in the tumors of DR-fed and AL-fed mice. Equal amounts of protein were loaded into each lane (25 μg), and the other conditions were as described in Section 2. Values are expressed as normalized means of three to four independent tissue samples per group $\pm$ SEM. There is no significant difference between values of DR-fed and AL-fed mice, Student's t-test. Two representative samples are shown for each tissue type.

It is well documented that prognosis is poor for most patients with malignant glioma [60]. The targeting of angiogenesis has become an important strategy in current therapy [61, 62]. The goal of antiangiogenic therapy is to reduce microvessel density and to increase vessel stabilization [9, 60–62]. We suggest that DR normalizes tumor vasculature by decreasing VEGF expression in the tumor. Our findings show that mature smooth-muscle-cell-covered vessels are more prominent in brain tumors under DR than in brain tumors under AL feeding.

5. Conclusions

The results indicate that dietary restriction promotes vessel maturation in an experimental mouse astrocytoma. This preclinical study shows that DR may be an effective nontoxic antiangiogenic therapy in brain tumors. DR may also improve drug delivery to brain tumors and may therefore be used in conjunction with drug therapy.

Abbreviations

CT-2A:	Mouse astrocytoma
DR:	Dietary restriction
AL:	Ad libitum
α-SMA:	Alpha smooth muscle actin
VSMC:	Vascular smooth muscle cell
VEGF:	Vascular endothelial growth factor
VEGF-R2:	Vascular endothelial growth factor receptor 2
PDGF-Rβ:	Platelet-derived growth factor receptor β.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

The Role of FoxC2 Transcription Factor in Tumor Angiogenesis

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Much has been learned about the mechanisms underlying tumor angiogenesis, and therapies that target vascular endothelial growth factor (VEGF) to limit tumor angiogenesis and subsequent disease progression have recently been approved. However, the transcriptional mechanisms that regulate pathological angiogenesis remain largely unknown. FoxC2, a member of the Forkhead box (Fox) transcription factor family, is critical for vascular formation during development, and recent studies have shown that FoxC2 is expressed in the endothelium of tumors in both humans and mice. In a B16 mouse melanoma model, Foxc2 deficiency reduced tumor growth and neovascularization and was associated with impairments in mural-cell coverage and increases in endothelial-cell apoptosis in tumor blood vessels. FoxC2 is also expressed by tumor cells in human breast, colonic, and esophageal cancer and participates in the epithelial-mesenchymal transition (EMT), a key process that leads to the invasion and metastasis of aggressive tumors. Collectively, these observations suggest that FoxC2 is essential for tumor angiogenesis and disease progression and that FoxC2 may be a viable target for cancer therapy.

1. Introduction

Tumor angiogenesis is a crucial contributor to tumor development and progression [1] because tumor blood vessels supply oxygen and nutrients to the tumor tissue and facilitate cancer cell invasion and metastasis. Tumor vessels are usually disorganized and excessively branched [2–4], and formation of the tumor vasculature involves complex interactions between tumor cells and their microenvironment that are controlled by numerous angiogenic factors secreted from both tumor cells and other cells in the tumor microenvironment. The mechanisms by which proangiogenic factors such as vascular endothelial growth factor (VEGF) promote tumor angiogenesis have been intensively studied [2–4]; however, the transcription factors that participate in this process remain incompletely understood. The FoxC2 transcription factor is a newly recognized regulator of tumor angiogenesis and metastasis, and this paper summarizes what is currently known about the role of FoxC2 in cancer.

2. Forkhead Transcription Factors in Cancer

The Fox (Forkhead box) transcription factors form a large family of proteins with similar DNA-binding domains that are evolutionarily conserved from yeast to humans [5–7]; other functional regions of Fox proteins, such as the transactivation and transrepression domains, are largely divergent. The consensus, seven-nucleotide core sequence bound by monomeric Fox proteins is 5′-(G/A)(T/C)(A/C)AA(C/T)A-3′ [8, 9], and Fox proteins are distributed among 19 subfamilies (FoxA to FoxS) based on similarities in their DNA-binding domains. Uppercase letters are used to designate human Fox genes (e.g., FoxC2), the first letter is capitalized for mouse genes (e.g., FoxC2), and the first and subclass letters are capitalized for chordates (e.g., FoxC2).

Fox proteins have many essential roles in embryonic development, and the mutation or dysregulation of FOX genes is often associated with disease, including cancer [10]. FoxM1 is expressed in glioma and pancreatic cancer cells and promotes angiogenesis by regulating VEGF expression

[11, 12], FoxO1/3/4 act as tumor suppressors in prostate cancer and leukemia [13, 14], and recent evidence suggests that FoxC2 is a key factor in tumor development and disease progression. Thus, the elucidation of *FOX* gene function will likely identify new strategies for the treatment of cancer.

3. Role of FoxC2 in Vascular Development and Angiogenesis

Murine FoxC2 is highly expressed in blood endothelial cells during embryonic development [16–19]. FoxC2 contributes to arterial cell specification in the early developing embryo as a downstream regulator of the Notch signaling pathway [18, 20–22], and FoxC2 participates in angiogenesis [23, 24] through the activity of its downstream targets CXC-chemokine receptor 4 (CXCR4) and integrin $\beta 3$ [25, 26]. The transcriptional activity of FoxC2 in vascular endothelial cells is modulated by VEGF signaling [20], and FoxC2 is critical for the migration of endothelial cells toward VEGF or stromal-cell-derived factor 1 (SDF-1) and for the formation of microvessels in vitro [25, 26]. Recent studies have shown that the activity of FoxC2 and the Ets transcription factor Etv2 combine to regulate endothelial-specific gene expression during early development, including Flk1, Tie2, and VE-cadherin [27], and the Ets transcription factors are known to be important for tumor angiogenesis [28], but whether FoxC2 and Etv2 also coregulate tumor vessel formation has yet to be determined.

The importance of epigenetic changes during the regulation of angiogenesis, including the methylation or histone acetylation of angiogenesis-associated genes, is becoming increasingly apparent [29]; for example, the histone deacetylase (HDAC) SIRT1 induces the sprouting and branching of endothelial cells by deacetylating Foxo1 [30]. SIRT1 also regulates the angiogenic activity of endothelial cells by deacetylating the Notch1 intracellular domain (NICD) [31], and a Notch transcriptional activation complex containing the NICD physically and functionally interacts with FoxC2 [20]. Collectively, these observations suggest that FoxC2 may be involved in SIRT1-mediated transcriptional control of angiogenesis.

FoxC2 is also expressed in lymphatic endothelial cells during development [32, 33], and mutations in human FoxC2 are responsible for the autosomal dominant syndrome lymphedema distichiasis, which is characterized by the obstruction of lymph drainage in the limbs and by the growth of an extra set of eyelashes [34, 35]. Congenital lymphatic defects are also observed in FoxC2 mutant mice: heterozygous FoxC2 mutants display hyperplasia of the lymphatic vessels [36], and homozygous FoxC2 mutants have defective lymphatic valves and abnormal pericyte recruitment of lymphatic vessels [33]. The contribution of FoxC2 to lymphangiogenesis in pathological conditions such as cancer remains unknown.

4. FoxC2 in Tumor Angiogenesis

FoxC2 expression has been detected in the tumor endothelium of both human and mouse melanomas, which suggests that FoxC2 has a role in tumor angiogenesis (Table 1) [15]. Homozygous mutations of FoxC2 lead to perinatal lethality, so the role of FoxC2 in melanoma was confirmed by subcutaneously implanting mouse B16 melanoma cells in wild-type and heterozygous FoxC2 mutant (FoxC2+/–) mice. Tumor growth and angiogenesis were remarkably lower in the FoxC2+/– mutants (Figure 1) [15], which confirms that FoxC2 has an essential role in tumor angiogenesis. Matrix metalloproteinase (MMP) 2 expression was also diminished in B16 tumors from FoxC2+/– mice [15], which is consistent with evidence that MMP2 is important for tumor growth and angiogenesis [37, 38]. The expression of the lymphatic endothelial markers Prox1 and Lyve1 were unchanged [15], despite evidence that FoxC2 is expressed in lymphatic endothelial cells.

Table 1: Summary of FoxC2 expression and prognosis in human cancer.

Tumor type	Number	FoxC2 expression	Prognosis
Melanoma [15]		Vascular endothelial cells	Not evaluated
Breast, colon [15]		Adenocarcinoma	Not evaluated
Esophageal [39]	70	Cytoplasm of cancer cells	5-year survival: 70% FoxC2 (low); 30% FoxC2 (high)
Breast [40]	18	Highly aggressive basal-like tumors	Not evaluated
Breast [41]		$CD44^{high}/CD24^{low}$ cells	Not evaluated

VEGF-A expression was also impaired in B16 tumors from FoxC2+/– mice [15], and this decline likely limits tumor neovascularization. The cells responsible for the decline in VEGF-A expression have yet to be identified; however, autocrine VEGF signaling promotes endothelial cell survival [42], and smooth muscle α-actin- (αSMA-) positive cancer-associated fibroblasts (CAFs), which are known to express VEGF-A in the tumor microenvironment [43–45], were less common in tumors from FoxC2+/– mice than in tumors from wild-type mice [15]. CAFs also secrete stromal cell-derived factor 1 (SDF-1), which stimulates tumor growth by activating its receptor, CXCR4, on tumor cells and by functioning as a chemoattractant for the recruitment of bone-marrow-derived endothelial progenitor cells that subsequently promote angiogenesis [45]. FoxC2 is also known to regulate the CXCR4-dependent mobilization of endothelial cells and bone-marrow-derived endothelial progenitor cells [25, 46], so FoxC2 might have a bimodal influence on the contribution of bone-marrow-derived cells to tumor angiogenesis by increasing both cell mobilization and the SDF-1-mediated recruitment of mobilized cells to tumors. Furthermore, FoxC2 expression is induced by hypoxia after ischemia/reperfusion injury in the kidney [47], and the hypoxic gradient that develops in the tumor microenvironment increases SDF-1 expression in CAFs and CXCR4 expression in tumor cells [48, 49]; thus, FoxC2 could also

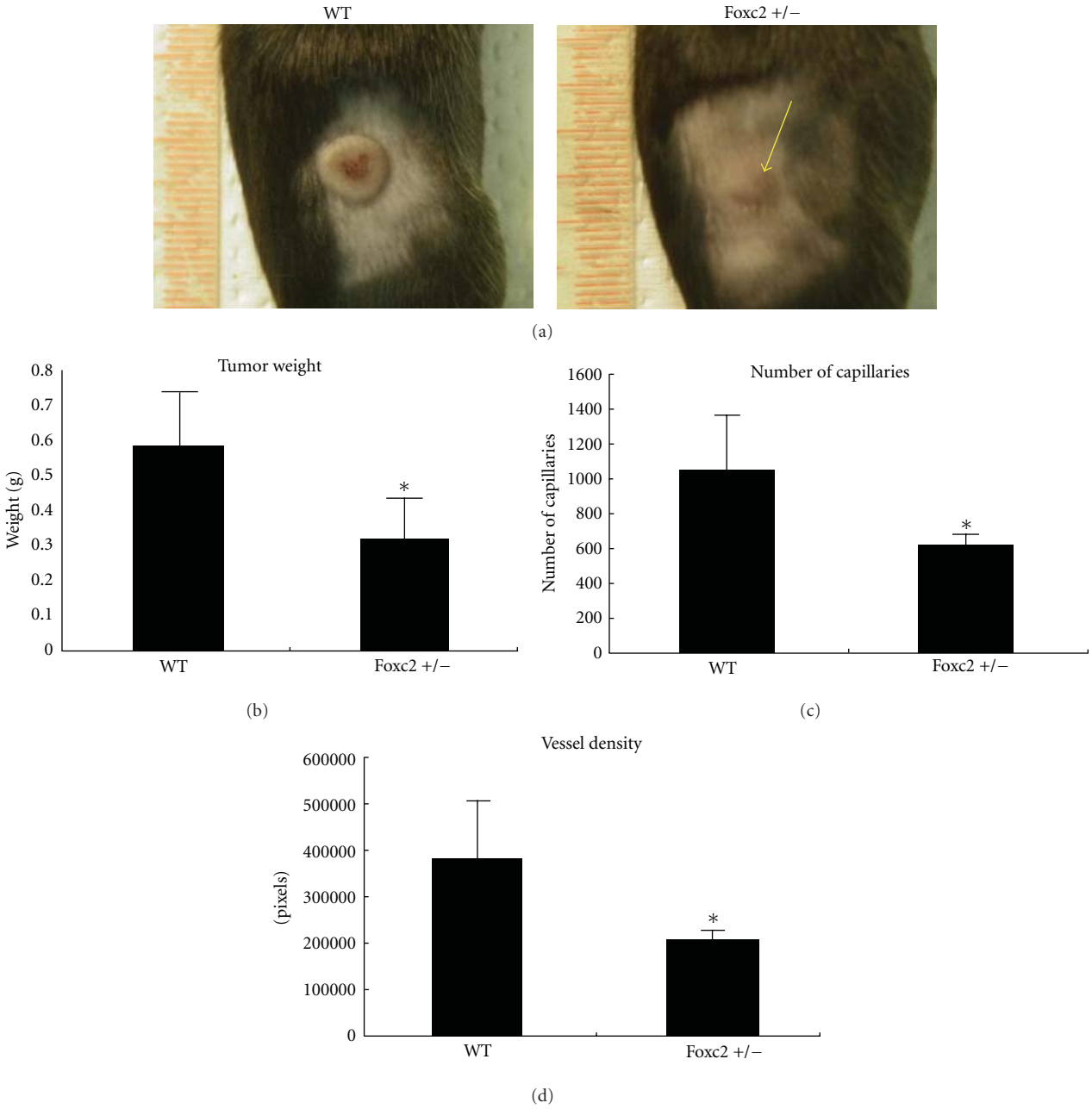

Figure 1: Tumor development and angiogenesis are impaired in FoxC2+/– mice. (a) Subcutaneous growth of B16 melanoma cells in WT and FoxC2+/– mice 11 days after injection. The yellow arrow identifies a smaller tumor in a FoxC2+/– mouse. (b) A difference in tumor weight between WT and FoxC2+/– mice was observed 11 days after the injection of B16 melanoma cells. The data are from five independent experiments and are presented as mean ± SD. Statistical significance was evaluated with Student's t-test. $^*P < 0.05$ versus WT. (c, d) Tumor angiogenesis is reduced in FoxC2+/– mice. (c) The total number of capillaries was calculated by counting PECAM-1-positive endothelial cells in B16 tumors. Statistical significance was evaluated with Student's t-test ($^*P < 0.05$ versus WT). (d) Total vessel density was calculated by measuring the evaluated with the Student's t-test PECAM-1-positive vessel area. Statistical significance was evaluated with Student's t-test. $^*P < 0.05$ versus WT. Adapted from Sano et al. [15].

contribute to tumor angiogenesis through a hypoxia-related mechanism.

Fibroblasts in the tumor microenvironment are a heterogeneous cell population, and the conventional markers αSMA and vimentin cannot identify all CAFs [50]. Furthermore, transforming growth factor- (TGF-) β1 can induce the phenotypic conversion of proliferating vascular endothelial cells into fibroblast-like cells; this endothelial-to-mesenchymal transition (EndMT) takes place at the invasive front of tumors in mice [51], and, consequently, antiangiogenic treatments may limit cancer progression, in part, by directly influencing the EndMT in tumor

vessels. The molecular mechanisms and signaling pathways that control EndMT have yet to be elucidated; however, CAFs can develop directly from cancer cells through the epithelial-to-mesenchymal transition (EMT) [52], which is regulated by FoxC2 (as discussed in the next section). CAFs can also evolve from resident fibroblasts, smooth muscle cells, pericytes, or bone-marrow-derived mesenchymal stem cells through a mesenchymal-to-mesenchymal transition (MMT), which is regulated by tumor-cell-derived factors such as TGF-β, platelet-derived growth factor (PDGF), and basic fibroblast growth factor (FGF) [53]; whether FoxC2 participates in the MMT is unknown.

The declines in tumor growth and vascularity observed in FoxC2+/– mice were also associated with abnormally low levels of PDGF-B, which is likely attributable to the impaired growth of smooth muscle cells surrounding the tumor blood vessels [15]. The precise role of PDGF signaling in tumor angiogenesis remains a subject of debate [54]; however, PDGF mediates the maturation and stabilization of blood vessels during normal vessel growth by regulating the interaction between endothelial cells and mural cells (e.g., vascular smooth muscle cells and pericytes) that are loosely associated with the tumor endothelium, and PDGF-B inhibition is associated with both the loss of mural cells and tumor-vessel regression [55]. The blockade of both VEGF and PDGF receptor-β signaling in tumor blood vessels with kinase inhibitors also induces tumor vessel regression [56, 57], and paracrine signaling between pericytes and the tumor endothelium [58] could increase the instability of the tumor vessels of FoxC2+/– mice by reducing VEGF and PDGF-B signaling [15].

5. FoxC2 in Cancer Cells

Tumor FoxC2 expression is not restricted to the vasculature (Table 1) [15]. T-MTA-6A tissue-array (NCI/NIH) analysis revealed the localization of FoxC2 protein in the majority of breast adenocarcinomas, including lobular and ductal adenocarcinoma, and in about half of colonic adenocarcinomas [15]. FoxC2 expression has also been reported in esophageal cancer cells [39], and the survival rate is significantly lower for patients with high levels of FoxC2 expression than for patients with low levels of FoxC2 expression, which suggests that FoxC2 could be used as a novel, independent prognosis factor for patients with esophageal cancer [39] (Table 1). Patients with high levels of FoxC2 expression also have higher levels of MMP2 and MMP9 expression [39].

FoxC2 is an important regulator of EMT, a key process that is often activated during tumor progression and metastasis [59]. FoxC2 expression is significantly correlated with highly aggressive basal-like breast cancers, and FoxC2 overexpression increases the metastatic potential of mouse mammary carcinoma cells to the lung [40]. The EMT-inducing transcription factors Twist, Snail, and Goosecoid, as well as TGF-β, which also induces EMT, can induce FoxC2 expression in epithelial cells [40, 60], and FoxC2 overexpression promotes mesenchymal differentiation, as well as the induction of MMP2 and MMP9 expression [40]. Furthermore, FoxC2 indirectly represses expression of the epithelial marker E-cadherin, whose loss is considered a hallmark of EMT [59], and directly downregulates p120-catenin, a regulatory protein that stabilizes E-cadherin at the adhesion junctions of epithelial cells [61]. Thus, accumulating evidence indicates that EMT-inductive signaling molecules, such as TGF-β, stimulate FoxC2 expression in carcinoma cells along with other EMT-promoting transcription factors and mediates mesenchymal differentiation, thereby leading to EMT and metastasis [59]. EMT has also been linked to the genesis of cancer stem cells [62]; for example, mammary epithelial cells have been shown to acquire properties associated with breast cancer stem cells, including the $CD44^{high}/CD24^{low}$ signature, after undergoing EMT and to express EMT-inducing transcription factors such as FoxC2 (Table 1) [41]. Notably, tumor cells undergoing EMT acquire a migratory phenotype by ectopically expressing mesenchymal genes, including FoxC2, which first function in the developing embryo, and tumor cells may adopt many other developmental signaling pathways that function in their ancestral precursor cells [63–65]. Thus, EMT may represent a critical point of convergence between the signaling paradigms that regulate development and tumor metastasis.

6. Future Perspectives

Though we are just beginning to understand how FoxC2 contributes to cancer development and related pathological conditions, its newly discovered role in tumor angiogenesis and metastasis suggests that FoxC2 could be an intriguing target for anticancer therapies. Drug therapies rarely target transcription factors directly, but a combination of advanced technologies, such as systems biology and computational chemistry, could lead to the development of viable transcriptional approaches for preventing cancer development and progression, as well as other conditions that involve pathological angiogenesis. For example, FoxM1 is currently being investigated as a target for cancer therapy [66], and several chemical inhibitors, including proteasome inhibitors, of FoxM1 have been reported [67–71], as well as a cell-penetrating peptide inhibitor of FoxM1 function [72]. The precise function of FoxC2 in the tumor microenvironment, including both cancer cells and endothelial cells, must be better understood before the development of FoxC2-based therapies can be considered and, consequently, studies focusing on the mechanisms controlled by FoxC2 are necessary. The identification of relatively small peptides that can selectively block the function of FoxC2 would also be a useful step toward designing FoxC2-specific inhibitors.

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Review Article

Angiogenesis in Head and Neck Cancer: A Review of the Literature

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Angiogenesis is a necessary process for tumor growth, progression and diffusion. In the last years many efforts have been made to understand the mechanisms necessary to the formation of new vessels in tumor tissue and how to integrate these findings in the treatment of different type of cancer. Thanks to these studies there are today many anti-angiogenic drugs with established activity in cancer and approved in clinical practice. Head and neck cancer is a common tumor worldwide that often has advanced stage at diagnosis and poor prognosis. Angiogenesis has a well recognized role in head and neck cancer progression and resistance to drugs and radiotherapy and many clinical trials has been conducted with antiangiogenic agents in this disease, even if they often showed limited efficacy. In this review we summarize the main trials published about angiogenesis in head and neck cancer with particular attention to factors involved in this process and the available data on the efficacy of treatment with anti-angiogenic agents in this disease.

1. Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common cancer with 500.000 diagnosis per year worldwide [1]. Patients with locally advanced disease have a chance of cure with multimodality treatments that involves surgery, radiotherapy, chemotherapy, and, in the last years, molecular targeted therapies [2]. Despite the advances in the treatment of locally advanced disease, more than 50% of patients will relapse. Furthermore, combining surgery, radiotherapy, and chemotherapy often leads to severe and permanent function deficits with a negative impact on patients' quality of life. On the other hand, patients with relapsed or metastatic disease have a worse prognosis with an overall survival of approximately 7–10 months [3]. New therapeutic protocols and agents should be developed to improve survival while limiting treatment-related toxicities.

Angiogenesis, the process that leads to the formation of new vessel, is a hallmark of tumor progression, and its role has been studied in many cancer types including HNSCC. Antiangiogenic agents are to date available and useful for the treatment of many tumors. In HNSCC; however, few clinical trials have yielded promising results when focusing on these new agents.

This paper is aimed at evaluating the angiogenic factors involved in HNSCC growth and progression and their therapeutic implications.

2. Angiogenesis in Head and Neck Cancer

Vascular endothelial growth factor A (VEGF-A) is the best known agent that induce angiogenesis. It is a vascular permeability factor that belongs to the platelet-derived growth factor (PDGF) superfamily, which also includes VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF) [4]. Hypoxia induces VEGF expression through the mediation of hypoxia-inducible factor (HIF-1α) [5]. There are many other factors involved in angiogenesis, such as epidermal growth factor (EGF), PDGF, prostaglandins, COX-2, and IL-6 [6]. The VEGF family of ligands plays its role through cell surface receptor tyrosine kinases, VGFR-1, VGFR-2, and VGFR-3 [7]. VEGFR-2 is the most important one through which VEGF exerts its mitogenic, chemotactic, and vascular permeabilizing effects on endothelial cell [4].

Moreover, VEGF interacts with a family of coreceptors called neuropilins (NRP-1 and NRP-2) [8, 9] that strengthen the link between VEGF and its receptors increasing their biological activity.

Overexpression of VEGF in HNSCC is associated with more advanced disease, increased resistance to cytotoxic agents, and poor prognosis [10–16]. In a meta-analysis of 12 studies including 1002 patients affected by cancer of oral cavity (70.8% of patients), pharynx (15.2%), and larynx (14%), VEGF expression was evaluated, and its positivity was associated with a twofold higher risk of death at 2 years [17].

Hasina et al. [18] demonstrated that there are different molecular mechanisms by which each tumor induce angiogenesis. Using sample collected from patients affected by HNSCC and sample of normal and dysplastic mucosa, they conducted an immunohistochemical analysis and gene expression profiling studies. They studied the expression of cytokines (CK) such as VEGF, IL-8/CXCL8, HGF, and FGF-2 in normal, dysplastic, and pathological tissues. These CK are well-known mediators of HNSCC angiogenesis. The authors observed that normal mucosa generally does not express VEGF, IL-8/CXCL8, FGF-2, and HGF and that, where present, the levels of these CKs are very low compared to dysplastic and pathological mucosa. The same CKs are more frequently expressed and at a higher levels in dysplastic oral mucosa. The incidence and the intensity of expression of VEGF, IL-8/CXCL8, FGF-2, and HGF are highest in HNSCC samples. Moreover, they validated the presence of two different clusters in relation to angiogenesis in HNSCC samples: tumors in Cluster A express high levels of VEGF and FGF-2 and low levels of IL-8/CXCL8 and HGF and are characterized by higher levels of microvessel density than tumors in Cluster B, expressing on the contrary low levels of VEGF and FGF-2 and higher levels of IL-8/CXCL8 and HGF. These data suggest that there are at least two different pathways in inducing angiogenesis in HNSCC. This hypothesis has an important therapeutic implication. In fact we can argue that the inhibition of a specific molecular pathways can block the angiogenesis process, and consequently the tumor growth, only if the target of the therapy is expressed by the tumor cells. In the same study the authors used three different HNSCC cell lines with different levels of expression of VEGF that were inoculated in nude mice. Then they treated the experimental models with anti-VEGF antibody, with nonspecific human IgG antibody, or with PBS (phosphate-buffered saline, a buffer solution isotonic and nontoxic to cells). The growth of tumor with high levels of VEGF was inhibited by anti-VEGF treatment while not influenced by nonspecific IgG or PBS. On the other hand anti-VEGF treatment had limited effects on the growth of tumor with low levels of VEGF. In this case no difference in tumor volume was found compared to those treated with nonspecific IgG or PBS. These data may have very important implications in clinical practice and support the need of better understanding the molecular alterations in each specific tumor in order to better select patients for targeted therapies.

3. Effect of Antiangiogenic Agents on Xenograft Models

Several studies report the activity of different molecules directed against the angiogenic process in head and neck models.

For example Miyazawa et al. [19] tested the effect of PTK/ZK (Vatalanib) on the initial stages of head and neck tumor angiogenesis. PTK/ZK is a small molecule inhibitor of VEGF receptors [20, 21]. The molecule has just been tested by Kim et al. [22] in anaplastic thyroid carcinoma xenografts in nude mice, and in that study it inhibited the phosphorylation of VEGFR-2 in the endothelial cells and reduced the microvessel density of the models. Miyazawa et al. tested the effects of PTK/ZK on neovascularization in vitro and in vivo. They inoculated experimental mice with different HNSCC lines and treated them with the oral administration of PTK/ZK or vehicle controls. They showed that animals treated with the small VEGF receptor inhibitor developed low microvessel density compared to those treated with vehicle control. Moreover, the models treated with PTK/ZK had a slower tumor progression than controls, even if the difference was not statistically significant.

Several preclinical data about the association between anti-EGFR and antiangiogenic treatments [23–25] and between radiotherapy and antiangiogenic drugs [26, 27] have been published recently. Moreover, in the last few years a study conducted by Bonner et al. [28] demonstrated the efficacy of the association of radiotherapy and a target therapy such as an anti-EGFR agent (cetuximab) in locally advanced disease. Few studies investigated the intriguing combination of the three approaches together. This association could be very interesting in clinical practice because the production of VEGF is inhibited, at least in part, by anti-EGFR agents while radiotherapy, through the induction of EGFR production in irradiated cells, can lead to neovascularization. So the combination of these three weapons could have synergistic effects.

In a study published in 2007, Bozec et al. [29] evaluated the efficacy of AZD2171, gefitinib, and radiotherapy. AZD2171 is an inhibitor of VEGFR-2, VEGFR-1, and VEGFR-3 in vitro [30], while gefitinib is an EGFR tyrosine kinase inhibitor with antiangiogenic activity [23]. The effects of the combination of the two drugs on tumor growth and of the combination of the drugs with radiotherapy were tested on human head and neck tumor xenografts. The investigators used a cell line, CAL33, that had high levels of EGFR end VEGF. Mice inoculated with CAL33 tumors were treated with vehicle alone, AZD2171 or gefitinib alone or in combination, or with the two drugs combined with radiotherapy. The treatment with AZD2171 and gefitinib showed better antitumor effects than either treatment alone, but tumor regrowth after discontinuation was observed. On the other hand, the triple combination (two drugs plus radiotherapy) had the best antitumor effects with a prolonged activity after treatment discontinuation.

The same authors conducted a similar study [31] using bevacizumab, monoclonal antibodies directed against VEGF, erlotinib, an EGFR tyrosine-kinase inhibitor, and

radiotherapy on head and neck orthotopic models. They tested the efficacy of the three treatments, given alone or in combination, on mice inoculated with CAL33 tumors. Treatment with each single agent did not show a significant activity on tumor growth while the combination of the three treatments had the best antitumor activity with supra-additive effects (combined ratios 2.3). An evaluation of vascularization marker was conducted in the same study and showed that the triple combination led to a decrease in cell proliferation and neoangiogenesis (lower Ki-67 and VEGFR-2 expression).

Then the same authors [32] tested the in vivo efficacy of the combination of sunitinib, a multitargeted tyrosine kinase inhibitor with great anti-VEGF activity, cetuximab, and radiotherapy. CAL33 cell lines were injected in mice that were then treated with vehicle or cetuximab and/or sunitinib and/or radiotherapy. In this study the treatments given alone showed a significant antitumor effect compared with controls. The best result on tumor growth was obtained by the triple combination. In fact at the end of the treatment with cetuximab, sunitinib, and radiotherapy, no tumor cells were detectable in all treated animals ($P < 0.001$ versus control).

Myoung et al. [33] conducted a study using the combination of paclitaxel and thalidomide on xenotransplanted oral squamous cell carcinoma. Thalidomide is able to inhibit neovascularization and tumor growth [34–37] while paclitaxel is an antitumor agent with antiangiogenic activity [38–41]. In this study a human oral squamous cell carcinoma line was inoculated into nude mice subsequently treated with thalidomide, paclitaxel, or control vehicle. Paclitaxel showed a significant activity on tumor growth, while thalidomide did not show any effect. It is worthwhile noting that the two drugs had remarkable effects on the immunohistochemical expression of VEGF and CD31, which was also reduced by the administration of paclitaxel and thalidomide. A similar reduction in the production of VEGF mRNA suggested a good activity of these drugs against neovascularization. The study suggests that the inhibition of angiogenesis is not enough to suppress oral squamous cell carcinoma growth and that probably antiangiogenic treatments have to be integrated with other different approaches.

4. Effect of Antiangiogenic Agents in Clinical Trials

Sorafenib and sunitinib are two tyrosine kinase inhibitors with activity against VEGFR2, VEGFR3, and the PDGF receptors that have been tested in different studies in patients with recurrent or metastatic HNSCC.

Three studies were reported with sunitinib. In the first study [42], 22 patients with recurrent or metastatic HNSCC who had received no more than two prior chemotherapy regimens were treated with sunitinib administered in 6-week cycles at 50 mg/day for 4 weeks followed by 2 weeks off. Patients were divided into 2 cohorts according to the Eastern Cooperative Oncology Group Performance Status (ECOG-PS): patients with ECOG-PS 0-1 in cohort A, patients with ECOG-PS 2 in cohort B. the primary endpoint was objective tumor response for group A (15 patients) and feasibility for group B (7 patients). In cohort A partial response (PR) was reported in only one patient, while no response was observed in cohort B. Stable disease (SD) was observed in 25% of patients. The median overall survival (OS) was 21.1 weeks for patients in Cohort A and 19.1 weeks for patients in cohort B. The main grade 3 hematologic toxicities reported were lymphopenia (18%), neutropenia (14%), and thrombocytopenia (5%). The only grade 4 hematologic toxicity observed was thrombocytopenia occurring in one patient. The most common nonhematologic grade 3 toxicities were fatigue and anorexia (23% of patients). Grade 3 hypertension occurred only in one patient in cohort B. Grade 4 hemorrhage was reported in one patient (gastrointestinal bleeding). Nonfatal hemorrhagic events were seen in 8 patients; in 1 of these patients a superficial tumor bleeding was observed. Even if sunitinib was well tolerated, accrual was closed at interim analysis as nonsignificant antitumor activity was demonstrated.

Another study was conducted by Fountzilas et al. [43] who treated 17 patients affected by metastatic or recurrent HNSCC with sunitinib in first-line setting. The primary endpoint of the study was objective response rate (ORR) while the secondary endpoints included time to tumor progression (TTP), OS, safety, and tolerability of sunitinib as monotherapy. Fourteen patients were assessable for response. Three patients (18%) had stabilization of disease while 11 patients (65%) showed progression. No objective responses were observed. Median TTP was 2.3 months, and median OS was 4 months. The most common grade 3 toxicity was fatigue that occurred in 7 patients (41%), while grade 3 hemorrhagic events were described only in 1 patient (6%). Bleeding of any grade was reported in 10 patients (59%). The study was discontinued because the drug proved to be barely active.

These 2 studies showed that sunitinib 50 mg/day for 4 weeks followed by 2 weeks rest is well tolerated but has no significant antitumor activity in monotherapy.

In the third study [44], sunitinib 37.5 mg daily, given continuously until disease progression or unacceptable toxicity, was tested on 38 patients with recurrent or metastatic HNSCC refractory to platinum-based treatment or unfit for platinum-based regimens. No more than 2 prior lines of chemotherapy were permitted. The primary endpoint was the rate of disease control (RDC), defined as complete response (CR) or PR or SD at 6 to 8 weeks after treatment initiation. RDC was 50% (1 patient with PR and 18 patients with SD). The median PFS was 2 months while the median OS was 3.4 months. The most frequent grade 3/4 toxicities were fatigue (32%), anorexia (16%), thrombocytopenia (13%), and diarrhea (8%). Serious hemorrhagic events of head and neck vessels (grades 3–5) were reported in 5 patients (one grade 3, one grade 4, and 3 grade 5); four of these patients were previously irradiated in the head and neck area. In conclusion this study showed a limited activity of sunitinib in the treatment of recurrent or metastatic HNSCC while reporting a significant risk of severe hemorrhage.

Sorafenib is the other small tyrosine kinase inhibitor tested in the same setting. It is a multitarget drug with activity against the EGFR-Ras-Raf-Mek-Erk signaling pathway and against VEGF-VEGFR.

In the first study, published in 2007 [45], Elser et al. conducted a single-arm phase II study in patients affected by recurrent or metastatic HNSCC (including nasopharyngeal carcinoma) that had previously received no more than one systemic treatment. The trial enrolled 28 patients, treated with sorafenib 400 mg twice daily continuously, 27 evaluable for efficacy. The primary objective was ORR. The ORR was 3.7%, while 37% of patients achieved a stabilization of disease as best response. Median TTP was 1.8 months; median OS time was 4.2 months. The most common grade 3 toxicities were lymphopenia (17%) and fatigue (7%).

The other published study, with sorafenib in first-line setting, was conducted on patients with persistent, recurrent, or metastatic HNSCC [46]. The primary endpoint was response probability. Sorafenib was administered as continuous treatment, 400 mg twice daily. Forty-one patients were eligible for response; one patient had a confirmed PR (2%). The estimated median PFS was 4 months, and the estimated OS was 9 months. The most common grade 3 adverse events were hand-foot syndrome (7.3%), stomatitis (4.8%), and nausea (4.8%). The only grade 4 event was a cerebral ischemia caused by asymptomatic pulmonary embolism.

The two studies demonstrated that sorafenib is well tolerated in this population of patients. No significant activity was demonstrated in terms of response rate, but we must consider that these novel drugs have often a cytostatic effects with limited cytotoxic activity. It is interesting to note that the trials conducted on chemonaive patients showed PFS and OS comparable to those achieved with more toxic and aggressive regimens based on platinum and taxanes.

A further antiangiogenic agent tested in recurrent and metastatic setting is the monoclonal antibody bevacizumab directed against VEGF. It was administered in association with erlotinib, an anti-EGFR inhibitor, to patients with recurrent or metastatic HNSCC never treated or previously treated with one line of chemotherapy [47]. It was a phase I/II study in which the authors used the association of an anti-vascular agent and an anti-EGFR one; there are in fact several trials in other cancers demonstrating that the use of these type of drugs together improves efficacy [48–53]. The phase I study was designed to determine the maximum tolerated dose of bevacizumab when associated to erlotinib: erlotinib was given at dosage of 150 mg/daily, while bevacizumab was administered in escalating dose cohorts. The primary objective of the phase II study was ORR and TTP; in this phase bevacizumab 15 mg/kg was administered every 3 weeks. Forty-eight patients enrolled were evaluable for response. An objective response (PR or CR) was reported in 7 patients (15%), while 15 patients (31%) maintained stability of disease. Four patients achieved a complete response with a duration of response that lasted up to 17 months in one patient. The median PFS was 4.1 months, and the OS was 7.1 months. The treatment was well tolerated. Grade 3 adverse events reported were esophagitis (1 patient), diarrhea (1 patient), and lymphopenia (1 patient). There was one grade 4 hemorrhage. In this study the association was well tolerated with an interesting activity if compared to trials with antiangiogenic agents used alone. The authors also conducted an exploratory study to investigate biomarkers that could predict clinical outcomes and find that high phosphorylated VEGFR2/VEGFR2 and endothelial cells phosphorylated EGFR/EGFR ratios in baseline tumor specimen can identify patients with the greatest probability of response to erlotinib and bevacizumab.

With regard to locoregionally advanced disease, few studies have just been published.

Seiwert et al. [54] added bevacizumab to fluorouracil and hydroxyurea-based chemoradiotherapy in patients with relapsed previously irradiated HNSCC or with poor prognosis newly diagnosed disease. It was a phase I study to determine the maximum tolerated dose of bevacizumab when added to chemoradiotherapy. Forty-three patients were enrolled: 29 patients (67.4%) were previously irradiated, while 14 patients (32.6%) were newly diagnosed. Dose-limiting toxicities were reached at level 3 (bevacizumab 5 mg/kg), so at level 4 (bevacizumab 10 mg/kg), the dosage of chemotherapeutic agents alone was reduced. The treatment was well tolerated with grade 3 mucositis occurring in 69.8% of patients and grade 3 radiation dermitis in 11.6%. The adverse events probably related to bevacizumab were grade 3 hypertension in 3 patients, 1 allergic rash reaction, 2 deep vein thrombosis, 1 stroke, and 2 fatal hemorrhages. The median OS of the patients enrolled was 10.7 months. Patients with no prior radiation had a significantly longer OS (40.1 months) than those previously irradiated (10.3 months). This study demonstrates that bevacizumab 10 mg/kg every two weeks can be safely integrated to fluorouracil and hydroxyurea-based concomitant chemoradiotherapy: the rate of severe complication was similar to those reported in trials with different agents in cohorts of patients with the same characteristics [55–68].

Two more interesting studies were presented in the form of abstract at the 2009 ASCO annual meetings as preliminary results.

In the first trial [68] the authors treated 60 patients with newly diagnosed locoregionally advanced HNSCC with two courses of induction chemotherapy repeated every 21 days consisting of carboplatin AUC 6 day 1, paclitaxel 200 mg/mq day 1, 5 fluorouracil 200 mg/mq/day continuous infusion every 3 weeks, and bevacizumab 15 mg/kg day 1 followed by radiotherapy and concomitant paclitaxel 50 mg/mq/weekly, bevacizumab 15 mg/kg weeks 1 and 4, and erlotinib 150 mg daily for 7 weeks. Forty-one patients (85%) completed all treatments with an objective response rate of 77%. This study has a short followup but interesting 18 months PFS of 85% and 18 months OS of 87%. Severe toxicity during induction chemotherapy was neutropenia (46%), neutropenic fever (6%), mucositis (14%), diarrhea (14%), and hand/foot syndrome (11%), while during concomitant treatment severe mucositis was experienced by 76% of patients.

In another trial [69] bevacizumab 15 mg/kg days 1, 15, and 43 and cisplatin 50 mg/mq days 1, 2, 22, 23, 43, and 44

were added to definitive IMRT in patients with previously untreated, stage III/IV, HNSCC. All patients completed the treatment with a locoregional control rate of 100% (3 patients developed distant metastases). Estimated one-year PFS was 83% and estimated one-year OS 88%. The main severe toxicities were mucositis (76%), nausea (24%), vomiting (17%), neutropenia (41%), hemoglobin (17%), hyponatremia (14%).

Table 1 summarize the main published clinical trials on anti-angiogenetic drugs.

5. Mechanism of Resistance to Antiangiogenic Treatment and Future Direction

In many clinical trials anti-angiogenetic drugs have a limited efficacy, especially in terms of overall survival. Some authors [70, 71] demonstrated in their laboratories that VEGF-targeted drugs inhibit the growth of primary tumors but may shorten survival of mice by promoting tumor invasiveness and the metastatic process. Ebos et al. [70] tested the role of sunitinib in developing metastasis in mice models. They selected sunitinib because of the schedule of administration in clinical practice (4 weeks on/2 weeks off) and the preliminary observations that tumor regrowth can occur during rest period [72]. They showed that sunitinib inoculated in different schedules and doses and with different tumor cell models can lead to opposite results on tumor growth. For example, sustained treatment of pre-established tumors inhibits its growth, while short-term treatment prior to tumor inoculation results in the acceleration of metastasis and reduction in survival. Pàez-Ribes et al. demonstrated in the same issue of Cancer Cells [71] that two different mouse models of tumors, pancreatic neuroendocrine cancer (PNET) and glioblastoma multiforme, can develop an adaptive and evasive response to an efficacious antiangiogenic treatment. This leads to a more aggressive behavior, increased dissemination, and distant metastasis progression. In PNET models two different drugs were tested, sunitinib and a specific VEGFR2 inhibitor. Sunitinib had significantly better efficacy than the competitor but surprisingly led to the development of more invasive tumors.

So, how can these different effects of treatment with angiogenic inhibitors be explained? First of all these agents act by inducing hypoxia, but tumor cells are often able to survive in hypoxic conditions thanks to the ability of producing energy in the absence of oxygen [73]. So hypoxia selects those cells that are more malignant and less sensitive to treatment with these classes of agents [74].

Moreover, tumors can activate more vascular supply mechanisms through upregulation of proangiogenic stromal cells (fibroblasts, pericytes, mesenchimal and hematopoietic cells) that contribute to the vasculature scaffold. Antivascular agents cause acute hypoxia that leads to the accumulation of endothelial progenitors cells at the tumor margins [75]. Both macrophages and neutrophils in proximity of hypoxic tissues can contribute to angiogenesis, escaping the mechanism of action of the drugs [76, 77]. Furthermore hypoxia caused by antiangiogenic treatments causes an increase in bone marrow-derived cells consisting in vascular progenitors and pro-angiogenic monocytic cells (monocytes, hemangiocytes VEGFR-1+ and CD11b+ myeloid cells) [78–82], all involved in the activation of angiogenesis-expressing cytokines, growth factors, and proteases [83, 84]. CD11b+Gr1+ cells are well known for their ability to confer resistance to anti-VEGF in mouse models. These cells derive from bone marrow, are present at high level in tumor and peripheral blood of tumor-bearing animals [85], and produce several angiogenic factors, such as Bv8 [86].

In addition VEGF-inhibitors induce an inflammatory state characterized by the production of several cytokines (PlGF, G-CSF, IL-6, erythropoietin, osteopontin) that stimulate angiogenesis and metastasis in a VEGF-independent manner [87]. Another possible mechanism is that anti-VEGF agents or the cytokines induced by their action could inhibit the action of pericytes on tumor vessels, making them more leaky and immature and facilitating the intravasation of tumor cells and metastatic spread [88].

The results achieved with antiangiogenic treatment are sometimes controversial, but we must take into account the several variables involved, such as VEGF levels, vessel number, and function, VEGF-dependence of tumor vascularization, pericyte action, recruitment and activation of bone marrow-derived cells, target and duration of treatment, and the combination with different cytotoxic agents. In conclusion it is time to further investigate how to optimally use these agents, with the aim of blocking the tumor growth while suppressing prometastatic effects.

6. Discussion

The review of the main studies published in the last years confirms the central role of angiogenesis in the growth and progression of head and neck tumor [18]. Moreover, most of the published data point to the relationship between VEGF overexpression, more advanced disease at diagnosis, and poor prognosis [10–17].

Despite the importance of angiogenesis in head and neck cancer, few antiangiogenic agents have shown relevant activity in this clinical setting and have been approved for the treatment of this disease.

The reason depends mainly on the fact that many studies have been conducted on xenograft models. First of all some data suggest the existence of two different pathways in angiogenesis [18], so it is mostly important to understand as better as possible the pathogenetic process in each patient, in order to select the correct therapeutic target.

Secondly, the lack of activity can be explained considering that some authors [33] demonstrated that angiogenesis inhibition is probably not enough to completely arrest the growth of tumors; then we should likely combine this approach with cytotoxic drugs or other treatment such as radiotherapy or anti-EGFR agents [29, 31, 32].

Furthermore, there are many preclinical data that suggest that antiangiogenic treatments could be effective on primary tumors' growth while promoting the developing of more aggressive disease with a greater prometastatic behavior [70–72].

Table 1: Clinical trials with antiangiogenic drugs.

Regimen	Setting	No. of patients	Results
Sunitinib [42]	Recurrent/metastatic (first line)	22	RDC 33% (terminated after interim analysis)
Sunitinib [43]	Recurrent/metastatic (first line)	17	Terminated for lack of efficacy
Sunitinib [44]	Recurrent/metastatic (platinum refractory)	38	RDC 50%. mPFS 2 months, mOS 4 months
Sorafenib [45]	Recurrent/metastatic (second line)	28	ORR 37%. mOS 4.2 months
Sorafenib [46]	Recurrent/metastatic (first line)	41	mPFS 4 months. Estimated OS 9 months
Erlotinib+bevacizumab [47]	Recurrent/metastatic (first/second line)	48	RR 15%. mPFS 4.1 months. mOS 7.1 months
Bevacizumab+fluorouracil+hydroxyurea and radiotherapy [54]	Locally advanced relapsed or poor prognosis newly diagnosed	43	mOS 10.7 months
Carboplatin+paclitaxel+5 fluororuracil+ bevacizumab followed by radiotherapy+ paclitaxel+bevacizumab+erlotinib [68]	Locally advanced (first line)	60	ORR 77%. 18 months PFS 85%, 18 months OS 87%
Bevacizumab+cisplatin+IMRT [69]	Locally advanced (first line)	42	Locoregional control rate 100%. Estimated 1 year PFS 83%; estimated 1 year OS 88%

RDC: rate of disease control; mPFS: median progression-free survival; mOS: median overall survival; ORR: overall response rate.

As for the trials concluded in patients with relapsed or metastatic disease, the drugs that are more extensively studied are TKI inhibitors, Sunitinib and Sorafenib.

Sunitinib [42–44] as monotherapy has shown limited activity in these patients, and so no more studies are warranted. Sorafenib [45, 46] did not give encouraging results with regard to objective response but interesting data of PFS and OS when used as first-line treatment. Few studies have just been concluded and published in patients with locoregionally advanced disease [67, 68], and the results of these trials have to be confirmed with a longer followup.

Finally, it should be interesting to investigate whether the expression of angiogenic factors can be used as predictive. To date only one study [47] with a combination of an antiangiogenic agent and an anti-EGFR inhibitor reported a possible role of a molecular biomarker that could predict a greater possibility of response to an antiangiogenic treatment. Further studies are needed to understand the mechanisms of response and resistance to angiogenesis inhibitors, how to integrate antiangiogenic therapies in the treatments of patients affected by HNSCC, and how to identify those most likely to respond, in order to offer the best treatment for each patient while limiting toxicities.

Conflict of Interests

The authors have no conflict of interests to declare, and no fee has been received for preparation of this paper.

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Research Article

In Vivo Time-Course Imaging of Tumor Angiogenesis in Colorectal Liver Metastases in the Same Living Mice Using Two-Photon Laser Scanning Microscopy

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In vivo real-time visualization of the process of angiogenesis in secondary tumors in the same living animals presents a major challenge in metastasis research. We developed a technique for intravital imaging of colorectal liver metastasis development in live mice using two-photon laser scanning microscopy (TPLSM). We also developed time-series TPLSM in which intravital TPLSM procedures were performed several times over periods of days to months. Red fluorescent protein-expressing colorectal cancer cells were inoculated into the spleens of green fluorescent protein-expressing mice. First- and second-round intravital TPLSM allowed visualization of viable cancer cells (red) in hepatic sinusoids or the space of Disse. Third-round intravital TPLSM demonstrated liver metastatic colonies consisting of viable cancer cells and surrounding stroma with tumor vessels (green). *In vivo* time-course imaging of tumor angiogenesis in the same living mice using time-series TPLSM could be an ideal tool for antiangiogenic drug evaluation, reducing the effects of interindividual variation.

1. Introduction

Angiogenesis is a fundamental process for the production of new blood vessels during reproduction, embryonic development, and wound healing [1]. It is also a hallmark of tumor growth and metastasis, and high levels of tumor angiogenesis are associated with advanced tumor growth, distant metastases, and an adverse prognosis in human cancers, including colorectal cancer (CRC) [2].

CRC is the second most frequent cause of cancer-related deaths worldwide. Although significant progress in the treatment of metastatic CRC has increased the median overall survival (OS) to around 24 months [3, 4], the 5-year OS in patients with stage IV CRC with liver metastasis remains below 10%, despite intensive multidisciplinary therapies [5]. There is therefore an urgent need to understand the mechanisms of colorectal liver metastasis and tumor angiogenesis.

Multiphoton microscopy, including two-photon laser scanning microscopy (TPLSM), has been introduced to tumor biology during the last decade and has become a common instrument in the biological laboratory [6–8]. We have established a new method for *in vivo* real-time TPLSM imaging of intra-abdominal gastrointestinal disease using green-fluorescent-protein- (GFP-) expressing mice [9].

In vivo real-time TPLSM imaging of colorectal liver metastasis formation is achieved by inoculating red-fluorescent-protein- (RFP-) expressing cell lines into the spleens of GFP mice. This involves fixation of the liver using an organ-stabilizing system to minimize microvibration of the observed area caused by heart beat and respiratory

movements, thus allowing the liver to be visualized at higher magnifications in the living mice.

We also established a time-series TPLSM technique consisting of several intravital TPLSM observations at different time points over prolonged experimental periods to allow the dynamics of liver metastasis formation to be followed in the same living mice over periods of months.

In this study, *in vivo* real-time dual-color imaging of colorectal liver metastasis formation with tumor angiogenesis was performed using intravital and time-series TPLSM.

2. Materials and Methods

2.1. Animals. GFP-expressing nude mice (C57BL/6-BALB/c-nu/nu-EGFP) were purchased from AntiCancer Japan (Osaka, Japan). GFP nude mice (20–22 g) were bred, housed in groups of six mice per cage, and fed with a pelleted basal diet (CE-7, CLEA Japan Inc., Tokyo, Japan). Mice had free access to drinking water. They were kept in the animal house facilities at Mie University School of Medicine under standard conditions of humidity (50 ± 10%), temperature (23 ± 2°C), and light (12/12 h light/dark cycle), according to the Institutional Animal Care Guidelines. The experimental protocols were reviewed and approved by the Animal Care and Use Committee at Mie University Graduate School of Medicine.

2.2. Human CRC Cell Line. The RFP-expressing human CRC cell line (RFP-HT29) was purchased from AntiCancer Japan. RFP-HT29 cells were grown in monolayer cultures in RPMI 1640 (Sigma-Aldrich, Inc., St. Louis, Mo, USA) supplemented with fetal bovine serum (10% (v/v), GIBCO BRL, Tokyo, Japan), glutamine (2 mM), penicillin (100,000 units/L), streptomycin (100 mg/L), and gentamycin (40 mg/L) at 37°C in a 5% CO_2 environment. For routine passage, cultures were spilt 1 : 10 when they reached 90% confluence, generally every 3 days. Cells at the fifth to ninth passage were used for liver metastasis experiments.

2.3. Experimental Liver Metastasis Model. RFP-HT29 cells were inoculated into the spleens of GFP nude mice, as a xenogeneic tumor model. RFP-HT29 cells at the fifth to ninth passage were harvested with trypsin/EDTA and washed in serum-containing RPMI 1640 medium to inactivate any remaining trypsin. The cells were centrifuged and resuspended in phosphate-buffered saline (PBS). Finally, the cells were adjusted to 1×10^7 cells/mL for single-cell suspensions. GFP nude mice were anesthetized by intraperitoneal injection of chloral hydrate (Sigma, St Louis, Mo, USA). Under direct vision, 1×10^6 cells were injected into the spleen using a 30-gauge needle through a small incision in the left lateral abdomen of anesthetized GFP nude mice.

2.4. Surgical Procedures for Intravital TPLSM (Figure 1). After inoculation, GFP nude mice were anesthetized by intraperitoneal injection of chloral hydrate. Body temperature was kept at 37°C throughout the experiments using a heating pad. The upper midline laparotomy was made as short as possible (<15 mm). The left lateral lobe of the liver was identified and exteriorized through the laparotomy. The liver lobe was then put onto an organ-stabilizing system (Japanese Patent Application number; P2007-129723) using a solder lug terminal with an instant adhesive agent (KO-10-p20, DAISO, Japan). The organ stabilizer minimized the microvibration of the observed area caused by heart beat and respiratory movements. Stabilization and fixation of the liver lobe represented a critical but technically difficult part of the intravital TPLSM procedure. After the application of PBS to the observed area, a thin cover glass was placed gently on the liver surface. After intravital TPLSM, the exteriorized liver lobe was gently removed from the organ-stabilizing system using a release agent (KO-10-p8, Daiso, Japan), to prevent liver injury. A sodium hyaluronate and carboxymethylcellulose membrane (Seprafilm Adhesion Barrier, Genzyme Corporation, Cambridge, Mass) was placed between the liver and the abdominal wall to prevent postoperative dense adhesion.

2.5. Time-Series TPLSM for Time-Course Imaging. The process of tumor angiogenesis during colorectal liver metastasis was observed in the livers of the same living mice repeating TPLSM at multiple time points using the above-mentioned surgical procedures until nondissecting adhesions formed between the liver and the abdominal wall. In preliminary experiments, the intravital TPLSM images at four or more time points were too unclear to observe colorectal liver metastases. If there was no dense fibrous adhesion between the liver and the abdominal wall, intravital TPLSM with clear image could be performed four or more times over intervals ranging from days to months. In reality, the time-series TPLSM consisted of three times intravital TPLSM procedures performed on the same mouse (see below; Section 2.8). The intervals of three time points (2 hour, 24 hour, and 8 week after inoculation) were also determined by preliminary experiments before this study. Precautions to prevent postoperative intraperitoneal infection were taken during the entire surgical procedure of time-series TPLSM.

2.6. TPLSM Setup. The procedures for TPLSM setup were performed as previously described [9]. Experiments were performed using an upright microscope (BX61WI; Olympus, Tokyo, Japan) and a FV1000-2P laser-scanning microscope system (Fluoview FV1000MPE, Olympus, Tokyo, Japan). The use of special stage risers enabled the unit to have an exceptionally wide working distance. This permitted the stereotactically immobilized, anesthetized mouse to be placed on the microscope stage. The microscope was fitted with several lenses with high numeric apertures to provide the long working distances required for *in vivo* work and with water-immersion optics. The excitation source in TPLSM mode was Mai Tai Ti: sapphire lasers (Spectra Physics, Mountain View, Calif), tuned, and mode-locked at 910 nm. The Mai Tai produces light pulses of about 100 fs width (repetition rate 80 MHz). Laser light reached the sample through the microscope objectives, connected to an upright microscope (BX61WI; Olympus, Tokyo, Japan). A mean laser

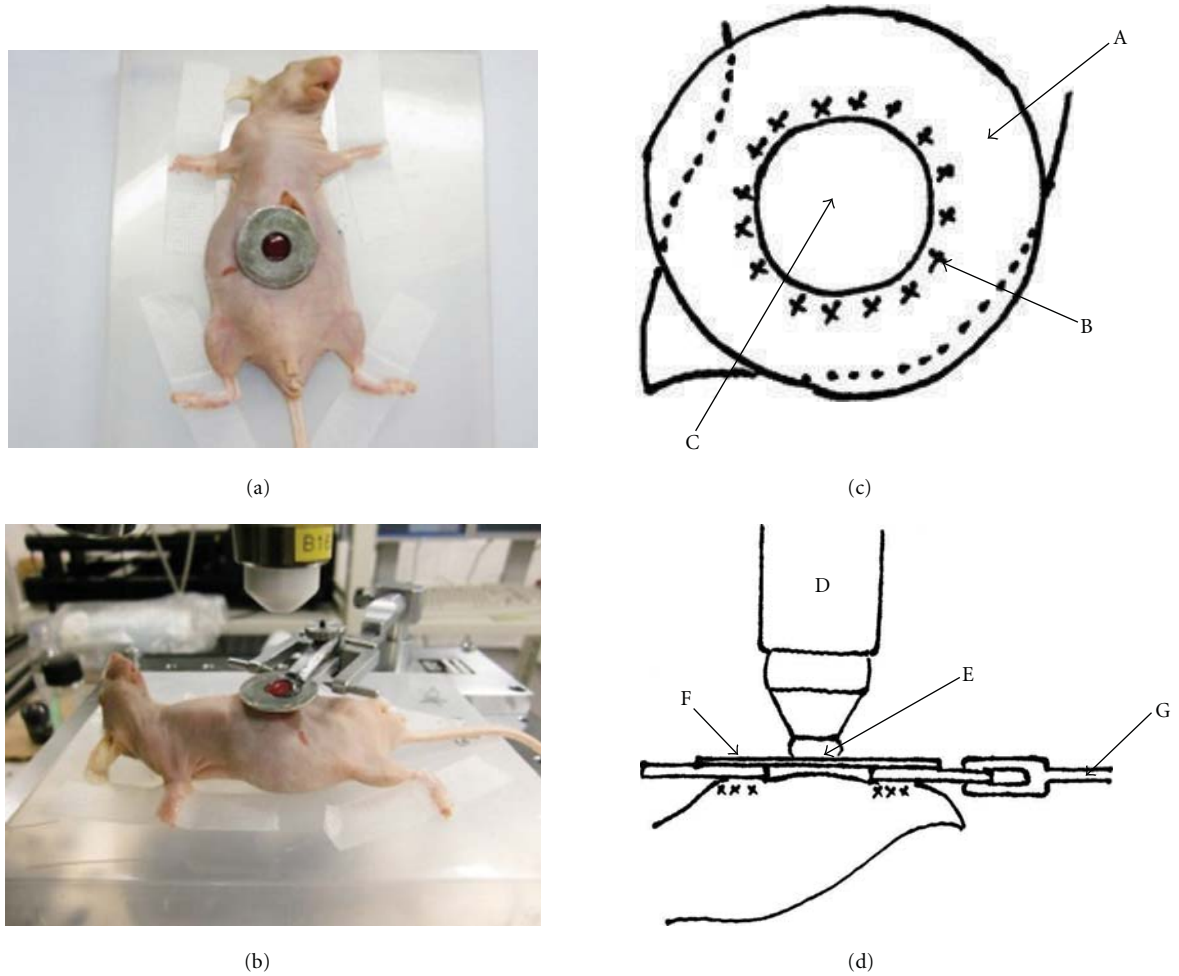


Figure 1: Overview and schematic drawing of liver-lobe fixation and intravital TPLSM setup. (a) Exteriorization of the left lateral lobe following upper midline laparotomy, followed by fixation of the left lateral lobe to a solder lug terminal using an instant adhesive agent. (b) Placement of an anesthetized mouse on the stage and setup of the organ-stabilizing system. (c) A solder lug terminal was adhered in a waterproof manner to the surface of the left lateral liver lobe using an instant adhesive agent. (d) PBS was applied to the observation area, and a watertight thin cover glass was placed over the area. The solder lug terminal was held on by the organ-stabilizing system. A: solder lug terminal, B: instant adhesive agent, C: observation area, D: objective, E: water immersion, F: cover glass, G: organ-stabilizing system.

power at the sample was between 10 and 40 mW, depending on the depth of imaging. Microscope objective lenses used in this study were 4x UPlanSApo (numerical aperture of 0.16), 10x UPlanSApo (numerical aperture of 0.4), and 60x LUMPlanFI/IR (water dipping, numerical aperture of 0.9, working distance 2 mm), respectively. Data were analyzed using a FV10-ASW (Olympus, Tokyo, Japan). TPLSM images were acquired with 512×512 pixels spatial resolution, from 210 μm field of view dimension, using a pixel dwelling time 4 μs. Two-photon fluorescence signals were collected by an internal detector (nondescanned detection method) at an excitation wavelength, to enable the simultaneous acquisition of EGFP signal and RFP (DsRed2) signal. In fact, an excitation wavelength of 1050 nm is optimum for DsRed2 as reported by Kawano et al. [10], although an excitation wavelength of 910 nm is optimum for EGFP. Therefore, it is difficult to excite DsRed2 at 910 nm amply. To overcome this difficulty, we have selected and used the tumor cells in which expression level of DsRed2 is so high that we can identify the DsRed2-labeled tumor cells clearly even with the 910 nm excitation. Color-coded green and red images were imaged at the same time and subsequently merged to produce single images.

2.7. Imaging of Colorectal Liver Metastasis Using Interval TPLSM. The surface of the liver lobe was initially screened at lower magnifications by setting out the *X/Y* plane and adjusting the *Z* axis manually to detect the optimal observation area containing RFP-expressing cancer cells (at least five areas). Each area of interest was subsequently scanned at a higher magnification (water-immersion objective 60x with or without 2x zoom) by manually setting the *X/Y* plane and adjusting the *Z* axis (either automatically or manually) to obtain high-resolution, clear TPLSM images. The scanning areas were 200×200 μm (600x) or 100×100 μm (600x with 2x zoom), respectively. The imaging depth or imaging stack was

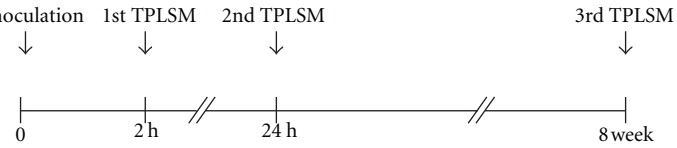


Figure 2: Experimental protocol and timing of intravital TPLSM. First-round intravital TPLSM at 2 hours after inoculation. Second-round intravital TPLSM at 24 hours after inoculation in the same mouse, for imaging the early events of colorectal liver metastasis. Third-round intravital TPLSM at 8 weeks after inoculation in the same mouse, for imaging-established liver metastases and tumor angiogenesis.

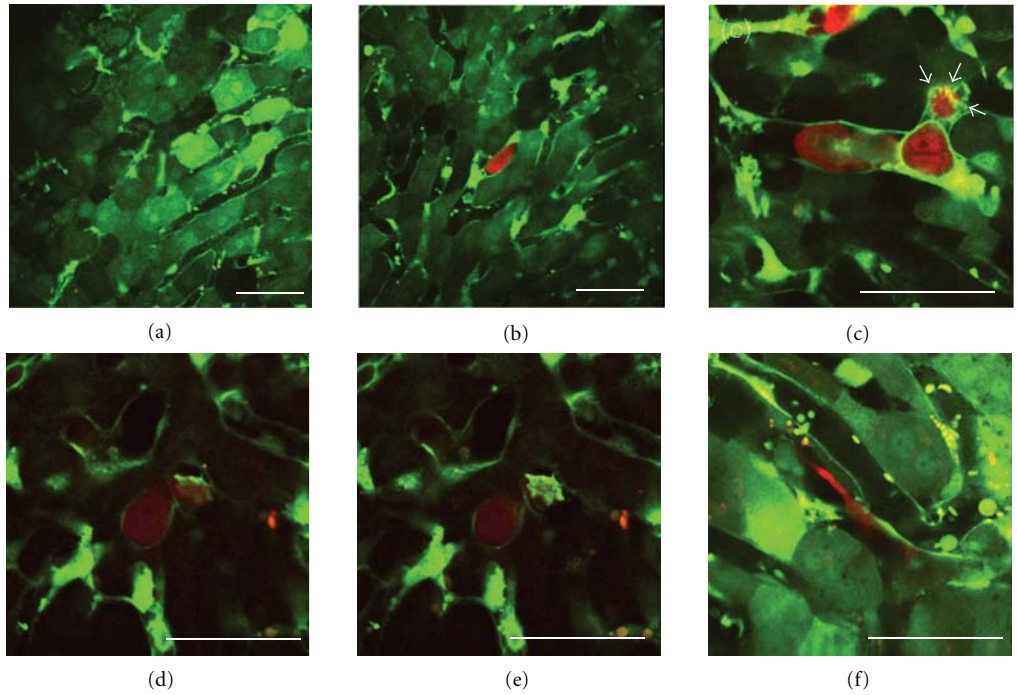


Figure 3: Imaging of early events of colorectal liver metastasis by intravital TPLSM. (a) Normal liver. (b) Arrest of a tumor cell in the hepatic sinusoid (2 hours after inoculation). (c) Tumor-cell-induced platelet aggregation (white arrows, 2 hours after inoculation). (d) Phagocytosis by a Kupffer cell (24 hours after inoculation). (e) Phagocytosis by a Kupffer cell at different depth. (f) Extravasation of a tumor cell (24 hours after inoculation) (bar, 50 μm).

determined arbitrarily to allow real-time three-dimensional visualization of colorectal liver metastasis *in vivo*. The laser power was adjusted according to the imaging depth. When imaging at larger depths, we increase the laser-power level (up to 100%) manually using laser power level controller. To image the optimal simultaneous imaging of EGFP and RFP (DsRed2), detection sensitivity (brightness by HV) was adjusted manually for EGFP (450–500) or RFP (550–600), respectively.

2.8. Experimental Schedule for Time-Series TPLSM. RFP-HT29 cells were inoculated into the spleens of GFP nude mice. Colorectal liver metastasis formation was observed by 8 weeks in this xenogeneic tumor model (data not shown). As shown in Figure 2, first-round intravital TPLSM was performed to observe the early stages of colorectal liver metastasis (tumor cell arrest/adhesion and tumor-cell-induced platelet aggregation) at 2 hours after inoculation. Second-round intravital TPLSM was also performed to observe the early stages of colorectal liver metastasis (Phagocytosis by Kupffer cells and extravasation of cancer cells) at 24 hours after inoculation in the same mouse. Established liver metastases were observed during third-round intravital TPLSM, performed 8 weeks after the inoculation of RFP-HT29 cells into GFP nude mice. With regard to these time points (2 hour, 24 hour, and 8 week after inoculation), we have performed preliminary experiments to determine the optimal time points. The intravital TPLSM images at four or more time points were too unclear to observe colorectal liver metastases because of abdominal fibrous adhesion. Thus, all mice were imaged at three time points. At the end of the experiments (8 weeks after inoculation), the whole liver was harvested and subjected to histopathological analysis.

2.9. Immunohistochemical Analysis of Cytokeratin 20. The lack of immunological cross-reactivity with other cytokeratins means that CK 20 has become an important tool for delineating the origin of metastatic human adenocarcinomas

arising from an unknown primary source. Mouse livers were removed and fixed in 4% formaldehyde in PBS (pH 7.4) for 24 hours, processed, and embedded in paraffin wax according to standard procedures. Formalin-fixed, paraffin-embedded tissue was sliced at a thickness of 3 μm, and the sections were placed on silane-coated slides. After deparaffinization and dehydration, the sections were autoclaved for 10 minutes in 10 mM sodium citrate buffer for antigen retrieval. They were blocked and incubated with primary antibody overnight at 4°C. Primary monoclonal anti-human cytokeratin 20 antibody (Clone KBsB20.8; DakoCytomation, Denmark) was used at a dilution of 1 : 50 for implementation of the labeled streptavidin-biotin method (LASB2 kit/HRP, DakoCytomation). Cytokeratin 20 was detected using Envision reagents (Envision kit/HRP, DakoCytomation). The sections were counterstained using hematoxylin. Negative controls were run simultaneously with preimmune immunoglobulin.

3. Results

3.1. The Development of Intravital TPLSM for In Vivo Real-Time Imaging of Colorectal Liver Metastasis. Figure 1 shows an overview and schematic drawing of liver-lobe fixation and intravital TPLSM setups.

The key steps were (1) optimal longitudinal laparotomy; (2) choice of left lateral lobe of the liver for fixation using an organ-stabilizing system; (3) liver fixation using a solder lug terminal and an instant adhesive agent; (4) adjustment of detector gain for simultaneous dual-color imaging of RFP-expressing cells in the liver of GFP mice; (5) release of the liver lobe from the solder lug terminal using a release agent and placement of a Seprafilm Adhesion Barrier to the abdomen for interval TPLSM.

A success rate approaching 100% for intravital and interval TPLSM can be achieved after a practice period.

3.2. Imaging of Early Colorectal Liver Metastasis by Intravital TPLSM. Intravital TPLSM imaging can be represented as a time-lapse two-dimensional movie and also as a *z*-stack three-dimensional movie from the liver surface to approximately 100–200 μm depth. The imaging depth for three-dimensional visualization of the *in vivo* real-time metastatic events and tumor angiogenesis was determined arbitrarily and depended in part on the positioning of the liver lobe using the organ-stabilizing system or on the laser power.

To observe the early events of colorectal liver metastasis at higher resolution and in dual color, at least five areas containing RFP-expressing cancer cells were initially identified at lower magnifications. Each area of interest was subsequently observed at higher magnification. The scanning areas were 200 × 200 μm or 100 × 100 μm, respectively. If necessary, additional five areas were observed by manually setting the *X/Y* plane and either automatically or manually adjusting the *Z* axis.

Table 1 shows the incidence of the early events of colorectal liver metastasis using this model. The phenomena of tumor cell arrest/adhesion and tumor-cell-induced platelet aggregation [11, 12] were frequently observed at 2 hours after inoculation (Figure 3(b), 3(c)). Phagocytosis by Kupffer cells and extravasation of cancer cells were observed within 24 hours after inoculation (Figure 3(d), 3(e), 3(f)) but were rare events.

Table 1: Early events of colorectal liver metastasis in the portal route model.

Early event	RFP-HT29 cells in GFP nude mice
Tumor-cell arrest/adhesion	20/20* (100%)#
Tumor-cell-induced platelet aggregation	15/20 (75%)
Extravasation from hepatic sinusoids	1/20 (5%)
Phagocytosis by Kupffer cells	2/20 (10%)

*Number of mice having the indicated phenomenon/number of mice inoculated with the indicated cells.
#Percentage.

Table 2: Rate of liver metastasis formation and observation by intravital TPLSM.

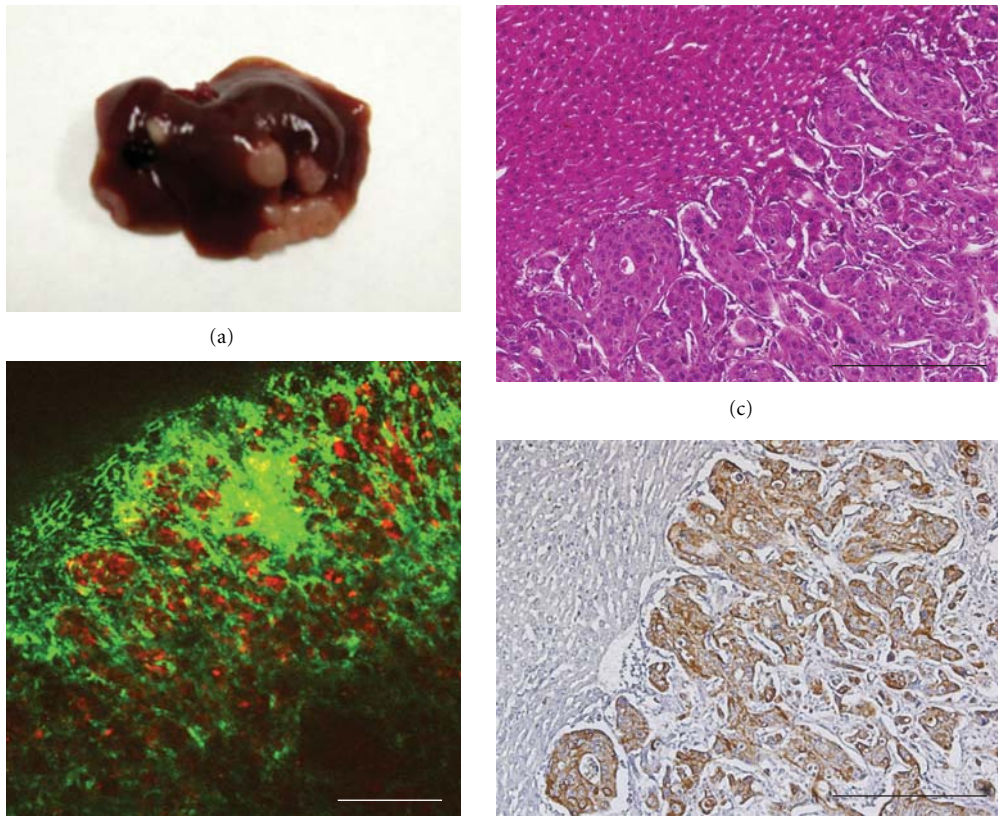
	RFP-HT29 cells in GFP nude mice
Liver metastases after intrasplenic inoculation	70%[a] (12/17)[b] at 8 weeks
Observation of metastasis by intravital TPLSM	67%[c] (8/12)[d] at 8 weeks

[a]Percentage of liver metastases after intrasplenic inoculation.
[b]The number of mice with liver metastases/the number of mice avaiable at the indicated time points.
[c]Percentage of successful observation of metastatic colonies by intravital TPLSM.
[d]The number of mice successfuly observed metastatic colonies by intravital TPLSM/the number of mice with liver metastases.

3.3. Imaging of Liver Metastatic Colonization and Tumor Angiogenesis by Intravital TPLSM. One mouse died of unknown causes within 24 hours of inoculation. Second-round intravital TPLSM was performed successfully in the remaining 19 GFP nude mice (19/19; 100%). Two more mice had died of colorectal liver metastases by 8 weeks after inoculation, before the scheduled third-round intravital TPLSM. The remaining 17 GFP nude mice were successfully imaged by third-round intravital TPLSM (17/17; 100%).

Among these 17 GFP nude mice, 12 (70%) had liver metastases with localized growth patterns at 8 weeks after inoculation. Only the left lateral lobe of the liver could be imaged using our experimental protocol, and four mice had liver metastases outside the observation area. As a result, only eight (67%) of the 12 mice were successfully imaged by third-round intravital TPLSM (Table 2).

Several metastatic nodules had been formed by RFP-HT29 cells approximately 8 weeks later (Figure 4(a), 4(b), 4(c)). Anti-human cytokeratin 20 antibody was used for the conformation of RFP-HT29 cells in the xenogeneic liver metastasis model. RFP-HT29 cells in micrometastatic



(a) (b) (c) (d)

Figure 4: Liver metastatic colonization by RFP-expressing HT29 cells. (a) Macroscopic findings. (b) Intravital TPLSM image (bar, 300 μm). (c) Microscopic hematoxylin-eosin staining (bar, 500 μm). (d) Microscopic cytokeratin 20 immunostaining (bar, 500 μm).

colonies (brown colored cells) were shown immunohistochemically (Figure 4(d)).

Each nodule was composed of tumor cell clusters and dilated/tortuous tumor vessels (Figures 5, and 6). A flow of aggregated platelets was frequently observed within the tumor vessels, suggesting the existence of a hypercoagulable state in the tumor microenvironment (data not shown).

4. Discussion and Conclusion

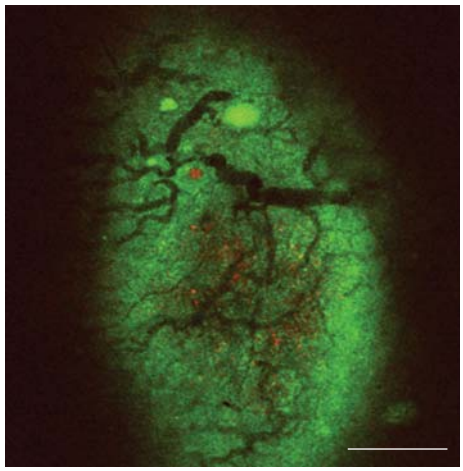
We have established a new method for investigating tumor metastasis and angiogenesis using intravital TPLSM. Using our model, the real-time development of colorectal liver metastases can be imaged *in vivo* at high optical resolution and in dual color (red: cancer cells, green: host cells) using intravital TPLSM. It is also possible to perform time-course imaging of metastasis and angiogenesis in the livers of the same living mice using time-series TPLSM, which consists of three times intravital TPLSM procedures performed at different time points over periods of days to months.

These techniques enabled us to observe metastatic nodules containing viable cancer cells (red) and tumor vessels in the surrounding stroma (green) in the same living mice at different time points.

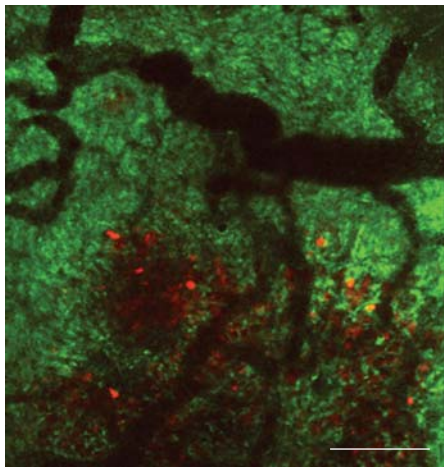
In this study, red-colored cancer cells were visualized in real-time in the green-colored liver structures of GFP mice *in vivo*. Tumor cell arrest/adhesion, tumor-cell-induced platelet aggregation, tumor cell extravasation, and phagocytosis by Kupffer cells could be observed using both time-lapse and *z*-stack imaging techniques (data not shown). Liver metastatic nodules composed of tumor cell clusters and dilated/tortuous vessels could also be observed in the same mice approximately 8 weeks later.

Previous studies of tumor angiogenesis using multiphoton microscopy have imaged tumor blood vessels by injecting a fluorescent contrast agent [6–8, 13, 14], and blood cells including leukocytes, erythrocytes, and platelets, or endothelial cells could therefore not be visualized. However, blood cells, except for erythrocytes and endothelial cells, can be visualized at higher optical resolutions and at higher magnifications using our TPSLM techniques. The interaction between intravascular cancer cells and endothelial cells or blood cells during the early stage of liver metastasis, as well as metastatic tumor growth with tumor angiogenesis during the late stage of liver metastasis, could therefore both be observed in the same living mice.

However, this method has several limitations that need to be overcome. The high magnification means that only a narrow observation field can be viewed, which represents only a fraction of the total tumor volume. The frequency of observations that can be made using time-series TPLSM is also limited. In addition, exteriorization of the liver and the

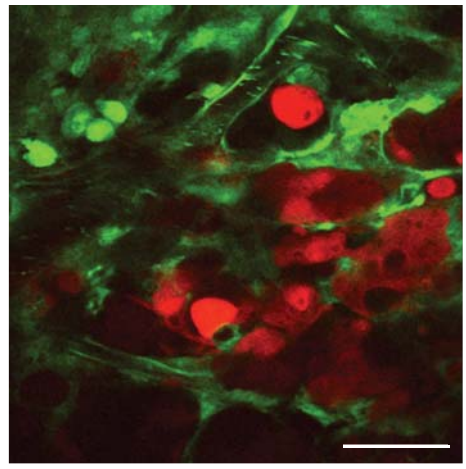


(a)

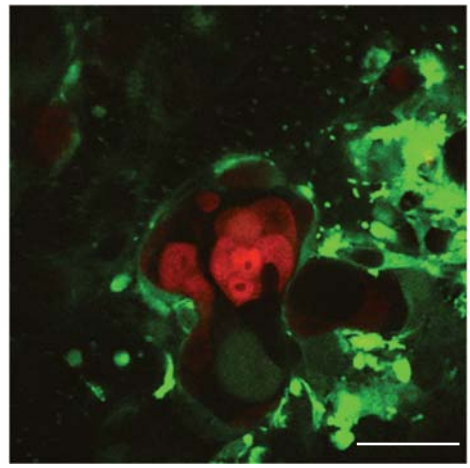


(b)

Figure 5: Imaging of liver metastasis and tumor angiogenesis by intravital TPLSM. (a) Liver metastatic nodule with dilated and tortuous tumor vessels (×40, bar, 750 μm); (b) (×100, bar, 300 μm).



(a)



(b)

Figure 6: High-resolution optical imaging of liver metastatic nodule by intravital TPLSM. (a) Liver metastatic nodules were composed of tumor cell clusters and dilated/tortuous vessels (×600, bar, 50 μm). (b) Dilated and tortuous tumor vessels were observed among the clusters of several tumor cells. A flow of aggregated platelets was frequently observed within the tumor vessels (×600, bar, 50 μm).

development of fibrous adhesions between the abdominal wall and liver surface may affect the physiological condition of the living liver.

Tumor-host interactions play an important role in tumor metastasis and angiogenesis [1, 15] and are thus a therapeutic target in human cancers [2, 16]. *In vivo* visualization of real-time tumor-host interactions at the cellular or subcellular level with high spatial resolution therefore represents a valuable means of studying tumor angiogenesis in a preclinical tumor model.

Like a TPLSM, second harmonic generation (SHG) imaging is also a nonlinear microscopy technique which directly visualizes the collagen assembly. Thus, SHG microscopy also becomes an interesting application to study the tumor-stromal interaction by imaging the stromal collagen. In the future study, we need to use SHG imaging as well as TPLSM for analyzing the tumor-host interaction including tumor metastasis and angiogenesis.

High-resolution, dual-color, *in vivo* real-time visualization of tumor metastasis and angiogenesis using intravital and time-series TLPSM can help to improve our understanding of spatiotemporal tumor-host interactions during metastatic processes in the organs of living animals. It may also provide an ideal method for antiangiogenic drug evaluation, reducing the effects of interindividual variation.

Acknowledgments

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Review Article

Tumor Angiogenesis: Pericytes and Maturation Are Not to Be Ignored

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Angiogenesis, an essential component of tumor growth and survival, is regulated by complex interactions between several cell types and soluble mediators. Heterogeneous tumor vasculature originates from the collective effect of the nature of carcinoma and the complexity of the angiogenic network. Although the application of angiogenesis inhibitors in some types of cancers has shown clinical benefits, predictive markers to assess treatment effects have yet to be established. In this review, we focus on tumor vessel maturity as a potential marker for evaluating treatment response.

1. Introduction

Like normal tissues, malignant tissues are dependent on an adequate blood supply. Unlike normal tissues, however, angiogenesis is reactivated under pathological conditions, such as wound healing and malignancies [1]. Inflammation, metabolic stress and hypoxia are three major conditions involved in angiogenesis [2]. As malignant cells grow, the "demand" for nutrients and oxygen necessitates new "supply" routes, that is, new blood vessels. Early studies in this field have revealed that a tumor mass cannot exceed 1 mm^3 without angiogenesis [3]. Although endothelial cells (ECs) are typically quiescent in humans [4], they can proliferate once the angiogenic switch turns on. This switch is off or differentially regulated in normal tissues based on the equilibrium between positive and negative angiogenic regulators. Upon receiving dominant proangiogenic stimuli from malignant cells or the tumor microenvironment through several effectors, such as vascular endothelial growth factors (VEGFs), platelet-derived growth factor (PDGF), placenta-derived growth factor (PlGF), hypoxia-inducible factor-1 (HIF-1α), angiopoietin-2, transforming growth factor β (TGF-β), or interleukin-8, ECs from preexisting vessels become activated. Activated ECs modify their interaction with perivascular cells (pericytes, PCs) and release proteases to degrade the surrounding basement membrane and extracellular matrix to facilitate EC proliferation and sprouting into the matrix [5]. Endothelial precursor cells (EPCs) from bone marrow also integrate with these growing vessels [6].

2. Tumor Vessel Maturity

Sprouting microvessels establish a plexus that keeps all tumor cells within a distance of 100–200 μm from the blood supply [7]. This new architecture is considered "immature" and differs from normal (or mature) vascular structures in many ways. Immature vessels lack vascular organization and hierarchy and are unevenly distributed in tumor tissue. They are usually irregularly shaped, tortuous and dilated. As a result of impaired cell-to-cell attachment, abnormal basement membranes, and increased permeability, the new microvessels are leaky and cannot sustain balanced intravascular pressure gradients, which can lead to interstitial hypertension. ECs in the tumor vasculature are dependent on cell survival factors (e.g., VEGF) for survival in contrast to ECs in normal tissues [8]. Pericytes, which stabilize ECs and mediate EC survival and maturation in normal vasculature both through direct cell contact with ECs and paracrine signaling, are also abnormal in the tumor vasculature [9]. Pericytes are usually absent in the tumor vasculature or

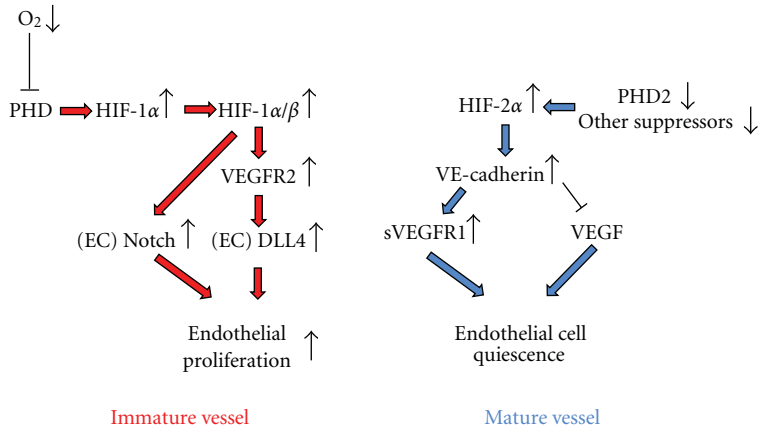

Figure 1: Hypoxia inducible factors (HIFs) and their role in endothelial cell proliferation. HIF-1α is hydroxylated by prolyl hydroxylase domain proteins (PHDs) and degraded in proteasomes under oxygenated conditions. When the oxygen level decreases, PHD activity is reduced, which leads to the accumulation of HIF-1α. Upon formation, the HIF-1α/β complex activates the transcription of numerous genes. Hypoxia and HIF-1α enhance the expression of VEGFR2, which induces DLL4 expression in the tip cell. Furthermore, DLL4 interacts with the Notch intracellular domain and increases its activity, which increases endothelial cell proliferation. Upregulation of HIF-2α due to lower degradation activates the junctional protein vascular endothelial cadherin (VE cadherin). VE cadherin induces a normalized endothelial phenotype by inhibiting VEGF-driven proliferation and upregulating the soluble isoform of the VEGF-trap VEGFR1 [12].

have loose associations with ECs, leaving most of the tumor microvessels immature. The basement membrane is rich in collagen type IV and a source of growth factors for ECs and PCs in normal vessels. In tumor vessels, the basement membrane has defects in its structure and composition and can provide a productive environment for metastatic malignant epithelial cells and proliferating ECs [10].

ECs are proliferative in a diverse manner based on immunostaining for proliferation molecules in cancer tissues [11].

During tumor growth, the tumor vasculature develops as the angiogenic switch is intermittently turned on and off [12]. The imbalance between tumor cell growth and vascular formation may often cause a collapse of tumor cells and the new vasculature. Hypoxic areas exist heterogeneously in the majority of human solid cancer tissues [13]. HIFs are a family of transcription factors that are activated in response to hypoxic tumor tissue in different ways (Figure 1).

3. Angiogenesis in Human Cancer

Breast cancer is the most common cancer among women and is studied as an angiogenic carcinoma due to the high expression levels of proangiogenic factors, such as VEGFs, HIF-1α, TGF-β, or thymidine phosphorylase (TP), in carcinoma cells [14]. Various components of the angiogenic pathway have been studied as prognostic and predictive factors in breast cancer. Distinct patterns of vascularity (using an EC marker) in ductal carcinoma *in situ* (DCIS) might be useful to identify patients who are at risk of relapse [15]. Bone marrow metastasis as a direct result of interactions between carcinoma cells and the vascular network has been shown to have a higher prevalence in breast cancer patients with high-grade vascular tumors [16]. Angiogenesis has been used to predict the likelihood of tumor response to adjuvant chemotherapy or hormone therapy. Heterogeneous characteristics of biological markers, such as estrogen and progesterone receptors (ER and PgR, resp.) as well as human epidermal growth factor receptor-2 (HER-2), define the clinical outcome of breast cancer patients. Patients with ER-positive tumors have been shown to have prolonged disease-free survival and an increased likelihood of tumor response to endocrine therapies [17]. After the introduction of trastuzumab, a humanized monoclonal antibody directed against the extracellular domain of ErbB [18], HER-2-targeted therapies has changed the disease course of originally aggressive type of HER-2-positive breast cancer [19]. Triple-negative breast cancer (ER-, PgR-, and HER-2-negative; TNBC) comprises a heterogeneous subgroup of tumors characterized by an aggressive clinical course and poor survival and is not amenable to hormone therapy or HER2-directed agents [20].

Breast cancer is classified into distinct molecular subtypes based on expression profiling using DNA microarrays [21]. These subtypes are luminal A, luminal B, (HER2)-overexpressing, normal breast tissue-like, basal-like and the more recently identified subtype claudin-low [22]. These subtypes respond differently to therapy and are associated with different outcomes. The shortest survival times have been observed in patients with the basal-like and HER2-overexpressing subtypes [23]. Highly proliferative tumors, including those that are negative for the estrogen, progesterone, and HER2/neu receptors, have enhanced angiogenesis, which supports rapid growth and early metastases and have been found to have high levels of VEGF [24]. Many of the molecules involved in neovascularization pathways have become targets for antiangiogenic drugs, which are under evaluation in clinical trials or are currently administered in clinics [25]. Although many of these therapies, such as

bevacizumab (anti-VEGF-A), are being used in combination with other therapeutic modalities, such as chemotherapy and endocrine therapy [26], studies are ongoing to find the optimal method to elucidate tumor response and overcome therapeutic resistance to antiangiogenic treatments. Genomic activation of VEGF-A is higher in TNBCs compared to other subgroups of breast cancer and suggests a specific role for bevacizumab treatment in this subgroup [27].

Although several distinguishing molecular features have been characterized in basal-like cancer cells, the microenvironmental features of this type of cancer have not been well characterized. A recent study on cocultures of breast cancer cell lines with fibroblasts has identified stromal interactions that distinguish basal-like from luminal-type breast cancers [28].

In a recent study on 1788 primary invasive breast cancers, VEGF expression is correlated with intrinsic subtypes with a higher frequency in luminal-type B, HER2, and basal-like types but not luminal-type A [24]. In another study of 564 tissue microarrays from primary tumors from premenopausal breast cancer patients who had been randomized to adjuvant tamoxifen or no adjuvant treatment, TNBCs show increased protein expression of epithelial growth factor receptor (EGFR) and VEGFR2, whereas the expression of VEGF-A is not a specific biomarker of TNBCs [27].

A pathological examination using CD34 of one thousand early-stage primary breast cancer specimens has shown that basal-like breast cancer and TNBCs had significantly higher microvessel densities (MVDs) than the nonbasal and non-TNBC groups [29]. In basal-like breast cancers, high MVD was associated with a larger tumor size and a higher grade. However, this association is not apparent in the TNBC group. CD34 has been reported to be present on endothelial progenitors [30]. Despite the fact that other morphological characteristics of the tumor vasculature are not available for evaluation, higher CD34-positive vascular structures might show a trend for the presence of immature vessels in basal-like breast cancers and TNBCs in this study. In addition, investigators have shown that in TNBCs and basal-like breast cancers, vascular invasion almost entirely consists of lymphatic vessels. Basal-like breast cancers have been reported to preferably metastasize to the brain and liver [31] and have been interpreted as distinct blood-borne metastases. However, the results from the aforementioned study might be explained by other molecular features of the interactions between basal-like malignant cells and endothelial cells.

In an *in vitro* study on breast cancer cell lines with different expression levels of ERα, Ang-1 mRNA and protein levels are higher in MDA-MB-231 cells (ERα-negative cell line) compared to those of MCF-7 cells, S30 cells, and HMEC (all ERα-positive cell lines) [32]. E2 treatment significantly attenuates Ang-1 mRNA and protein expression levels in S30 cells. Ang-1, VEGF, and CD31 staining in tumor samples from animals that have been inoculated with S30 and MDA-MB-231 cells reconfirms decreased angiogenesis *in vivo* in tumors that originated from the ERα-positive cell line.

Circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) have a significant prognostic role in breast cancer patients [33]. These cells have heterogeneous biological characteristics that promote metastasis. In addition to the proangiogenic role of VEGF, VEGF stimulates tumor cell proliferation [34]. In a study of CTCs obtained from breast cancer patients, VEGF and its upstream regulators HIF-1 and phosphorylated-focal adhesion kinase (pFAK) are expressed in 73%, 56%, and 81% of detected CTCs, respectively [35]. Although the biological significance of these findings remains unknown, they implicate a possible role for angiogenic characteristics in the metastatic behavior of CTCs.

A genomic study on 134 primary breast cancers and 27 regional or distant metastases showed a high expression of a 13-gene cluster (VEGF profile) containing *VEGF*, *angiopoetin-like 4* (*ANGPTL4*), and *adrenomedullin* (*ADM*) in tumors from patients with confirmed distant disease at the time of diagnosis (i.e., MetScore = 3) [36]. *VEGF* and *ANGPTL4* are endothelial cell growth inducers, and *ADM* is an inducer of lymphatic vessel growth [37, 38]. In addition, "perinecrotic" HIF-1α IHC staining correlates with the expression of the VEGF profile. Eight of these 13 genes had hypoxia response elements that are 2000 bp upstream of their start codons. The VEGF profile also correlates with the expression profile of three individual genes (*Snail, Twist,* and *HIF-1*α) and the intrinsic subtype of breast cancer.

BRCA-associated breast cancers are different from spontaneous breast cancers in many aspects, such as morphology, triple negativity, basal cytokeratin expression, and p53 mutations [39]. HIF-1α overexpression is more frequent in BRCA1-related breast cancer compared to that in sporadic cancer in a small series of 30 cases [40]. Elevated expression of HIF-1α and the loss of prolyl hydroxylase enzyme 3 (PHD3) and factor inhibiting HIF (FIH) in the nucleus have been observed in 125 BRCA-associated breast cancers [41]. PHD3 and FIH are responsible for the HIF-1α degradation and modulation observed in BRCA1-mutated breast cancers. This observation might explain how the BRCA1 tumors enhance hypoxic drive.

The number of microvessels that are positive for vasohibin-1 (a negative feedback regulator of angiogenesis) and vasohibin-1 mRNA levels in 17 breast ductal carcinomas *in situ* (DCIS) is significantly lower compared to those of 22 invasive ductal carcinomas [42]. This difference has not been observed when analyzing CD31. However, the number of vasohibin-1-positive microvessels and vasohibin-1 mRNA levels shows significant correlations with the Ki-67-labeling index and a high nuclear and histological grade in DCIS cases.

Multiple roles of COX-2 in tumor angiogenesis, such as VEGF production, the promotion of vascular sprouting, migration, and tube formation, have been well studied [43]. COX2 expression occurs in malignant cells and under preneoplastic conditions, such as esophageal dysplasia [44]. In a study of 49 DCIS samples without any invasive component, the investigators have shown that VEGF expression is significantly associated with COX-2 expression [45]. This result is in agreement with a xenograft model in a human DCIS study that observed that COX2 upregulation in DCIS xenografts increased VEGF and MMP14 expression [46].

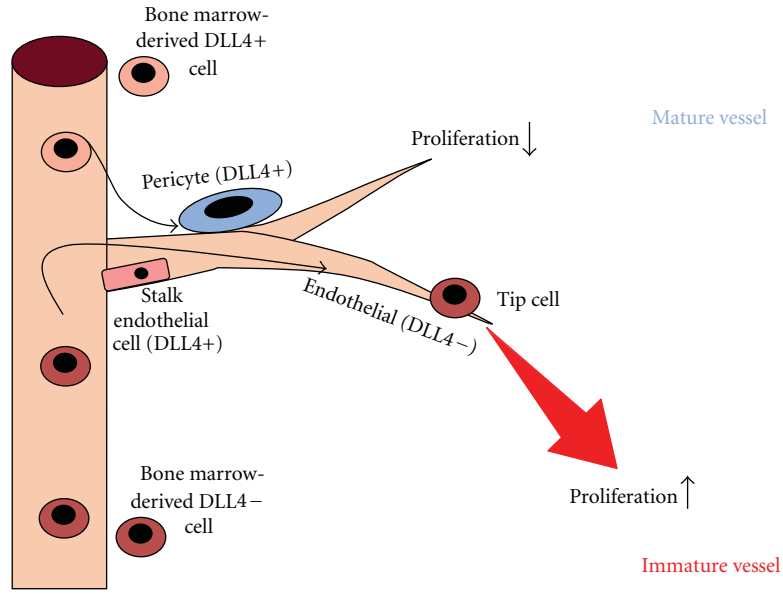

Figure 2: Delta-like ligand 4 (DLL4) and its expression in bone marrow-derived cells. The expression level of DLL4 dictates the role of bone marrow-derived cells in the neovasculature. DLL-4-Notch signaling controls tip cell versus stalk cell fate in endothelial cells and has a regulatory effect on pericyte formation [66].

4. Antiangiogenic Therapies and Pericytes

One of the main mechanisms of action of antiangiogenic agents is vascular normalization [47]. These drugs change the balance of pro- and antiangiogenic factors in the tumor tissue and fix the delivery system to ensure that oxygen and therapeutic drugs are effectively distributed to a larger number of tumor cells. In other words, they help the immature tumor vasculature mature. One of the well-studied factors that maintains vascular maturity is the close association between PCs and ECs [48]. Activated ECs sprout and form an endothelial tube, which is a lumen with an EC lining. These ECs stop proliferating and secrete PDGF to recruit PCs and progenitor PCs (e.g., from bone marrow), which express PDGF receptors (PDGF-Rs) [49]. Recruited PCs proliferate and encapsulate these new channels. Newly formed vessels that are enveloped with PCs mature and stop remodeling [50]. Pericytes stabilize the neovessels and are crucial for EC survival by locally releasing VEGF and angiopoietin-1 [51]. Therapies that target VEGF have been observed to selectively prune ECs that are not covered by PCs [52]. Paracrine EC-PC signaling that is mediated by members of the PDGF family may account for the relative resistance of more mature vessels to anti-VEGF therapies [53]. Although bevacizumab has shown clinical efficacy in the treatment of several tumor types, no treatment-predictive-markers have been established in clinics [54].

Hypertension (HTN) has been reported to be a common event associated with bevacizumab treatment, possibly due to EC-derived nitric oxide reduction and consecutive vascular smooth muscle constriction, which increases vascular resistance [55]. In a large meta-analysis, the incidence of HTN in bevacizumab-treated cancer patients was 23.6% with 7.9% of patients with grade 3-4 of HTN [56]. Recent studies on metastatic colon cancer [57], renal cell carcinoma [58], lung cancer [59], and breast carcinoma [60] have shown an association between bevacizumab-related HTN and a better outcome, which suggests that HTN may be a predictive factor. An exploratory retrospective analysis of samples from the AVF2119 g phase III trial has shown no progression-free survival (PFS) benefits from the addition of bevacizumab to neoadjuvant capecitabine in metastatic breast cancer patients and has revealed that subgroups with low expression of endothelial neuropilin-1 (NRP1), TP, VEGF-C, or endothelial delta-like ligand 4 (DLL4) showed trends toward PFS benefits [61]. This result is compatible with the study on rectal carcinoma showing that bevacizumab upregulated NRP1 in tumor-associated-macrophages [62]. NRP1 is a co-receptor for VEGF and is expressed on ECs, tumor cells and vascular smooth muscle cells [63]. Preclinical studies have shown that combining bevacizumab treatment with anti-NRP1^{B} treatment resulted in additive effects to inhibit tumor growth [64]. DLL4 expression on ECs activates the Notch signaling pathway, which results in the regulation of tumor angiogenesis in a VEGF-independent manner [65]. DLL4 is expressed by ECs, EPCs, and bone marrow-derived α-smooth muscle actin (α-SMA)-positive mural cells (e.g., pericytes) (Figure 2) [66]. Disruption of DLL4 signaling in combination with anti-VEGF treatment has shown additive effects on tumor growth [67]. Compared to 64.5% of DLL4-negative vessels, 98.7% of DLL4-positive tumor vessels are surrounded by α-SMA-positive pericytes in bladder cancer [68].

Monocyte chemoattractant protein-1 (MCP-1) is a protein that is produced by breast cancer cells and stromal cells and participates in VEGF-mediated angiogenesis [69]. We

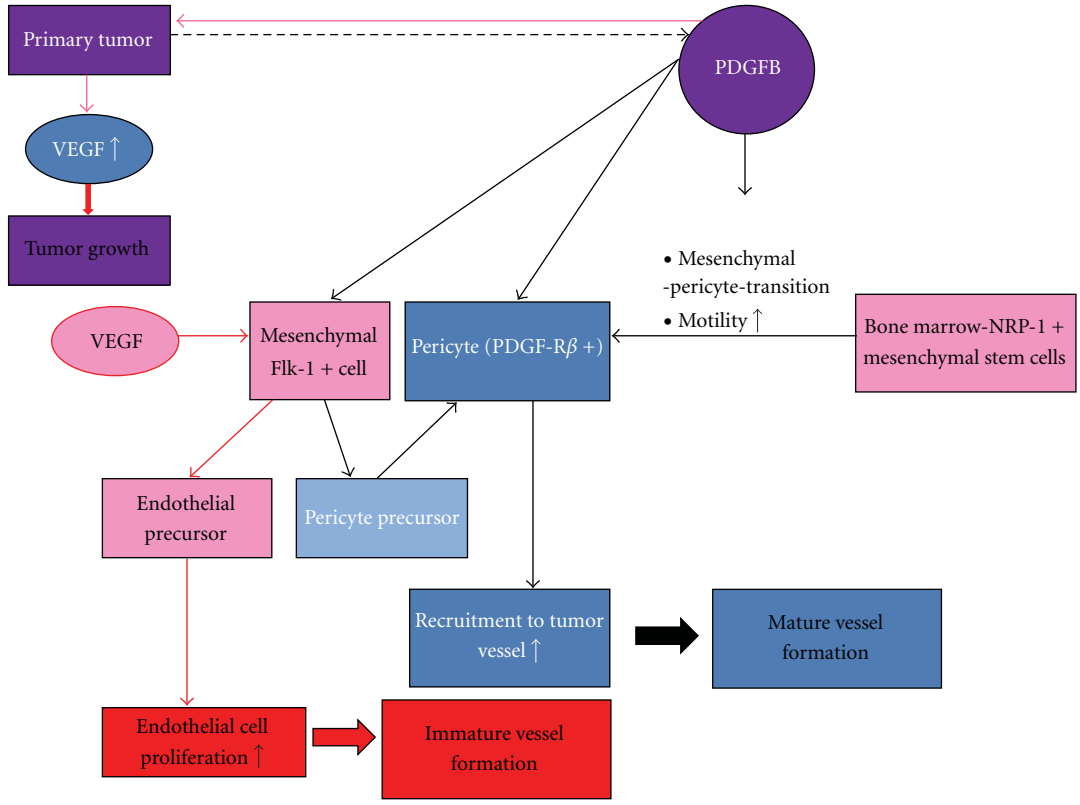

Figure 3: Tumor cell-derived PDGF-B induced pericyte differentiation. Incorporation of pericytes into newly formed vessels is one of the key steps to terminate angiogenesis. Studies in animal models have shown that PDGF-B is responsible for (1) differentiation of pericytes from mesenchymal stem cells through the PDGF-B-NRP-1 signaling pathway (2) increased recruitment and attachment of newly differentiated pericytes into newly formed tumor vessels. In addition, Flk1+ cells differentiate into endothelial cells or pericytes upon stimulation by VEGF or PDGF-B [72].

have previously shown that MCP-1 alone or in combination with VEGF is a significant prognostic factor in breast cancer [69]. MCP-1 is a direct gene target for TGF-β in ECs and mediates the angiogenic effect of TGF-β by promoting the recruitment of mural cells to ECs [70]. Although the immunoregulatory role of MCP-1 in breast tumor metastasis has been shown [71], its involvement in tumor vessel maturation might be another factor in metastasis promotion.

An *in vitro* study using mouse embryonic mesenchymal stem cells has shown that tumor cell-derived PDGF-B plays an important role in the differentiation and recruitment of PCs through NRP1 signaling (Figure 3) [72]. Less aggressive cell lines, such as MCF-7 cells (a noninvasive breast cancer cell line), have been shown to cause more recruitment and attachment of PCs to blood vessels compared to aggressive cell lines, such as MDA-MB-231. Another animal study has shown that tumor-derived PDGF-BB upregulates the transcription of stromal derived factor-1α (SDF-1α) in ECs [73]. EC-derived SDF-1α forms a chemotactic gradient that recruits CXCR4+ pericytes and smooth muscle progenitor cells during cancerous vascular remodeling. Another malignant cell-derived factor involved in the normalization of the tumor vasculature is PlGF [74]. This growth factor only binds to VEGFR1, forms a heterodimer with VEGF that inhibits angiogenesis, and leads to vascular remodeling by forming pericyte-enriched vascular networks (Figure 4).

The combination of anti-VEGF-R and anti-PDGF-R antibodies enforces tumor vessel regression by interfering with PC-mediated EC survival mechanisms [75]. Other studies have revealed that inhibiting PDGF-R improves tumor drug uptake in experimental tumor models [76]. Hepatocyte growth factor (HGF), a mesenchymal-derived protein, is a potent chemokine that regulates the growth and motility of many cell types, such as vascular smooth muscle cells [77]. Upon activation by angiopoietin-1, ECs produce HGF, which in turn leads to the migration of smooth muscle cells toward ECs [78]. XL880 (foretinib, GSK1363089) and XL184 (cabozantinib) are small molecule inhibitors that potently block multiple RTKs including VEGFR and the receptor of hepatocyte growth factor, c-Met. In a mouse model of pancreatic islet tumors, treatment with XL880 or XL184 led to rapid, widespread, and progressive regression of the tumor vasculature and reduced pericyte numbers [79].

5. Quantification of Angiogenesis

In clinical routines, the response to antitumor therapies (chemo- or radiotherapy) is usually assessed by measuring

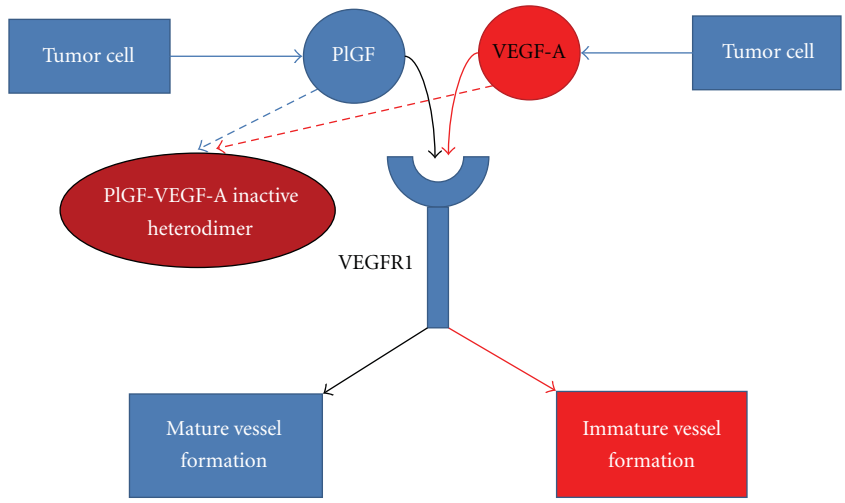

Figure 4: Negative regulation of angiogenesis by the PlGF-VEGFR1-mediated signaling pathway. In PlGF-producing mouse Lewis lung and human tumors, the tumor vasculature that is induced by PlGF-1 and -2 is covered by pericytes and is less leaky compared to the tumor vasculature that is induced by VEGF-A. This result might be caused by the formation of angiogenically inactive PlGF-VEGF heterodimers [74].

tumor size [80]. However, strategies with more specificity are needed to provide information about tumor vitality. Imaging of angiogenic and antiangiogenic behaviors is an essential component to evaluate antiangiogenic therapy. Various quantitative imaging techniques, such as positron emission tomography (PET) [81], magnetic resonance imaging (MRI) [82], or X-ray-computed tomography (CT) [83], are being used to evaluate changes in the tumor vasculature following the administration of angiogenesis inhibitors. However, these techniques can only reflect the physiological changes and require further development before they can be accepted as surrogate endpoints. These techniques require the use of blood-pool contrasting agents to characterize tissue blood volume, perfusion, and vessel permeability [84]. Although small vessels have been visualized using these methods in animal models [85], their clinical application is limited due to the long scanning time or experimentally constructed agents. Therefore, biopsy and histopathological evaluation are currently the accepted gold standards in clinical trials. MVD has been a well-studied marker to assess neoangiogenesis in several malignancies, such as breast carcinoma. Endothelial cells express different cell surface markers as a function of developmental age [86]. Differentiated endothelial cell specific markers (e.g., CD31 or CD34) are commonly used for tissue analysis by single immunohistochemical staining and MVD analysis. A histological examination of 512 breast cancer samples has shown that stromal PDGF-R expression significantly correlates with less favorable clinicopathological parameters (e.g., histopathological grade, estrogen receptor negativity, and high HER2 expression) and shorter survival rates [87].

Although the single immunohistochemical staining method allows the analysis of morphological aspects of angiogenesis, such as vessel size, shape, and density, these criteria might not be sufficient to assess vascular function. Double immunohistochemical staining using an EC marker and a PC marker (e.g., α-SMA) can be used to evaluate the morphology and the maturity of tumor microvessels by differentiating between the vessels with or without PCs. Our studies have shown that this method is easily applicable to tissue samples from formalin-fixed paraffin-embedded pathological archives (Figure 5). Our studies of predictive factors and prognostic values of microvessel maturity have used EC-PC double staining in primary breast cancer patients and are ongoing in patients who have undergone neoadjuvant chemotherapy. We believe that this new method has great potential to evaluate the prognostic and predictive role of angiogenesis from a new prospective. Brain metastases from nonsmall cell lung carcinomas have a higher proportion of mature vessels compared to primary tumors independent of the vascular pattern of the primary tumor [88]. This discordance between the vascular characteristics

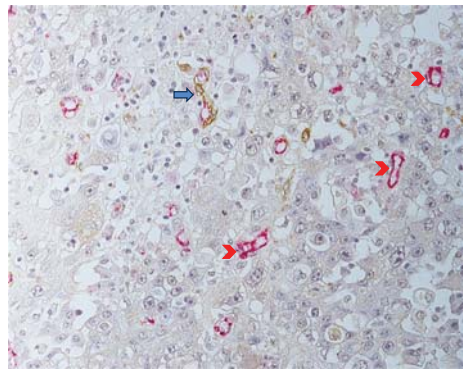

Figure 5: Double-immunostaining of pericytes and endothelial cells in breast cancer. Double immunostaining for CD31 (red) and α-SMA (brown) is shown. The blue arrow indicates a vessel that is CD31+/α-SMA+; the red arrowheads indicate vessels that are only CD31+. Magnification $\times$200.

of primary and secondary cancers suggests that the maturity of microvessels in primary tumors should be considered when assessing angiogenesis as a prognostic or predictive factor. In another study of a small number of breast cancer samples ($n = 50$), blood vessel maturity was assessed by the positivity of LH39 at the lamina lucida of mature microvessels [89]. Mature blood vessels are defined by staining with antibodies to LH39 and CD31, using double immunohistochemistry, whereas immature blood vessels are characterized by positive CD31 staining. The vascular maturity index (VMI) is defined as the percentage of the fraction of mature vessels (LH39-positive)/total number of vessels (CD31-positive). TP expression but not VEGF expression is correlated with a low VMI showing intense vascular remodeling in TP-expressing cancers. TP is highly expressed in breast cancer cells and inflammatory cells in the stroma [90]. Its antiapoptotic and proangiogenic roles have been well studied. Therefore, the assessment of vessel maturity and microvessel count may identify patients who might benefit more from specific chemotherapy or antiangiogenic therapies.

Circulating endothelial cells (CECs) and circulating progenitor cells (CPCs) are novel surrogate markers of vascular disruption and repair in cancer [91]. In a study of 160 breast cancer patients, CECs are significantly higher in the poor prognostic group based on the Nottingham Prognostic Index (NPI), whereas CPCs are lower in the poor prognosis group [92]. Although vascular invasion and tumor size are independently associated with CECs, Her-2 status positively predicts CPCs. Circulating endothelial cell analysis in 46 advanced breast cancer patients treated with metronomic cyclophosphamide, capecitabine, and bevacizumab demonstrates that a high level of CECs indicates an active vascular turnover and predicts a prolonged clinical benefit for treatment, whereas low CEC counts are evident during tumor progression [93]. Decreased levels of CECs are accompanied by increased levels of VEGF-A and basic fibroblast growth factor, which suggests a switch toward a different type of cancer vascularization. High levels of CECs, which indicate active vascular remodeling, are shown to be associated with therapeutic responses. However, low CEC counts in cases of tumor progression might indicate a more stable microvasculature in these tumors (i.e., mature microvessels).

6. Conclusion

Combining different chemotherapeutic agents and angiogenic inhibitors normalizes the tumor vasculature, and the essential role of PCs in microvessel maturity and the concomitant histological evaluation of EC-PC interactions, and tumor microvessel morphology seems to be inevitable. In addition, the influence of lymphangiogenesis and EC interactions with tumor cells that express angiogenic receptors should also be investigated. Many new angiogenic inhibitors target pathways that are involved in the recruitment of pericytes to tumor microvessels. Therefore, it is essential to assess PCs in parallel with ECs when studying the tumor vasculature. This evaluation, which can be performed in a diagnostic pathology laboratory, can be used as a decision-making tool to select patients who might benefit from antiangiogenic therapies.

Abbreviations

EC: Endothelial cell
PC: Pericyte
VEGF: Vascular endothelial growth factor
PDGF: Platelet-derived growth factor
HIF: Hypoxia-inducible factor
ER: Estrogen receptor
PgR: Progesterone receptor
HER-2: Human epidermal growth factor receptor-2
TNBC: Triple-negative breast cancer.

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Research Article

Lack of Efficacy of Combined Antiangiogenic Therapies in Xenografted Human Melanoma

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Antiangiogenic therapy is theoretically a promising anticancer approach but does not always produce significant tumor control. Combinations of antiangiogenic therapies are therefore being investigated as strategies to enhance clinical benefit. Common targets for angiogenic blockade include endothelial specific receptors, such as Tie2/Tek, which signal blood vessel stabilization via recruitment and maturation of pericytes. Here, we report on the effects of targeted Tie2 antiangiogenic therapy (TekdeltaFc) in combination with nontargeted metronomic cyclophosphamide (LDM CTX) (reported to also act in antiangiogenic fashion) in xenografted human melanoma. Individually, these therapies showed transient antitumor activity, but, in combination, there was no significant reduction in tumor growth. In addition, while TekdeltaFc caused the expected increased pericyte coverage in treated blood vessels, LDM CTX alone or in combination with TekdeltaFc resulted in increased levels of VEGF production. Collectively, our data highlight the complexity of molecular interactions that may take place when antiangiogenic regimens are combined.

1. Introduction

Cytotoxic chemotherapeutic drugs may have antiangiogenic properties when administered metronomically at doses significantly lower than the maximum tolerated dose (MTD) and as such appear to have less severe or even absent cytotoxic side effects [1]. Cyclophosphamide (CTX), a nitrogen mustard alkylating agent, is clinically the most studied drug in a low-dose metronomic chemotherapy setting [2, 3]. In contrast to MTD, metronomic administration of low-dose cyclophosphamide induces selective apoptosis of genetically stable endothelial cells in tumors (hence circumventing drug resistance) [4, 5]. In addition, studies suggest that this effect is due to overproduction of Thrombospondin-1, a well-known, highly specific, and potent endogenous inhibitor of angiogenesis [6, 7]. Interestingly, an inverse relationship between Thrombospondin-1 and VEGF production has been reported in cultured ovarian epithelial cells and LDM-CTX has been shown to decrease VEGF levels in patients with breast cancer [8], suggesting a possible relationship between VEGF expression and LDM-CTX-induced Thrombospondin-1 expression. In general, the antitumor effects of cancers treated with LDM chemotherapy are even more pronounced when combined with an antiangiogenic inhibitor that targets endothelial cells specifically [9, 10]. Tie2 receptors are primarily found on endothelial cells and are constitutively expressed in normal vasculature [11]. Thus, it was surprising when our laboratory identified blood vessels within human cancers that lacked Tie2 expression. In particular, malignant melanoma had the highest percentage (~15%) of Tie2-negative blood vessels of all cancer types evaluated [12]. Differential expression of vascular growth factor receptors, such as Tie2, may be due to tumor microenvironment conditions such as hypoxia and hypoglycemia, which occur as a result of an imbalance between the oxygen supply and consumption and altered energy demand [13–15].

Heterogeneous expression of Tie2 in tumor vasculature suggested a role for Tie2 in tumor angiogenesis, however, elucidating the functional significance of Tie2 expression in tumors required the use of a specific Tie2 inhibitor TekdeltaFc, which is an artificial extracellular domain of Tie2

[16]. Since angiopoietins bind with high affinity to Tie2 extracellular domain, we reasoned that this inhibitor should efficiently interfere with angiopoietin-mediated Tie2 activity. In addition, we hypothesized that the tumor microenvironment is at least partially responsible for the lack of Tie2 expression observed in malignant melanoma blood vessels and investigated the possibility that Tie2 heterogeneity is due to severe hypoxia or hypoglycemia.

Excessive tumor angiogenesis has been associated with poor prognosis in metastatic melanoma [17]. Thus, antiangiogenic therapies targeting the tumor microvasculature have been intensively used in clinical trials in combination with the standard chemotherapeutic regimens. In one such study, antiangiogenic low-dose paclitaxel in combination with celecoxib caused significant disease stabilization of more than 6 months in 15% of metastatic melanoma patients [18]. Clinical benefits were also observed in melanoma patients treated with anti-VEGF therapy, bevacizumab (Avastin), combined with carboplatin and paclitaxel [19], or in combination with interferon-α-2b [20]. However, to date, the effect of Tie2 inhibition on melanoma angiogenesis, alone or in combination with other antiangiogenic strategies, has not yet been explored.

Recent publications suggest a clear advantage of simultaneously using multiple antiangiogenic therapies in combination with metronomic, low-dose chemotherapy, thus targeting more than one endothelial cell signaling pathway [5, 21, 22]. To determine if the response to targeted antiangiogenic therapy such as Tie2 inhibition can be enhanced by nontargeted antiangiogenic therapy such as LDM CTX, we examined the impact of this combined approach on human malignant melanoma cancer cell xenografts.

2. Methods

2.1. Cell Lines and Reagents. The human melanoma cell line WM239 was originally isolated from a patient's metastatic lesion [23]. Cells were maintained in Dulbecco's modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen), sodium pyruvate (Invitrogen), and gentamicin (Invitrogen) in a humidified atmosphere at 37°C in 5% CO_2. Tie2 inhibitor, murine TekdeltaFc, was provided by Amgen. Cyclophosphamide (CTX) was purchased from Sigma Aldrich.

2.2. Growth of Tumor Xenografts. All procedures described below were done according to the guidelines and recommendations of the Canadian Council of Animal Care and approved by the University of Guelph Local Animal Care Committee. Tumor xenografts were established in *RAG1*$^-$ immune-deficient mice [24] by injecting 100 μL of 0.1% BSA/PBS solution containing 1×10^6 WM239 melanoma cells subcutaneously into the right flank. Tumor growth was measured twice weekly and tumor size estimated using the equation: volume = length $\times$ width^2 $\times$ 0.5. Once tumors reached at least 100 mm^3, mice were randomly allocated into one of four treatment groups each containing 8 mice. Mice were treated for 14 days as follows: group 1 received 250 μg TekdeltaFc as 250 μL i.p. every 3 days; group 2 received low-dose cyclophosphamide in drinking water (equivalent to 30 mg/kg/day; water was changed twice weekly); group 3 received both TekdeltaFc every 3 days and low-dose cyclophosphamide in drinking water; group 4 control mice received 250 μL i.p. injections of sterile PBS every 3 days and untreated drinking water. Tumor growth was measured for the duration of the trial every 3-4 days.

One hour prior to euthanasia, mice were injected i.p. with 150 mg/kg Hypoxyprobe-1 (Chemicon International Inc.). Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Tumors were dissected from the surrounding tissue and cut into pieces, embedded in OCT cryomatrix (Fisher Scientific), and snap frozen in liquid nitrogen, fixed in 4% paraformaldehyde (USB Corporation) for 24 h and paraffin embedded, or snap frozen in liquid nitrogen and stored at -80°C for future protein isolation.

2.3. Quantification of Tumor Hypoxic and Necrotic Areas. Paraformaldehyde-fixed paraffin-embedded 8 μm thick sections were deparaffinized, and sodium citrate antigen retrieval (10 mM, pH = 6.0, boiled for 8 minutes then cooled in buffer at RT for 15 min) was performed. Following antigen retrieval, sections were washed and blocked first with Dako protein-free block (Dako) for 15 minutes and then with 5% normal goat serum (Sigma-Aldrich) for 30 minutes. Next, 3% hydrogen peroxide (Fisher Scientific) was used for 15 minutes to block endogenous peroxidase activity, then sections were washed and incubated in mouse Hypoxyprobe-1 antibody (Chemicon International) (1 : 50) overnight at 4°C, followed by goat antimouse biotinylated secondary antibody (1 : 200) for 30 minutes. Sections were washed and treated with R.T.U. Vectastain Elite ABC reagent (Vector) for 30 minutes followed by incubation with substrate reagent diaminobenzidine (DAB) for 2 minutes. Sections were then rinsed with water, counterstained using Mayer's hematoxylin solution (diluted 1 : 1 with water) (Sigma-Aldrich) for 1 minute, and mounted using Aquapolymount (Polyscience). In total, two blocks from each of twenty tumors were evaluated (five from each of the four treatment groups). Images were captured in a blinded fashion using 20x magnification objective of a Leica DMLB compound light microscope fitted with a Q imaging QICAM fast1394 digital camera using Q-Capture software, depending on the size of the tumor, certain sections were subdivided into one to four fields of view. Hypoxic regions were identified by strong brown reaction product, and necrotic regions were identified as those adjacent to hypoxic zones and lacking intact, well-defined nuclei (as seen with hematoxylin staining). Optimas 6.0 software (Optimas, Houston) was used to quantify areas of hypoxia and necrosis in each section and percentage of hypoxia/necrosis or hypoxia and necrosis per section area was calculated.

2.4. Quantification of Tie2 Expression and Microvessel Density (MVD). To evaluate Tie2 expression patterns as well as determine MVD, two tissue blocks from each tumor were assessed. Cryosections, 8 μm thick, were cut using a cryostat adjusted to -20°C. Once cut, sections were stored at -80°C

until further use. For immunofluorescent staining, sections were air dried at RT, fixed in cold methanol/acetone (50 : 50) for 10 min at −20°C, and then air dried. Then, sections were rehydrated in PBS and blocked using 10% normal goat serum (Sigma-Aldrich) for 1 h at room temperature followed by mouse anti-Tie2 (1 : 100; BD Biosciences) for 1 h at room temperature and incubation with goat antimouse Cy3 conjugated secondary antibody for 20 minutes (1 : 200; Jackson ImmunoResearch). Sections were blocked using Dako protein-free block (Dako) for 15 minutes, incubated overnight at 4°C using rat anti-CD31 antibody (1 : 50; Hycult Biotechnology), followed by donkey antirat FITC secondary antibody for 30 minutes (1 : 100; Jackson ImmunoResearch) and 2 minutes in DAPI (4′,6-diamidino-2-phenylindole) (Dako) nuclear stain. Slides were then washed briefly in water and mounted using Fluorescent Mounting media (Dako). Entire sections were examined by epifluorescence microscopy using the 20x objective in a semiblinded fashion. MVD was determined by dividing the total number of blood vessels per field of view, to obtain a value expressed as number of blood vessels per mm^2.

2.5. Quantification of Blood Vessel Pericyte Coverage. To evaluate the degree of pericyte coverage of tumor blood vessels, two blocks from each tumor were assessed. Cryosections, 8 μm thick, were fixed as previously described, then rehydrated in PBS, permeabilized using 0.05% Tween-PBS for 10 minutes and blocked using 10% normal goat serum (Sigma-Aldrich) for 30 minutes at RT. Sections were incubated in a mixture of rabbit anti-PDGFR-β (1 : 100; Cell Signaling Technology) and biotinylated rat anti-CD31 (1 : 60; Hycult) primary antibodies overnight at 4°C. Sections were washed in PBS and incubated in a mixture of goat antirabbit secondary antibody conjugated to Cy3 (1 : 200; Jackson ImmunoResearch) and streptavidin conjugated to Alexa350 (1 : 150; Invitrogen) for 40 minutes at room temperature followed by rabbit anti-desmin primary antibody for 30 minutes (1 : 100; Abcam, Cambridge, Mass, USA) conjugated to Alexa488 using the rabbit antibody labeling kit according to manufacturer's protocol (Invitrogen). Sections were mounted in Fluorescent Mounting Media (Dako) and examined using epifluorescence microscopy as previously described. Five random fields were captured, and blood vessels were enumerated as $CD31^{positive}$/$desmin^{positive}$/PDGFR-$\beta^{positive}$ or $CD31^{positive}$/$desmin^{negative}$/PDGFR-$\beta^{negative}$ and expressed as percentage per section.

2.6. Measurement of VEGF Levels in Tumor Xenograft Lysates. Commercially available human and mouse VEGF ELISA kits (both R & D Systems) were used to quantify VEGF levels in lysed tumor xenografts from each of the treatment groups (control, TekdeltaFc, LDM CTX and TekdeltaFc + LDM CTX). Briefly, frozen tumor pieces (3–5 per group) were defrosted on ice and lysed using a disposable tissue grinder and cell lysis buffer (Cell Signaling). Protein was collected as previously described and VEGF ELISA performed according to the manufacturer's protocol. Values were expressed relative to total tumor protein.

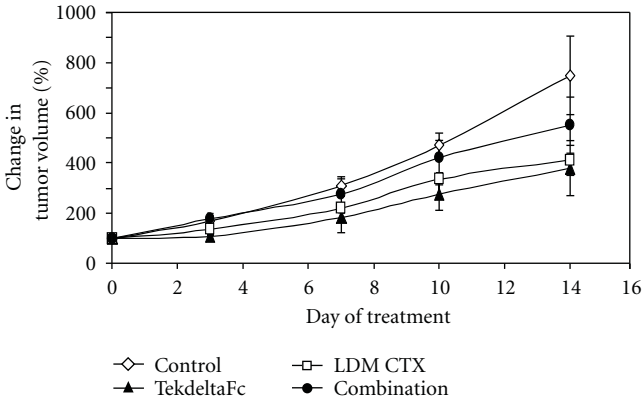

Figure 1: In vivo xenografts of human melanoma cancer cells treated with TekdeltaFc and LDM CTX. Relative tumor growth for each treatment group: Control, TekdeltaFc, low-dose metronomic cyclophosphamide (LDM CTX), and combination for two weeks. There were no statistically significant differences in tumor growth between treatment groups at any time points ($P > 0.05$).

2.7. Statistical Analysis. Calculation of preliminary summary statistics such as mean, standard deviation, and standard error was completed using Microsoft Excel (Microsoft). On all samples, Grubbs' test, also called the ESD method (extreme studentized deviate), was used to determine significant outliers. Once outliers, if any, were identified and omitted from the analysis; ANOVA was performed to determine the significance within and between groups ($P < 0.05$). Further, the least significant difference (Tukey) test was used if there was significant difference between groups. Data were presented as mean and standard error.

3. Results

3.1. Effects of LDM CTX and TekdeltaFc in Melanoma Xenografts. While CTX LDM and TekdeltaFc showed a reduction in tumor growth compared to control, these differences were not statistically significant (Figure 1; $P > 0.05$). Surprisingly, there was also no additive effect when CTX LDM and TekdeltaFc were combined, as this dual target antiangiogenic regime also had no significant effect on tumor growth compared to control at any time point (Figure 1; $P > 0.05$). When tumor sections were evaluated for cellular responses (Figure 2(a)), we found no significant differences in the proportions of viable or hypoxic regions between treatment groups (Figure 2(b); $P > 0.05$). However, significantly lower amounts of tissue necrosis, and necrosis plus hypoxia were observed in tumors treated with CTX LMD when compared to control and combination-treated tumors (Figure 2(b); $P < 0.05$).

We quantified microvessel density (MVD) as well as the proportion of Tie2-negative vessels in tumor xenograft sections stained for CD31 and Tie2 (Figure 3(a)). The average number of Tie2 negative blood vessels was about 12.5%, which confirmed our previous finding of Tie2 vascular heterogeneity in melanoma [12]; there were no significant differences between treatment groups ($P > 0.05$). Although

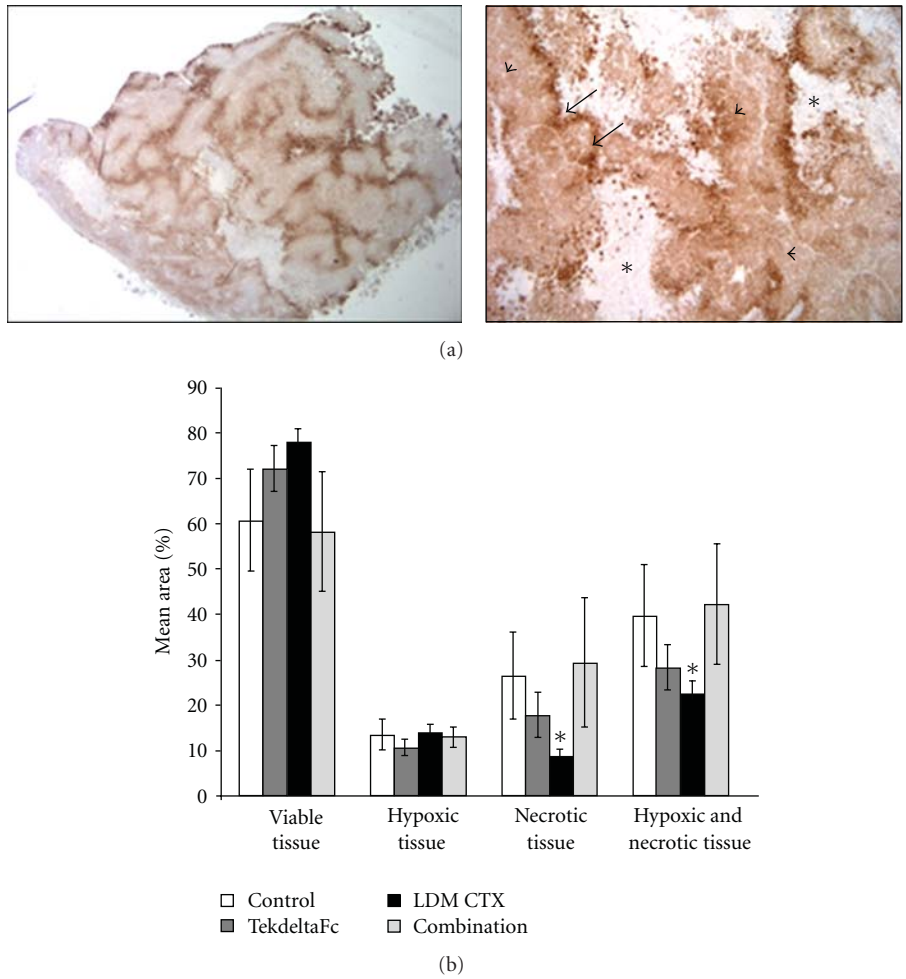

Figure 2: Quantification of hypoxic and necrotic areas in tumor xenografts treated with TekdeltaFc and LDM CTX. (a) Representative low (5x, left) and high (20x, right) magnification images of hypoxia immunostaining for Hypoxyprobe adducts and tissue necrosis in tumor sections. Dark brown areas are hypoxic (arrow), light brown areas are viable tumor (arrowhead), and areas lacking brown reaction product and containing degraded nuclei are necrotic tissue (asterisk). (b) Quantification of the mean percent viable tissue, hypoxia, necrosis, or hypoxia and necrosis in each treatment group, showing significant differences in proportions of necrotic, and necrotic plus hypoxic areas in LDM-CTX-treated tumors compared to control and combination ($^*P < 0.05$).

decreased MVD was observed in LDM-CTX-treated tumors compared to TekdeltaFc treatment group, this was not statistically significant ($P > 0.05$) (Figure 3(b)).

3.2. TekdeltaFc Significantly Increased Pericyte Coverage of Tumor Blood Vessels Compared to Low-Dose CTX Treatment. Triple immunofluorescence was utilized on frozen sections of xenografts with antibodies to desmin and PDGFR-β to detect vascular mural cells [25]. Vessels were categorized as Desmin/PDGFR-β^{positive} or Desmin/PDGFR-β^{negative} (Figure 4(a)). We observed a statistically significant difference ($P < 0.05$) between treatment groups. TekdeltaFc-treated tumors had statistically significant ($P < 0.05$) increased pericyte coverage compared to all other groups (Figure 4(b)).

3.3. TekdeltaFc and LDM CTX Increased VEGF Expression. Tumor pieces from each treatment group were lysed and VEGF expression analyzed and expressed as pg/mL, normalized for total protein in the tumor lysate. Human VEGF levels were significantly increased ($P < 0.05$) in TekdeltaFc and LDM CTX combined treated tumors compared to all of the other treatment groups. In addition, human VEGF concentration was significantly higher ($P < 0.05$) in LDM CTX compared to control group (Figure 5(a)). Although murine VEGF levels were highest in LDM-CTX-treated tumors, ANOVA showed that overall there were no significant differences in murine VEGF levels ($P > 0.05$; Figure 5(b)).

4. Discussion

Tumor angiogenesis is an attractive therapeutic target, since it is shared by most commonly occurring, and perhaps all, types of human cancers [26]. Considering the importance of vascular growth in tumor progression, approaches targeting tumor endothelium using antiangiogenic therapies may

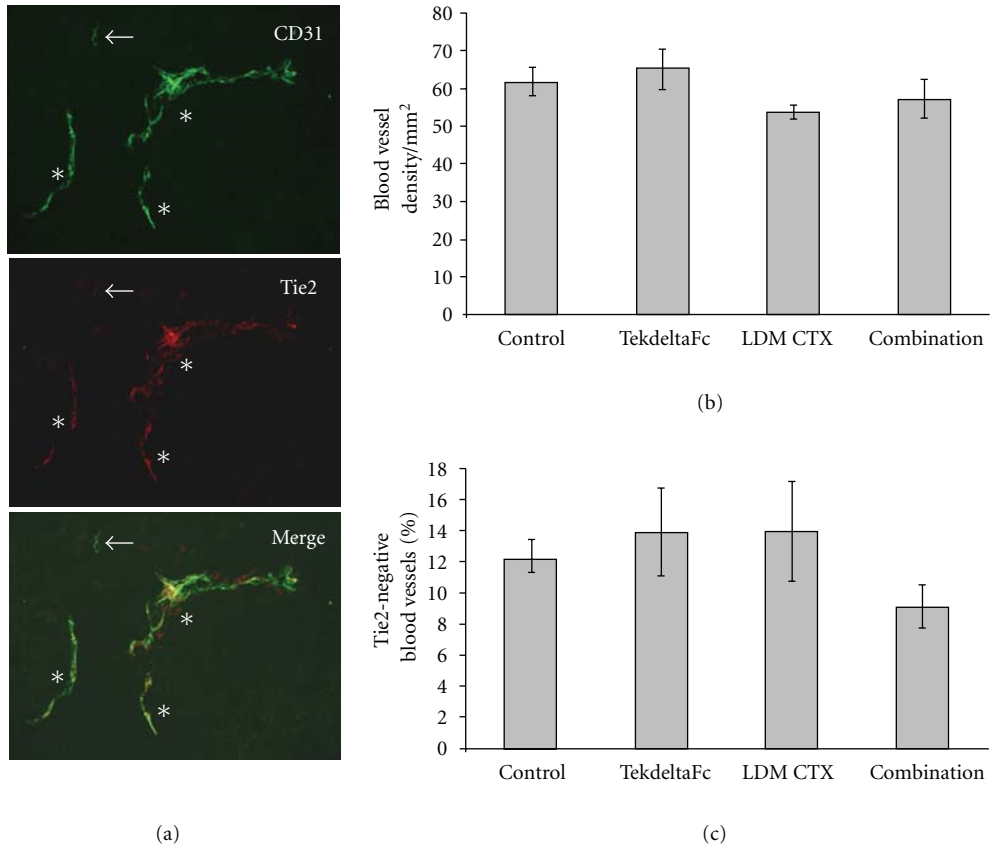

Figure 3: Dual immunofluorescence staining for CD31 and Tie2. (a) Tie2 was fluorescently labeled red using Cy3 while CD31 was fluorescently labeled green using FITC. Representative Tie2-positive blood vessels are marked with an asterisk while representative Tie2-negative blood vessels are labeled with arrows. (b) Quantification of average blood vessel densities (as number of CD31-positive vessels/mm^2) between treatment groups. There were no statistically significant differences in blood vessel density between different treatment groups ($P > 0.05$). (c) Quantification of % Tie2-negative blood vessels in tumor xenografts treated with TekdeltaFc and/or LDM CTX. Graph depicts the average % of Tie2-negative blood vessels per treatment group; the difference between groups is not statistically significant ($P > 0.05$).

provide a long-term and more effective control of disease compared to cytotoxic chemotherapy. One of the advantages of using antiangiogenic agents is that, under physiological conditions, normal endothelial cells are quiescent compared to tumor endothelial cells that are actively proliferating and migrating, which minimizes possible side effects on normal endothelium [27]. In addition, endothelial cells are genetically more stable than cancer cells and antiangiogenic agent delivery is less complicated by not having to penetrate large bulky masses. Finally, antiangiogenic therapies can have different modes of action—interfering with angiogenic ligands, their receptors or downstream signaling, upregulation/delivery of endogenous inhibitors, or by directly affecting tumor vasculature [28]. More recently, it has become apparent that cancer cells recruit a variety of bone-marrow-derived cells which are also able to contribute to the vasculature in direct and indirect ways [29].

The mechanism of action of TekdeltaFc (the extracellular domain of murine Tie2/Tek receptor fused to the Fc portion of murine IgG) involves binding with high avidity to both Ang1 and Ang2 [16]. A similar inhibitory molecule, ExTek, was shown to function as a potent inhibitor of Tie2 by sequestering available angiopoietin, and by binding to Tie2 receptors, inhibiting phosphorylation and downstream signaling molecules related to cell survival [30–32]. Interestingly, ExTek decreased the number of lung metastasis in a murine melanoma model [33]. Other studies employing different versions of the Tie2 extracellular domain as an inhibitor achieved similar effects in different tumor models [34–36].

In our study, while LDM CTX had a more profound effect on tumor blood vessel density compared to TekdeltaFc treatment, these differences were not significant. LDM CTX may have had an indirect effect on tumor growth by significantly decreasing the mobilization of circulating endothelial cells from the bone marrow, as previously reported [37]. Interestingly, treatment of tumors with these antiangiogenic agents did not result in increased hypoxia/necrosis of the tumor tissue. This could be explained by some of our previous work which showed that WM239 cells can develop reduced vascular dependence and therefore enhanced survival even if their blood vessel density decreases [38] or by the fact that reduced vascular density actually represents a "normalization" of the vascular bed, accompanied by

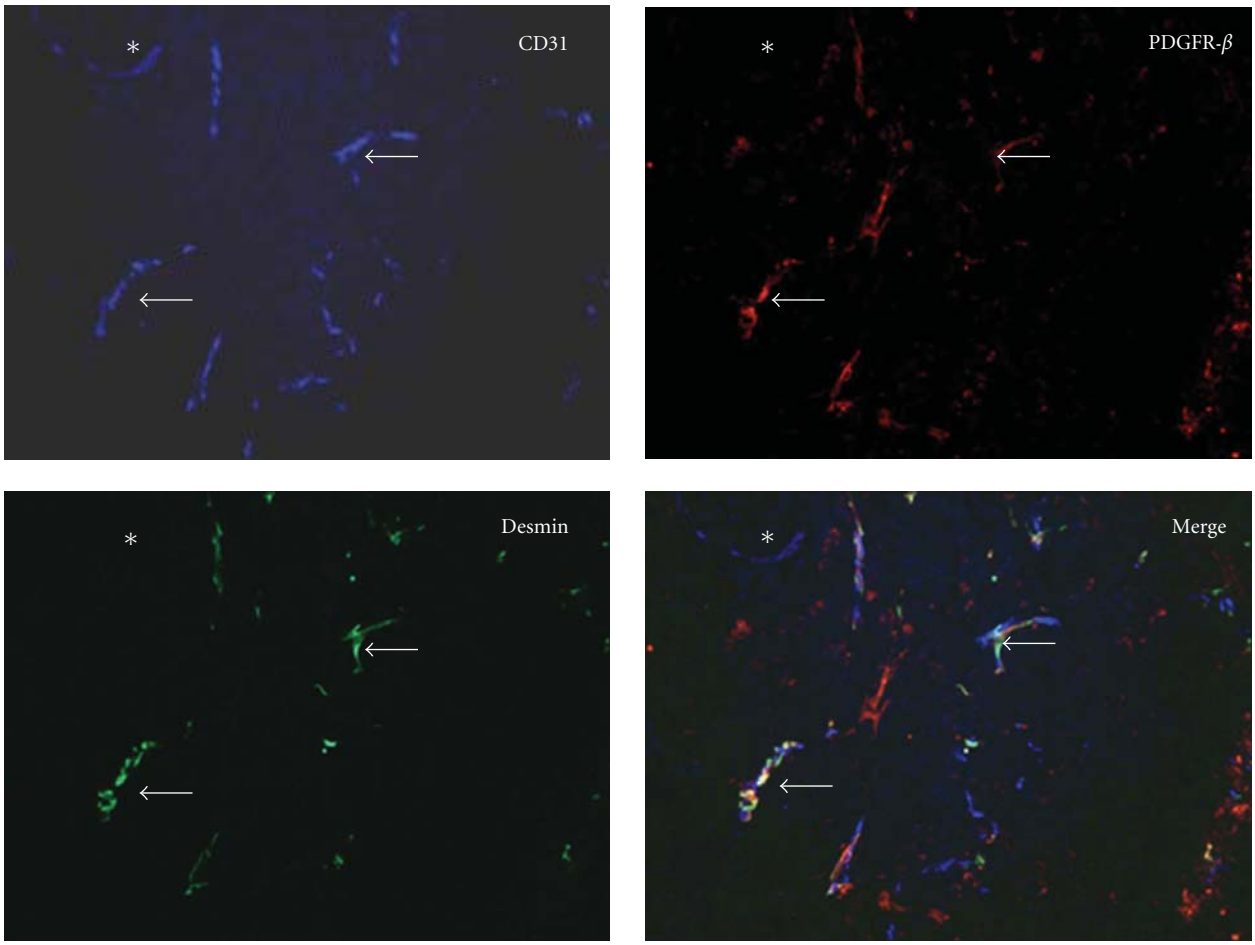

Figure 4: Immunostaining of blood vessels and pericytes in treated tumors. (a) Images show blood vessels immunofluorescently stained using antibodies to CD31 (Alexa 350; blue), desmin (Alexa 488; green), PDGFR-β (Cy3; red), and overlay of CD31/desmin/PDGFR-β. Asterisk marks blood vessel negative for mural cells (neither desmin nor PDGFR-β) while arrow indicates blood vessel with positive mural cell markers (desmin and PDGFR-β staining). (b) Quantification of blood vessel pericyte coverage in tumors treated with TekdeltaFc and/or LDM CTX. The percentage of "stable" blood vessels with pericyte coverage was significantly different between TekdeltaFc and all other treatment groups ($^*P < 0.05$).

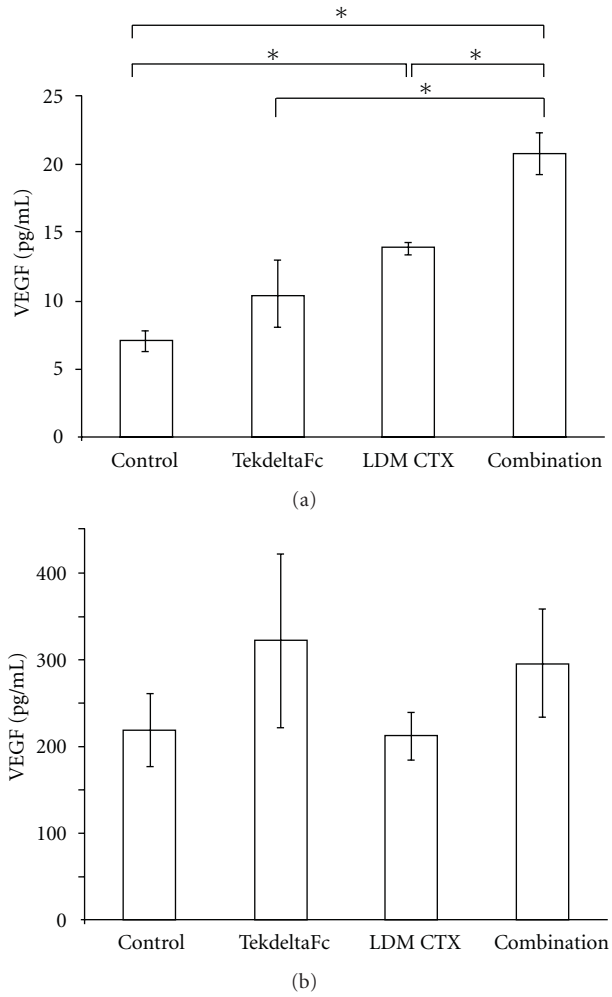

Figure 5: Expression of VEGF in tumor xenografts. Concentration of VEGF levels in tumor lysates measured by ELISA and expressed as pg/mL normalized to protein content in the tumor lysate. (a) There were significant differences in human VEGF levels between treatment groups ($^*P < 0.05$). The lowest human VEGF concentration was detected in control tumors while the highest VEGF concentration was detected in combined treatment group, showing that TekdeltaFc and LDM CTX therapy both individually and in combination significantly upregulated melanoma cell VEGF expression. (b) There were no significant differences in murine VEGF between treatment groups ($P > 0.05$).

improved perfusion [39]. Such mechanisms are consistent with the fact that LDM-CTX-treated tumors had the lowest amount of necrotic tissue compared to control or combined treatment groups.

We also observed that TekdeltaFc alone caused significant increases in desmin/PDGFR-β dual positive pericyte coverage of blood vessels compared to control, LDM CTX, or combination therapies. Antiangiogenic therapies have been previously shown to improve response to chemotherapy by inducing maturation of the blood vessels via increasing pericyte coverage [27, 40]. There is considerable variability in pericyte characteristics between tumor types, with some studies reporting that mature pericytes are PDGFR-β negative and desmin positive [40], while other studies report that tumor pericyte populations can have overlapping markers [41]. Interestingly, PDGFR-β-positive/desmin-negative cells were more likely to be detached from adjacent vessels [41]. In abnormal tumor vasculature, VEGF induces expression of Ang-2 from endothelial cells in the microvasculature [42, 43]. By binding to Tie2, Ang-2 becomes an autocrine regulator of endothelial cell function; whether it acts as an agonist [42, 44] or antagonist [45] is context dependent [46]. It is now known that in vitro Ang-2 inhibits the stabilizing effects of Ang-1, but weakly activates Tie2 if Ang-1 is absent [47]. In our tumor model, TekdeltaFc likely caused sequestering of abundantly expressed Ang-2, thus allowing Ang-1 to bind and phosphorylate Tie2, hence the observed increase in pericyte coverage. Vessel "normalization" has been associated with improved response to cytotoxic chemotherapy [21, 48], thus employing CTX at a maximally tolerated dose rather than a metronomic dose might be more effective.

Both TekdeltaFc and LDM CTX alone caused significant decreases in tumor volume at earlier time points, but they failed to do so after two weeks of treatment. This could be due to the fact that most advanced malignant tumors produce multiple angiogenic factors, and so targeting only angiopoietins (in TekdeltaFc treated tumors) or only VEGF (in LDM-CTX-treated tumors) may not be adequate for complete tumor control [8, 28, 49, 50]. Tumors may also have become resistant to TekdeltaFc or LDM CTX, allowing them to regrow after initial inhibition. Interestingly, tumor xenografts treated with TekdeltaFc and LDM CTX combination therapy grew at the same rate as controls, suggesting potential interference between these two therapies. This is in contrast to results with a neutralizing antibody against Ang2, which had potent antitumor effects in xenograft models, especially when combined with VEGF-targeted therapy [21]. Differences from our study may be due to the fact that, unlike anti-Ang2 antibody, TekdeltaFc affects Ang1 and Ang2 signaling. Differences may also be due to cancer type, as melanoma was not evaluated in the anti-Ang2 antibody study. Thus, although combination of antiangiogenic therapies is becoming a common practice [2, 22, 50, 51], our studies provide evidence that interactions may be complex and tumor type dependent.

Surprisingly, treated tumors in our study contained significantly higher levels of human VEGF than control tumors, and combined treated tumors contained the highest amount, consistent with a proangiogenic environment observed at the end of this trial. One of the modes of resistance to antiangiogenic therapy, usually occurring after a transient response phase, is upregulation of alternative proangiogenic pathways in tumors, such as fibroblast growth factor 1 and 2, ephrin A1, or Ang 1. These presumably compensate for the inhibited pathway and allow tumor regrowth [52]. In one such study, patients treated with VEGFR inhibitor cediranib had an increase in FGF2 expression in their relapse phase after successful but transient response [53]. It has also been shown in clinical trials that tyrosine kinase inhibitors can transiently increase the levels of proangiogenic factors, such as VEGF [54], as we observed in our present study. In fact, increases in proangiogenic factor VEGF in

the presence of antiangiogenic Tie2 inhibitor probably account for the lack of an observed decrease in blood vessel density in our treated tumors. Collectively, these data support the idea that two different antiangiogenic therapies may interact to stabilize the microvasculature, thus preventing vessel regression and tumor inhibition, an outcome that suggests that caution should be taken in designing such antiangiogenic combinations.

Acknowledgments

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Research Article

Bone Marrow Suppression by c-Kit Blockade Enhances Tumor Growth of Colorectal Metastases through the Action of Stromal Cell-Derived Factor-1

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Background. Mobilization of c-Kit$^+$ hematopoietic cells (HCs) contributes to tumor vascularization. Whereas survival and proliferation of HCs are regulated by binding of the stem cell factor to its receptor c-Kit, migration of HCs is directed by stromal cell-derived factor (SDF)-1. Therefore, targeting migration of HCs provides a promising new strategy of anti-tumor therapy. *Methods*. BALB/c mice ($n = 16$) were pretreated with an anti-c-Kit antibody followed by implantation of CT26.WT-GFP colorectal cancer cells into dorsal skinfold chambers. Animals ($n = 8$) additionally received a neutralizing anti-SDF-1 antibody. Animals ($n = 8$) treated with a control antibody served as controls. Investigations were performed using intravital fluorescence microscopy, immunohistochemistry, flow cytometry and western blot analysis. *Results*. Blockade of c-Kit significantly enhanced tumor cell engraftment compared to controls due to stimulation of tumor cell proliferation and invasion without markedly affecting tumor vascularization. C-Kit blockade significantly increased VEGF and CXCR4 expression within the growing tumors. Neutralization of SDF-1 completely antagonized this anti-c-Kit-associated tumor growth by suppression of tumor neovascularization, inhibition of tumor cell proliferation and reduction of muscular infiltration. *Conclusion*. Our study indicates that bone marrow suppression via anti-c-Kit pretreatment enhances tumor cell engraftment of colorectal metastases due to interaction with the SDF-1/CXCR4 pathway which is involved in HC-mediated tumor angiogenesis.

1. Introduction

Angiogenesis is one of the crucial steps in tumor progression and metastasis [1, 2]. Due to the lack of oxygen supply and the accumulation of toxic products, avascular tumors and tumor metastases cannot grow beyond a critical size of ~1-2 mm and thus will stay clinically occult [1, 3, 4]. Therefore, only a small proportion of circulating cancer cells finally forms macroscopic tumors [5]. Tumor vessels can grow by sprouting of preexisting host vessels, intussusception or incorporation of bone marrow-derived endothelial progenitor cells (EPCs) which are a subset of hematopoietic cells (HCs). This mechanism is called vasculogenesis and mimics embryonic angio-development [2].

Although EPCs incorporate into tumors in only small numbers, targeting EPCs has been shown to be effective in reducing tumor angiogenesis and tumor growth in experimental models [6]. One of the most important factors mediating survival and proliferation of HCs is the stem cell factor (SCF) which binds to the c-Kit receptor on the surface of HCs. Okamoto et al. have shown that bone marrow suppression by anti-c-Kit treatment induces a delay in tumor angiogenesis due to the inhibition of angiogenic sprouting in colon tumors and that c-Kit blockade suppresses tumor growth of subcutaneously implanted prostate carcinomas (PC3) by inhibition of tumor angiogenesis regulated by HCs [7].

Recruitment of HCs and EPCs to avascular areas is orchestrated by different angiogenic growth factors and cytokines [8], predominantly by the CXC-chemokine stromal cell-derived factor (SDF)-1 and its receptor CXCR4 [9, 10]. HCs and EPCs fluctuate to peripheral organs in antiphase with the expression of SDF-1 within the bone marrow microenvironment [11] and migrate alongside a chemotactic gradient towards higher concentrations of SDF-1, for example, sites of injury and tumor tissue [12–14]. Furthermore, it has been demonstrated that SDF-1-mediated recruitment of c-Kit-positive cells to the periphery is dependent on cofactors, including tumor necrosis factor-α and the endothelial nitric oxide synthase (eNOS) [12].

However, the impact of circulating HCs and EPCs on vasculogenesis and sprouting angiogenesis in tumor angiodevelopment is still controversially discussed in the literature [15–17], and the role of the SDF-1/CXCR4 pathway is not yet fully understood. Therefore, in the presented study we analyzed the influence of bone marrow suppression by anti-c-Kit treatment combined with SDF-1 neutralization on tumor cell engraftment and neovascularization using a murine model of colorectal tumor metastasis.

2. Materials and Methods

2.1. Tumor Cell Line and Culture Conditions. The CT26 cell line is an N-nitroso-N-methyl-urethane-induced undifferentiated adenocarcinoma of the colon, syngeneic with the BALB/c mouse. For our studies [18], the CT26.WT cells (ATCC CRL-2638, LGC Promochem GmbH, Wesel, Germany) were transfected with the enhanced GFP expression vector pEGFP-N1 (Clontech) with the use of CLONfectin (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. For the individual experiments, CT26.WT-GFP cells were grown in cell culture as monolayers in RPMI-1640 medium with 2 mM L-glutamine (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 10% fetal calf serum (FCS Gold, PAA Laboratories GmbH, Cölbe, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA Laboratories GmbH). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2, and only cells of the first three serial passages after cryostorage were used. At the day of implantation, tumor cells were harvested from subconfluent cultures (70 to 85%) by trypsinization (0.05% Trypsin and 0.02% EDTA, PAA Laboratories GmbH) and washed twice in phosphate-buffered saline solution (PBS).

2.2. Animals. Experiments were performed after approval by the local governmental ethic committee and conformed to the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (as described in 1998 in *Br J Cancer* 77: 1–10) and the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council; NIH Guide, Vol. 25, No. 28, 1996). Female BALB/c mice (Charles River Laboratories GmbH; Sulzfeld, Germany) with a body weight (BW) of 18–20 g were used. The animals were housed in single cages at room temperature of 22–24°C and at a relative humidity of 60–65% with a 12-hour light/dark cycle environment. The mice were allowed free access to drinking water and standard laboratory chow (Altromin; Lage, Germany).

2.3. Experimental Model—Dorsal Skinfold Chamber. To allow repetitive analyses of the microcirculation of the growing tumors, the dorsal skinfold chamber model was used for intravital microscopy as described previously in detail [19]. For operative procedures, animals were anesthetized by intraperitoneal injection of 90 mg/kg BW ketamine (Ketavet, Parke Davis; Freiburg, Germany) and 20 mg/kg BW xylazine (Rompun, Bayer; Leverkusen, Germany). The chamber, consisting of two symmetrical titanium frames, was positioned to sandwich the extended double layer of the dorsal skin. One layer of skin and subcutis was completely removed in a circular area of 15 mm diameter. The remaining layers, consisting of the epidermis, subcutaneous tissue, and striated skin muscle, were covered with a glass coverslip incorporated into one of the titanium frames. The animals tolerated the chambers well and showed no signs of discomfort or changes in sleeping and feeding habits. After a 48-hour recovery period, the animals were reanesthetized. For tumor cell implantation, the coverslip of the chamber was temporarily removed and 1×10^5 CT26.WT-GFP cells were implanted onto the surface of the striated muscle tissue within the chamber. Immediately after cell implantation, the chamber tissue was covered again with the coverslip [20–22].

2.4. Experimental Protocol. Animals were assigned to three different groups: the first group (cKit-Ab; $n = 8$) received a pretreatment with a monoclonal anti-c-Kit receptor antibody (ACK45, BD Biosciences, Heidelberg, Germany) by daily intraperitoneal injections starting 4 days before tumor cell implantation. The ACK45-antibody was given four times at a dose of 1 mg/kg BW daily as described by Okamoto et al. [7]. Animals of the second group (cKit/SDF1-Ab; $n = 8$) were also pretreated with ACK45 as described above. Additionally, these animals received 1 mg/kg BW of a monoclonal mouse anti-mouse SDF-1 antibody (MAB310, R and D Systems, Wiesbaden, Germany). MAB310 application was performed intraperitoneally, starting at the day of tumor cell implantation (d0) and repeated every second day thereafter until day 12. The third group (control; $n = 8$) served as control and received pre- and posttreatment the same amount of the corresponding isotype-matched IgG control antibodies (A95-1, BD Biosciences, and MAB002 R&D Systems). All animals underwent repetitive intravital microscopic analyses directly (d0) as well as 5, 8, 11, and 14 days after tumor cell implantation. At the end of the experiment (day 14), the chamber with the tumor tissue was harvested for histology and immunohistochemistry.

2.5. Intravital Fluorescence Microscopy. Intravital fluorescence microscopy was performed in epi-illumination technique using a modified Zeiss Axio-Tech microscope (Zeiss, Oberkochen, Germany) with a 100-W HBO mercury lamp.

Microscopic images were monitored by a charge-coupled device video camera (FK 6990, COHU, Prospective Measurements Inc., San Diego, CA, USA) and were transferred to a video system (VO-5800 PS, Sony, München, Germany) for subsequent off-line analysis. Tumor size, growth kinetics, migration of tumor cell, and neovascularization were analyzed using blue light epi-illumination (450 to 490 nm excitation wavelength and >520 nm emission wavelengths) [20–22].

2.6. Microcirculation Analysis. Microcirculatory parameters were assessed off line by frame-to-frame analysis of the videotaped images using a computer-assisted image analysis system (CapImage, Zeintl Software, Heidelberg, Germany). The fluorescent labeling of the tumor cells allowed precise delineation of the tumor from the surrounding host tissue. It also enabled for distinct identification of individual tumor cells to study tumor cell migration. At each observation time point, the surface of the fluorescently labeled tumor mass within the chamber was scanned for determination of the tumor size (given as tumor area in mm^2). Eight regions of interest (ROIs) were randomly chosen next to the tumor margin. The number of migrating cells was counted, and the distance to the tumor margin was measured (given in μm).

Microcirculation was quantified as described before in detail [22]. First, eight representative ROIs within the tumor margin were chosen and analyzed for microcirculation parameters. In these ROIs, the onset of angiogenesis, that is, the existence of angiogenic buds, sprouts, and newly formed blood vessels, was documented and scored 0 to 8, with 0 indicating existence of newly formed tumor microvessels in none of the ROIs and 8 indicating their existence in all of the ROIs. Functional capillary density (given in cm/cm^2) of the tumor microvessels was measured to quantify the angiogenic activity. This parameter was defined as the length of red blood cell perfused microvessels per observation area and was analyzed within the eight ROIs of the tumor margin and within four additional ROIs of the tumor center. Diameters of the newly formed tumor microvessels were measured perpendicularly to the vessel path (given in μm). To study vascular permeability of the newly formed tumor microvessels, petechial bleedings were documented in each of the ROIs, and given as percentage of all the ROIs analyzed [20, 21, 23].

2.7. Histology and Immunohistochemistry. At the end of the experiment (day 14), the tumor and the adjacent host tissue were harvested and immediately fixed in formalin. For light microscopy, formalin-fixed biopsies were embedded in paraffin. Sections of 5 μm were cut and stained with hematoxylin and eosin (HE) according to standard procedures to analyze tumor growth characteristics. Tumor cell invasion of the muscular layer on the surface of the dorsal skinfold chamber was quantified over the entire tumor basis and given as percentage of the length of the tumor basis.

To study tumor cell proliferation and apoptotic cell death, proliferating cell nuclear antigen (PCNA) and cleaved caspase-3 were stained using indirect immunoperoxidase techniques. Therefore, deparaffinized sections were incubated with 3% H_2O_2 and 2% goat normal serum to block endogenous peroxidases and unspecific binding sites. A monoclonal mouse anti-pan PCNA antibody (PC10, DakoCytomation, Hamburg, Germany) and a polyclonal rabbit anti-mouse cleaved caspase-3 antibody (Asp175, Cell Signaling Technology, Frankfurt, Germany) were used as primary antibodies. Goat anti-mouse and goat anti-rabbit POD-conjugated antibodies were used as secondary antibodies for streptavidin-biotin-complex peroxidase staining. 3,3′ diaminobenzidine (DakoCytomation) served as chromogen. Sections were counterstained with Hemalaun according to Mayer and examined by light microscopy.

To assess the expression of CD31 as a marker for endothelial cells, zinc fixative fixed paraffin sections of tumor tissue were used. After incubation with 3% H_2O_2 and 2% goat normal serum to block endogenous peroxidases and unspecific binding sites, deparaffinized sections were incubated with a rat anti-mouse CD31 antibody (MEC 13.3, BD Biosciences). A polyclonal goat anti-rat IgG antibody (BD Biosciences) was used as secondary antibody. Colorimetric detection was performed using 3,3′ diaminobenzidine (DakoCytomation) substrates. Sections were counterstained with Hemalaun according to Mayer and examined by light microscopy.

2.8. Flow Cytometric Analysis of CT26.WT-GFP Cells. FACScan (Becton Dickinson, Mountain View, CA, USA) analysis was performed to assess the expression of c-Kit on the CT26.WT-GFP cells in triplicate. Cells were fixed with 2% formalin, washed twice with PBS, resuspended in FACS buffer and incubated with an FITC-conjugated rat anti-mouse IgG2b c-Kit antibody (1 : 50; 553354, BD Biosciences) or an FITC-conjugated isotype-matched control antibody (553988, BD Biosciences). Cells were washed again and then maintained in 2% paraformaldehyde in PBS. Tumor cells were selectively analyzed for their fluorescence properties using the CellQuest data handling program (BD Biosciences) with assessment of 5000 events per sample. The flow cytometer was calibrated with fluorescent standard microbeads (CaliBRITE Beads, BD Biosciences).

2.9. Western Blot Analysis. To study protein expression patterns, additional Western blot analyses were performed on tumor specimen gained from the dorsal skinfold chamber. Therefore, 12 additional animals received CT26.WT-GFP tumor cell implantation in the dorsal skinfold chamber and were assigned to the three groups as described above. Eight animals were pretreated with the anti-c-Kit antibody (ACK45, BD Biosciences). Four of these pretreated animals received additional treatment with the neutralizing anti-SDF-1 antibody (MAB310, R and D Systems) starting at the day of tumor cell implantation. Four animals received the same amount of the corresponding isotype-matched control antibody (A95-1, BD Biosciences).

For whole protein extracts and Western blot analysis of the expression of vascular endothelial growth factor (VEGF), the chemokine receptor CXCR4 and eNOS, CT26.WT-GFP-tumors were completely removed from the skinfold

at day 5. The tumor tissue samples were homogenized separately in lysis buffer and a protease inhibitor cocktail (Sigma, Taufkirchen, Germany), incubated on ice and centrifuged at 16.000 ×g. The supernatant was saved as whole protein extract fraction. Protein concentrations were determined using the Lowry assay with bovine serum albumin as standard. Ten microgram protein per lane were separated discontinuously on sodium dodecyl sulfate polyacrylamide gels (10% SDS-PAGE) and transferred to a polyvinyldifluoride membrane (0.2 μm, BioRad, München, Germany). After blockade of nonspecific binding sites, membranes were incubated with an anti-VEGF antibody (A-20, Santa Cruz, Heidelberg, Germany), an anti-CXCR4 antibody (ab2074, Abcam, Heidelberg, Germany), or an anti-eNOS antibody (BD Transduction Lab., Heidelberg, Germany) followed by the corresponding secondary peroxidase-conjugated antibodies (GE Healthcare, Freiburg, Germany and Santa Cruz). Protein expression was visualized by means of luminol enhanced chemiluminescence (ECL, GE Healthcare) and exposure of the membranes to a blue-light-sensitive autoradiography film (Hyperfilm ECL, GE Healthcare). Signals were densitometrically assessed (Bio-Rad, Gel-Dokumentationssystem) and normalized to β-actin signals (mouse monoclonal anti-β-actin, Sigma) to correct for unequal loading.

2.10. Statistical Analysis. All values are expressed as means ± SEM. After proving the assumption of normality and homogeneity of variance across groups, differences between groups were calculated by a one-way analysis of variance (ANOVA) followed by the appropriate post hoc comparison, including correction of the alpha error according to Bonferroni probabilities to compensate for multiple comparisons. Overall statistical significance was set at $P < 0.05$. Statistical analysis was performed with the use of the software package SigmaStat (SPSS Inc, Chicago, Ill).

3. Results

3.1. Tumor Growth. All animals had an uneventful postoperative recovery and tolerated well the dorsal skinfold chamber implantation and the repetitive intravital microscopic analyses. The general conditions of the mice were not affected and no changes in feeding or sleeping habits were observed. The take rate of the CT26.WT-GFP cells within the dorsal skinfold chamber was 100%, and progressive tumor growth was observed in all groups throughout the entire observation period (Figures 1(a)–1(c)). Quantitative analysis of the increase of the tumor area revealed a significantly enhanced engraftment of tumor cells and a significant stimulation of tumor growth after pretreatment with the anti-c-Kit antibody compared to controls ($P < 0.05$). Interestingly, additional blockage of SDF-1 completely blunted this enhancement of tumor growth leading to similar tumor sizes as measured in controls ($P < 0.05$; Figure 1(d)).

3.2. Angiogenesis and Neovascularization. In control animals, newly developed microvessels could be detected in ∼50% of the ROIs by day 5 after tumor cell implantation. At day 8, angiogenesis was seen in almost 100% of the ROIs. Pretreatment with the anti-c-Kit antibody did not influence the onset of the angiogenic switch compared to controls. Of interest, blockade of SDF-1 after pretreatment with anti-c-Kit significantly delayed the angiogenic switch, with only ∼50% of the ROIs showing newly formed microvessels by day 8 and ∼70% by day 11 ($P < 0.05$). In this group, angiogenesis was observed in all ROIs only at the end of the observation period (Figure 2).

The differential effects of c-Kit blockade with or without additional SDF-1 blockade were also reflected by quantitative analysis of the microvascular densities of the newly formed tumor vessels. As characteristic for tumor vessels, the vascular network of the tumors consisted of irregularly shaped and chaotically arranged microvessels (Figures 3(a)–3(c)). Whereas no differences of microvascular density within the tumor vasculature could be observed between controls and anti-c-Kit pretreated animals, additional treatment with the anti-SDF-1 antibody significantly decreased the microvascular density within the tumor center compared to the other groups from day 8 until the end of the observation period (Figure 3(d)). Quantitative analysis of the functional microvascular density within the tumor margin showed similar results (data not shown). The functional microvascular density within the tumor margin and the tumor center was not significantly different within the individual treatment groups.

In contrast to the highly vascularized CT26.WT-GFP tumors within the intravital fluorescence microscopy, immunohistological staining for CD31 as a marker for endothelial cells displayed positive staining of only a few cells within tumor microvessels without significant differences between the three groups (data not shown).

As neovascularization is usually associated with vasodilation due to the action of VEGF, microvessel diameters within the tumor were analyzed. However, no overall differences between the diameters of the microvessels within the tumor margin and the tumor center could be observed (Table 1). During the 14 days observation period, diameters slightly increased in all groups until day 11, particularly in the control and anti-c-Kit-pretreated groups. Of interest, at day 14, microvessel diameters within tumors of mice treated with anti-c-Kit and anti-SDF-1 were markedly smaller compared to the other two groups (Table 1).

Petechial bleedings within the areas of angiogenesis of growing tumors are a characteristic indicator of the VEGF action on vascular permeability. To study this VEGF-induced increase of vascular permeability, we analyzed the petechial bleedings within the ROIs by intravital fluorescence microscopy. Microbleedings were observed in all tumors. At day 5, signs of petechial bleedings were observed in ∼6% of the tumor area of control tumors, whereas petechial bleedings occurred in up to 30% of the tumor area in animals pretreated with anti-c-Kit at that time. In all groups, petechial bleedings were most pronounced 8 days after tumor cell implantation (control: 15–20%, anti-c-Kit: ∼30%, anti-c-Kit/anti-SDF-1: 35–40%). Comparing the three groups, no statistical significance could be found due to the high

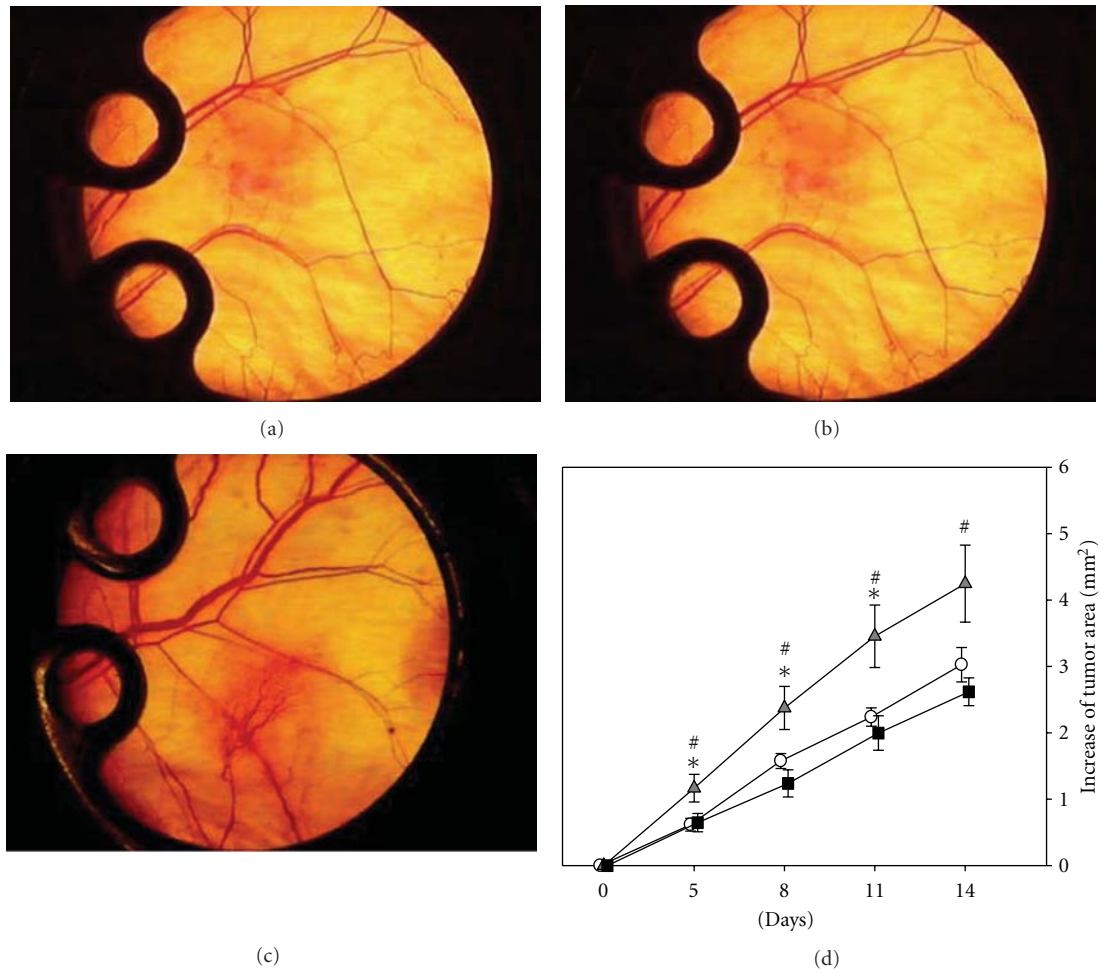

Figure 1: Time course of tumor growth of CT26.WT-GFP tumors in the dorsal skinfold chamber. Stereomicroscopy photographs of representative 14 days old tumors after either treatment with an isotype-matched control antibody (a), pretreatment with anti-c-Kit (b) alone, or pretreatment with anti-c-Kit followed by anti-SDF-1 treatment (c). Quantitative analysis of the tumor area (d) displayed progressive tumor growth in all groups. After anti-c-Kit pretreatment (grey triangles), tumor growth was significantly accelerated compared to controls (white circles). Of interest, additional neutralization of SDF-1 (black squares) completely blunted this anti-c-Kit-associated enhancement of tumor growth. Mean ± SEM; $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus anti-c-Kit. Original magnification (a)–(c) ×4.

Table 1: Microvessel diameters within the tumor margin and the tumor center of control animals, animals pretreated with anti-c-Kit and animals pretreated with anti-c-Kit followed by anti-SDF-1 treatment. All values are given in μm. Diameters slightly increased during the observation period. No significant differences could be observed between the tumor margin and center. Fourteen days after tumor cell implantation, microvascular diameters within the tumor center were significantly smaller in anti-c-Kit/anti-SDF-1 treated animals compared to controls and to anti-c-Kit treated animals.

	Time	Control	Anti-c-Kit	Anti-c-Kit/anti-SDF-1
Tumor margin	d5	12.42 ± 0.53	11.88 ± 0.77	13.02 ± 0.52
	d8	13.97 ± 0.48	14.60 ± 0.93	15.02 ± 0.57
	d11	14.73 ± 1.36	15.39 ± 1.00	14.84 ± 1.06
	d14	15.81 ± 1.20	15.24 ± 0.91	13.94 ± 0.75
Tumor centre	d5	13.91 ± 0.67	12.85 ± 0.83	13.22 ± 1.00
	d8	14.44 ± 0.52	13.93 ± 0.75	14.21 ± 0.47
	d11	15.35 ± 1.40	15.26 ± 0.98	15.66 ± 1.83
	d14	16.86 ± 1.10	16.84 ± 1.35	12.82 ± 0.67$^{*\#}$

Data are given as mean ± SEM; $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus anti-c-Kit.

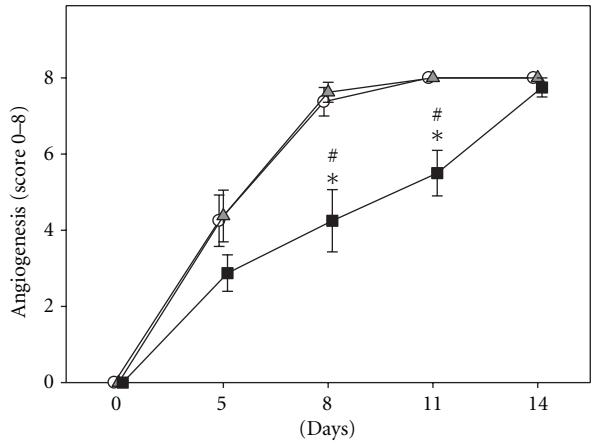

Figure 2: Semiquantitative analysis of the onset of angiogenesis within the CT 26.WT-GFP tumors. Animals were pretreated with anti-c-Kit (grey triangles) alone or anti-c-Kit and anti-SDF-1 neutralizing antibodies (black squares). Animals treated with isotype-matched control antibodies served as controls (white circles). In controls and anti-c-Kit pretreated animals, ~50% of the ROIs showed newly developed microvessels 5 days after tumor cell implantation. By day 8, almost 100% of the ROIs were vascularized. Treatment with anti-c-Kit and anti-SDF-1 resulted in a significant delay of angiogenesis with only ~50 and ~70% of the ROIs showing newly developed microvessels by days 8 and 11. Within these tumors, angiogenesis could be observed in all ROIs only at day 14. Mean ± SEM; $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus anti-c-Kit.

standard deviation within the individual groups (data not shown).

3.3. Tumor Cell Migration. As SDF-1 has been demonstrated to exert chemotactic effects on CT26.WT-GFP tumor cells, we additionally focused on migrating tumor cells next to the tumor margin. The migrating tumor cells were easily detectable due to their GFP labeling (Table 2). Starting at day 5 after tumor cell implantation, tumor cell migration was detectable in all tumors until the end of the observation period with slightly increasing distances from the tumor margin. The number of migrating tumor cells ranged between 20 and 25 per representative field during the whole observation period. Treatment with anti-c-Kit alone or additional blockade of SDF-1 did not impair tumor cell migration compared to controls (Table 2).

3.4. Tumor Cell Morphology, Proliferation, and Apoptotic Cell Death. Histological examinations on hematoxylin-eosin stained sections revealed solid tumor growth within the dorsal skinfold chamber. Signs of malignant tumor growth such as invasion of the adjacent host tissue by the tumor cells were detectable in each group (Figure 4). Quantitative analysis of tumor cell invasion showed a significant increase of tumor cell infiltration through the muscular layer of the dorsal skinfold chamber in tumors of animals which were pretreated with anti-c-Kit compared to controls ($P < 0.05$). Of interest, tumors of animals additionally treated with anti-SDF-1 showed a significant reduction of muscular infiltration compared to anti-c-Kit pretreated tumors ($P < 0.05$), resulting in invasive growth characteristics which were comparable to controls (Figure 4(c)).

PCNA as an indicator of cell proliferation displayed positive staining in 46.9 ± 3.1% of the tumor cells in control animals (Figure 5). After pretreatment with anti-c-Kit, the number of PCNA-positive tumor cells was significantly higher compared to controls ($P < 0.05$, Figures 5(a) and 5(c)). In contrast, additional blockade of SDF-1 resulted in a significantly lower number of PCNA-positive tumor cells compared to controls and to anti-c-Kit pretreated animals ($P < 0.05$, Figures 5(b) and 5(c)).

To study apoptotic cell death, immunohistochemical staining of cleaved caspase-3 products within the tumors was performed. Of interest, 14 days after tumor cell implantation, only a minor fraction of the total number of 351.3 ± 5.2 tumor cells within the high power fields (HPF) showed positive staining for caspase-3. Whereas 0.73 ± 0.13 tumor cells/HPF were positive for caspase-3 in controls, pretreatment with anti-c-Kit reduced apoptotic cell death within the tumors (0.26 ± 0.06 tumor cells/HPF). In tumors of animals additionally treated with anti-SDF-1 antibodies, this effect was even more pronounced (0.15 ± 0.03 tumor cells/HPF, $P < 0.05$).

3.5. C-Kit Expression and Western Blot Analysis. FACScan analysis demonstrated that 8.2 ± 0.8% of the CT26-GFP cells transfected with the enhanced GFP expression vector pEGFP-N1 was c-Kit receptor positive.

At day 5 after tumor cell implantation, the tumors of animals pretreated with anti-c-Kit antibodies showed a significantly higher expression of VEGF and CXCR4 compared to controls ($P < 0.05$; Figures 6(a) and 6(b)). Additional SDF-1 blockade did not further increase VEGF and CXCR4 expression. Furthermore, eNOS expression within the tumors was decreased by anti-c-Kit pretreatment with and without additional anti-SDF-1 treatment compared to controls (data not shown).

4. Discussion

The major finding of the present study is that bone marrow suppression by anti-c-Kit treatment significantly enhances tumor cell engraftment of colorectal tumors due to an increase of tumor cell proliferation and invasion. Additional anti-SDF-1 treatment neutralizes this increased tumor outgrowth by inhibition of tumor cell proliferation and tumor neovascularization. These findings suggest that the enhanced tumor growth under the conditions of bone marrow suppression induced by anti-c-Kit treatment is related to the SDF-1/CXCR4 pathway.

Bone-marrow-derived hematopoetic cells (HCs) contribute to physiological and pathological vessel formation. During tumor growth, the release of cytokines and chemokines mediates the recruitment of EPCs and HCs contributing to the early initiation and stabilization of newly formed blood vessels, so-called vasculogenesis [24–26]. However, their precise role has not been fully elucidated

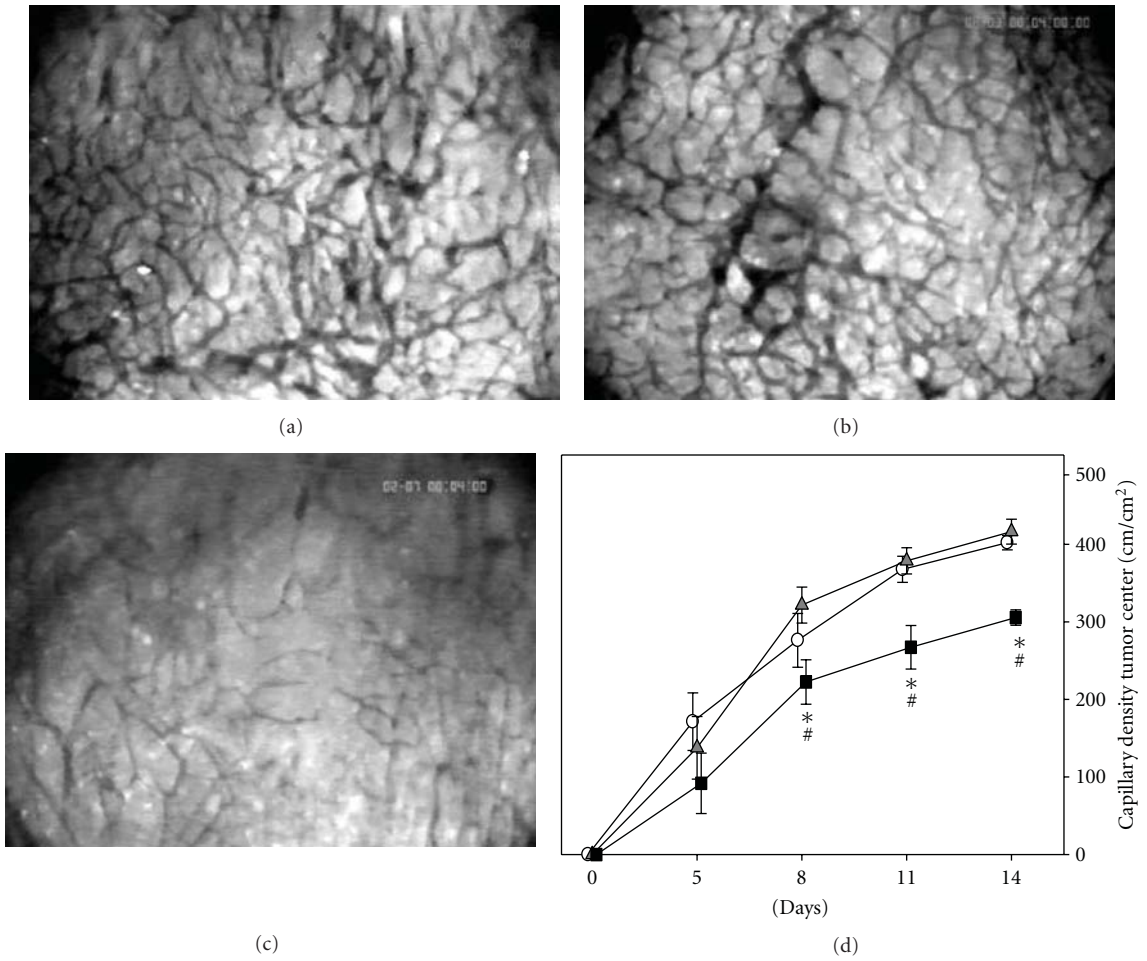

Figure 3: Time course of the functional capillary density within CT-26.WT-GFP tumors in dorsal skinfold chambers analyzed by intravital fluorescence microscopy. Representative fluorescence microscopic images show the network of chaotically arranged microvessels within the tumor center of control animals (a), animals pretreated with anti-c-Kit (b), and animals additionally treated with anti-SDF-1 (c) at day 11 after tumor cell implantation. Quantitative analysis of the functional capillary density (d) revealed a significant inhibition of tumor neovascularization within the tumor center after combined anti-c-Kit and anti-SDF-1 treatment (black squares) compared to controls (white circles). Anti-c-Kit pretreatment alone (grey triangles) did not significantly influence the extent of neovascularization. Mean ± SEM; $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus anti-c-Kit. Original magnification (a)–(c) ×40.

Table 2: Tumor cell migration in dorsal skinfold chambers of control animals, animals pretreated with anti-c-Kit, and animals pretreated with anti-c-Kit followed by anti-SDF-1 treatment during a 14-day observation period. Migrated tumor cells next to the tumor margin were observed from day 5 until the end of the observation time. Comparing all three groups, no significant differences could be observed concerning the total number of migrated cells as well as their distance [μm] from the tumor margin. Of interest, at the later time points, tumor cells were found at greater distances from the tumor margin.

	Time	Control	Anti-c-Kit	Anti-c-Kit/anti-SDF-1
Number	d5	22.08 ± 1.33	23.00 ± 1.33	19.91 ± 1.14
	d8	22.67 ± 1.33	23.14 ± 1.18	20.67 ± 1.07
	d11	22.42 ± 1.53	23.48 ± 1.05	20.80 ± 1.34
	d14	20.84 ± 1.71	23.96 ± 1.32	21.17 ± 1.30
Distance	d5	276.24 ± 16.08	291.82 ± 15.95	309.45 ± 50.93
	d8	337.94 ± 9.38	351.11 ± 23.57	323.38 ± 20.45
	d11	383.85 ± 15.80	423.32 ± 25.65	360.83 ± 18.58
	d14	421.50 ± 22.40	438.49 ± 32.01	458.51 ± 59.01

Data are given as mean ± SEM.

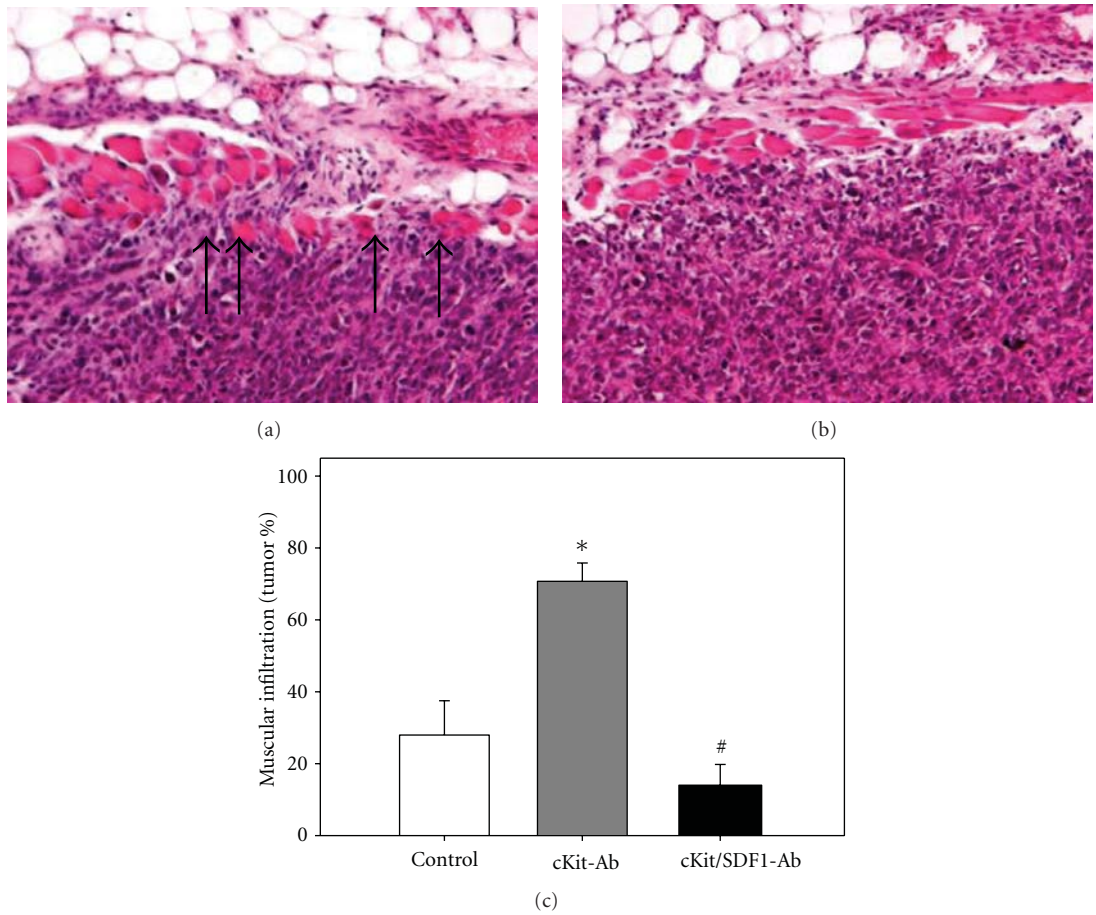

Figure 4: Hematoxylin-eosin staining of CT26.WT-GFP tumors shows solid tumor growth 14 days after tumor cell implantation within the dorsal skinfold chamber. Sections display tumor infiltration through the underlying muscular layer (marked by arrows) after pretreatment with anti-c-Kit (a) and lack of muscular infiltration after pretreatment with anti-c-Kit and additional neutralization of SDF-1 (b). Quantitative analysis of tumor cell invasion of the muscular layer is given as percentage of the length of the tumor. Tumors of animals pretreated with anti-c-Kit showed a significantly pronounced infiltration of the muscular layer compared to controls. Of interest, blockade of SDF-1 after anti-c-Kit pretreatment abrogated this anti-c-Kit-associated increase of tumor cell infiltration. Mean ± SEM; $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus anti-c-Kit. Original magnification (a, b) ×88.

yet. HCs and EPCs express stem cell markers such as c-Kit. Whereas many studies indicate that bone-marrow-derived EPCs incorporate into tumor neovessels [25, 27], HCs may promote vasculogenesis via paracrine release of angiogenic factors enhancing the recruitment and incorporation of EPCs into neovessels [28].

In the early phases of tumor growth, 50–90% of the neovessels within the tumor mass are derived from the bone marrow dependent on the tumor type [25]. However, recent reports have already doubted the impact of vasculogenesis from bone-marrow-derived cells for tumor neovascularization and claim an exclusive role for sprouting angiogenesis in tumor blood vessel development [15, 16]. In a model of syngeneic bone marrow transplantation, Patil et al. demonstrated that GPF-expressing c-Kit^{+} bone-marrow-derived progenitor cells are recruited to subcutaneously implanted Lewis lung carcinoma but do not directly contribute to microvascular structure. They, therefore, concluded that even if circulating HCs and EPCs home to sites of tumor growth, they do not contribute to tumor angiogenesis [29]. In a model of subcutaneously implanted prostate carcinoma, Okamoto et al. observed an accumulation of HCs around newly formed blood vessels, but did not find these cells to be part of the tumor microvessels themselves. Because of these findings, the authors postulated a stabilizing and supportive role of HCs for the developing vascular network [7].

In the present study, intravital fluorescence microscopy showed highly vascularized CT26.WT-GFP tumors 14 days after tumor cell implantation. However, immunohistological staining using the endothelial cell marker CD31 displayed positive staining of only a few endothelial cells within these tumors. This finding reflects the diverging structure between normal microvessels and tumor neovessels which consist of only cancer cells or a mosaic of cancer and endothelial cells [2].

Previous experimental studies in mice have shown that a depletion of the myeloid and erythroid cell lineages including EPCs and HCs from the bone marrow could be performed

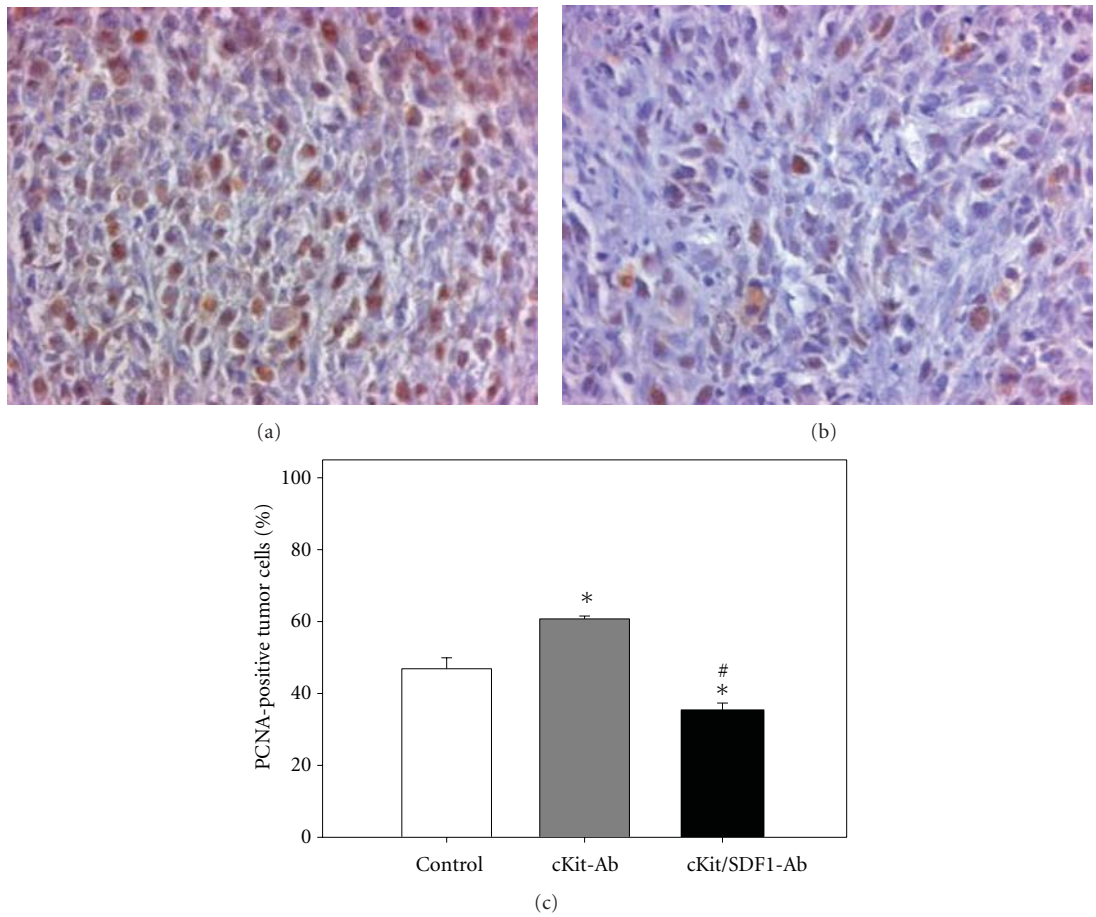

Figure 5: PCNA immunohistochemistry in CT26.WT-GFP tumors at day 14 after tumor cell implantation. Animals were pretreated with anti-c-Kit (cKit-Ab, (a)) alone or anti-c-Kit and anti-SDF-1 neutralizing antibodies (cKit/SDF1-Ab, (b)). Animals treated with an isotype-matched control antibody served as controls (control). Quantitative analysis demonstrated that anti-c-Kit pretreatment significantly increased the rate of proliferating tumor cells compared to controls (c). Of interest, additional neutralization of SDF-1 significantly decreased the amount of proliferating tumor cells compared to controls and anti-c-Kit-treated animals. Mean ± SEM; $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus anti-c-Kit. Original magnification (a, b) ×175.

by daily injection of 1 mg/kg ACK2, an antagonistic anti-c-Kit antibody, which blocks the function of c-Kit without cytotoxic side effects on c-Kit^{+} cells [30, 31]. Of interest, whereas no polymorphonuclear cells or erythroblasts were present in the bone marrow of these ACK2-treated animals, B lineage cells continued to grow to fill the space from which myeloid and erythroid progenitor cells were purged [30, 31]. Moreover, the reduction rate of the colony forming cells within the bone marrow was dependent on the amount of anti-c-Kit antibodies [30]. In the present study, we, therefore, used the anti-c-Kit antibody in a dosage of 1 mg/kg BW for the induction of bone marrow suppression over a time period of 4 days to avoid surgical complications and death of the animals resulting from the immune incompetence by bone marrow depletion.

As shown by Okamoto et al., bone marrow suppression by anti-c-Kit pretreatment over a time period of 4 days before subcutaneous implantation of colon tumor cells induced leucopenia which was still detectable 10 days after the last injection [7]. Although tumor growth and sprouting of tumor vessels were slightly reduced in these studies of Okamoto et al. during the first 5–7 days, tumor growth was rapidly reinitiated as the number of circulating HCs in the peripheral blood increased again [7]. Therefore, the authors concluded that HCs migrating into the tumor mass promote the initiation of tumor neovascularization. In our present study, although neoangiogenesis was not influenced after administration of anti-c-Kit, tumor growth was stimulated as a result of an increased tumor cell proliferation and invasion. This observation might be the result of immunological mechanisms that suppress tumor growth in animals with an intact immune system. C-Kit blockade for the induction of bone marrow suppression concurrently stimulates B lymphopoiesis [30, 31]. As the number of B cell precursors normally decreases in tumor bearing mice [32, 33], it must be speculated that in our experiment of bone marrow suppression, c-Kit blockade stimulates B cell genesis and thus increases tumor cell engraftment. Furthermore, B lymphopoiesis is associated with increased angiogenesis and cellular proliferation [34–36]. In our study, neoangiogenesis

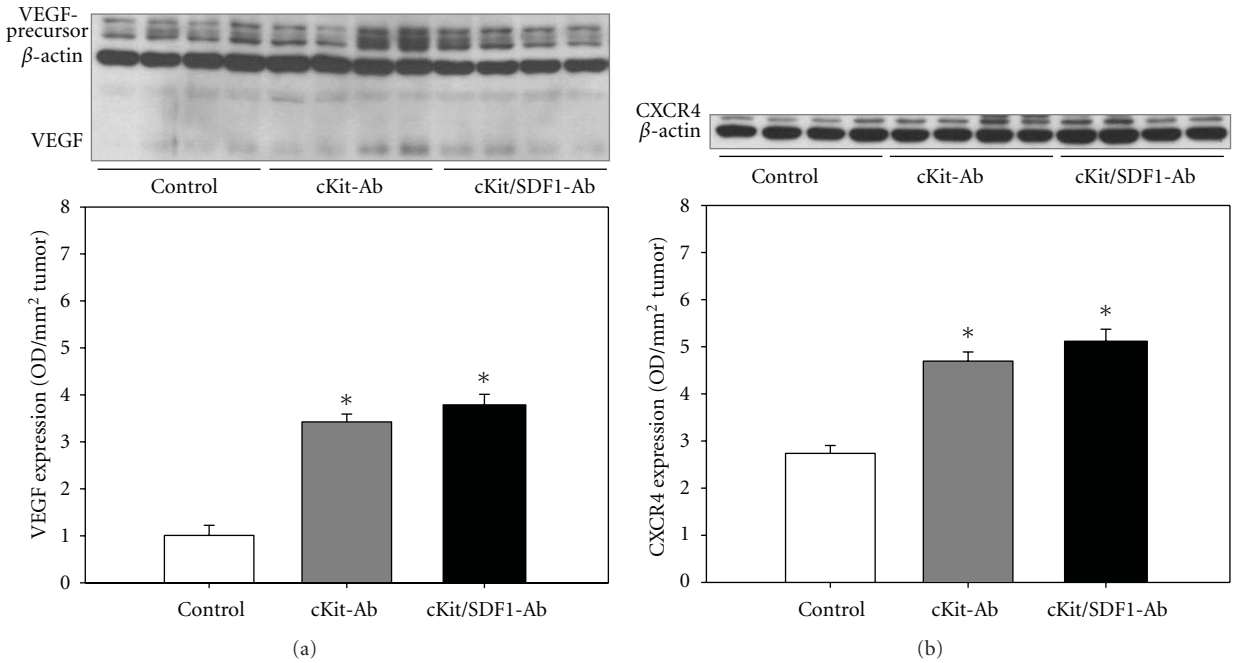

Figure 6: Western blot analysis of VEGF (a) and CXCR4 (b) expression within CT 26.WT tumors five days after tumor cell implantation. Animals were pretreated with anti-c-Kit (cKit-Ab) alone or additionally treated with a SDF-1 neutralizing antibody (cKit/SDF1-Ab). Animals treated with isotype-matched control antibodies served as controls (control). Quantitative analysis showed that VEGF expression was significantly higher after anti-c-Kit pretreatment than in control animals (a). Additional neutralization of SDF-1 had no further effect on the increased VEGF expression. CXCR4 was also significantly higher expressed after anti-c-Kit pretreatment compared to controls and was not further influenced by additional SDF-1 neutralization (b). Mean ± SEM; $^*P < 0.05$ versus control.

within the colorectal tumors of animals pretreated with anti-c-Kit was comparable to controls despite the lack of functional HCs which are necessary for angiogenesis and vasculogenesis. Thus, we hypothesize that the lack of HCs was compensated by local angiogenic factors or B lymphopoiesis-associated angiogenesis, resulting in a tumor neovascularization comparable to that observed in controls.

The SCF receptor c-Kit has been shown to be essential for the development of blood cells, melanocytes, germ cells, interstitial cells of Cajal in the gastrointestinal tract, and mast cells [37]. Furthermore, the majority of tumor cells, especially those of the neural axis, breast, lung, prostate, and colon show an aberrant c-Kit expression [38]. Especially gastrointestinal stromal tumors (GISTs) express c-Kit on the cell-surface, and mutations of Kit in these tumors results in an activation of Kit signaling, which leads to uncontrolled cell proliferation and resistance to apoptosis [39, 40]. Today, patients with GIST are treated with Imatinib, an inhibitor of certain protein tyrosine kinases including KIT, depending on the mitotic index of the GIST. Imatinib induces an arrest of tumor cell proliferation and causes apoptotic cell death. In established MCA26 tumors, Pan et al. showed that injection of anti-c-Kit antibodies markedly reduces tumor-induced immune tolerance exhibited by myeloid-derived suppressors in mice [41]. Furthermore, their experiments demonstrate that anti-c-Kit treatment can prevent tumor-specific T-cell anergy and development of T regulatory cells (Treg). In combination with an immune modulatory therapy of IL-12 plus 4-1BB activation, treatment with anti-c-Kit antibodies significantly improved the long-term survival of MCA26 tumor bearing mice [41]. In our study, only 8% of the CT26.WT-GFP cells were c-Kit receptor positive. Therefore, we do not expect a direct inhibitory effect of anti-c-Kit treatment on tumor cell proliferation.

Recruitment of HCs and EPC is predominantly mediated by SDF-1 and its receptor CXCR4 [9, 10] because HCs and EPCs migrate along a chemotactic gradient towards higher concentrations of SDF-1 [12–14]. Kaminski et al. have shown that almost 100% of bone marrow and peripheral blood c-Kit$^+$ cells are positive for CXCR4 [12]. In the present study, we could demonstrate that neutralization of SDF-1 after anti-c-Kit pretreatment significantly reduces neovascularization most probably by the inhibition of HC and EPC recruitment within the tumors. Additionally, in combination with anti-c-Kit pretreatment, SDF-1 neutralization is capable of decreasing tumor cell proliferation and invasion. Taken together, neutralization of SDF-1 counteracts the stimulating effects of bone marrow suppression by anti-c-Kit treatment on tumor cell engraftment and inhibits compensatory local and B lymphopoiesis-associated angiogenesis.

As described by Kaminski et al., the presence of SDF-1 chemoattractant activity and inflammatory endothelial activation by TNF-α is required for c-Kit$^+$ cells to form functionally relevant interactions with the endothelium in postcapillary venules [12]. Blockade of ICAM-1 and CXCR4 abolishes adhesion of c-Kit$^+$ cells to the vascular endothelium despite application of SDF-1 and TNF-α. Moreover, in their cremaster muscle microcirculation model, stem cell

adhesion was significantly reduced when eNOS was not present or systemic NOS inhibited [12]. As SDF-1 and TNF-α stimulation activates the endothelium by an increase of the CXCR4 expression leading to relevant stem cell attraction [12], our results indicate that the increase of CXCR4 and VEGF expression within the tumors represents a compensatory pathway after anti-c-Kit pretreatment. Neutralization of SDF-1 inhibits interactions of c-Kit positive cells with tumor vessels and as a consequence, leads to inhibition of tumor neovascularization.

In conclusion, bone marrow suppression by anti-c-Kit pretreatment significantly enhances tumor cell engraftment of colorectal tumors. As anti-SDF-1 treatment counteracts this increased tumor outgrowth by inhibition of neovascularization, the SDF-1/CXCR4 pathway seems to be crucial for tumor angiogenesis mediated by HCs and EPCs.

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Review Article

Tumor Angiogenesis as a Target for Dietary Cancer Prevention

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Between 2000 and 2050, the number of new cancer patients diagnosed annually is expected to double, with an accompanying increase in treatment costs of more than $80 billion over just the next decade. Efficacious strategies for cancer prevention will therefore be vital for improving patients' quality of life and reducing healthcare costs. Judah Folkman first proposed antiangiogenesis as a strategy for preventing dormant microtumors from progressing to invasive cancer. Although antiangiogenic drugs are now available for many advanced malignancies (colorectal, lung, breast, kidney, liver, brain, thyroid, neuroendocrine, multiple myeloma, myelodysplastic syndrome), cost and toxicity considerations preclude their broad use for cancer prevention. Potent antiangiogenic molecules have now been identified in dietary sources, suggesting that a rationally designed antiangiogenic diet could provide a safe, widely available, and novel strategy for preventing cancer. This paper presents the scientific, epidemiologic, and clinical evidence supporting the role of an antiangiogenic diet for cancer prevention.

1. Introduction

Cancer now affects as many as 24 million people worldwide, and results in over six million deaths each year [1]. In the United States, men and women have a 43% and 38% chance, respectively, of being diagnosed with any type of cancer during their lifetime [2]. Despite advances in the early detection of cancer, most malignancies are still diagnosed and treated at advanced stages, with a limited range of therapeutic options and poor overall survival. Simultaneously, cancer treatment costs are escalating, from $125 billion annually in the US in 2010 to a projected $207 billion by 2020 [3]. Cancer prevention is, thus, a key opportunity for managing the cancer pandemic. Unlike interventional approaches delivered to patients diagnosed with advanced cancer, preventative strategies must be suitable for healthy individuals and have low systemic toxicity, inhibiting microscopic tumor growth with minimal adverse effects on healthy tissues [4].

It is now well established that solid tumor growth is dependent upon angiogenesis, the growth of new blood vessels [5–10]. During early stages of tumorigenesis, the induction of angiogenesis by cancer cells is a critical event separating the preinvasive and dormant form of cancer from the invasive and metastatic phases of malignant growth. Multiple studies have demonstrated that the degree of tumor vascularity correlates positively with disease stage, the likelihood of metastases, and cancer recurrence [11, 12]. Angiogenesis also plays a role in hematogenous malignancies, such as leukemia, lymphoma, and multiple myeloma, as well as in premalignant myelodysplastic syndromes [13–17]. In these pathologies, vascular endothelial cells sustain and promote malignant cell growth by secreting paracrine survival factors [18, 19].

Antiangiogenic therapy has been validated as an effective cancer treatment strategy for a growing number of cancer types, including colorectal, renal, liver, lung, brain, pancreatic neuroendocrine tumors (NET), gastrointestinal stromal tumors (GIST), multiple myeloma, and myelodysplastic syndrome [20]. More than 120 novel antiangiogenic agents are in clinical, trials [20–22]. Importantly, a growing body of preclinical, clinical and epidemiological data is demonstrating that angiogenesis inhibition can be applied for achieving cancer prevention [23, 24]. This paper presents the scientific and clinical evidence supporting antiangiogenesis as a rational strategy for the prevention of cancer, exploiting factors that are naturally present in dietary sources.

2. The Physiological State of Angiogenesis Regulation

The human body contains 60,000 miles of blood vessels, including 19 billion capillaries. All normal cells in the body

are located no further than 100–200 μm from the nearest capillary, the diffusion limit of oxygen [25]. Capillaries not only deliver oxygen and micronutrients to tissues, but the endothelial cells comprising them secrete paracrine growth and survival signals that influence adjacent nonvascular cells [18]. Under physiological conditions, the rate of cell proliferation is balanced with the rate of cell death (apoptosis), so there is no net tissue growth. Expansion of tissue mass requires angiogenesis to support increased metabolic demand [9]. In normal healthy adults, angiogenesis is constitutively suppressed except for brief bursts during the female reproductive cycle (endometrial regeneration, corpus luteum formation), pregnancy (placentation), and wound healing (granulation) [26–30]. The physiological state is thus maintained in a constitutive state of suppressed angiogenesis by endogenous inhibitory mechanisms opposing the action of angiogenic growth and other stimulating factors.

2.1. Angiogenic Growth Factors. More than 30 endogenous molecules have been identified as angiogenic factors (Table 1). These share the ability to stimulate neovascularization *in vivo* and induce endothelial proliferation, migration, or capillary tube formation *in vitro*. Basic fibroblast growth factor (bFGF or FGF2) was the first angiogenic factor to be identified from a tumor extract, but vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is the best studied [30]. VEGF is a potent endothelial mitogen that increases vascular permeability, and also induces Bcl-2, promoting vascular survival [31–34]. VEGF is expressed by all human tumors studied and its receptors (VEGFR-1, -R2, -R3 and -R4) are expressed selectively on angiogenic endothelial cells [35]. Placental growth factor (PlGF) plays a specific role in pathological neovascularization by recruiting bone marrow-derived vascular stem cells to disease sites [36, 37]. Other factors include platelet-derived growth factor (PDGF), platelet-derived endothelial cell growth factor (PD-ECGF), interleukin-3 (IL-3), interleukin-8 (IL-8), transforming growth factor-β (TGFβ), and tumor necrosis factor-alpha (TNFα) [38, 39]. Other angiogenic factors are neuregulin, a ligand for the ErbB receptor, and keratinocyte growth factor (KGF or FGF-7) [40, 41]. Angiogenic factors are observed at low levels or undetectable in the circulation in normal, healthy subjects. By contrast, markedly elevated levels of factors such as bFGF, VEGF, and PD-ECGF are present in the serum, urine, and cerebrospinal fluid of cancer patients [42, 43].

2.2. Physiological Inhibition of Angiogenesis. Angiogenesis inhibitory activity was first discovered in studies of cartilage, a naturally avascular tissue [44]. Numerous endogenous antiangiogenic molecules have subsequently been identified, including troponin-1, tissue inhibitors of matrix metalloproteinases (TIMPs), chondromodulin I, connective tissue-growth factor (CTGF), decorin, metastatin, pigment epithelium-derived factor (PEDF) thrombospondin-1 and -2, interferons, tetrahydrocortisol-S, platelet factor-4, and protamine [45–60]. Other inhibitors, such as canstatin, tumstatin, and arresten, are present in the basement membrane surrounding established blood vessels [61–63].

Table 1: Angiogenic factors.

Angiogenin
Angiopoietin-1
Adrenomedullin
Del-1
Fibroblast growth factor-1 (acidic FGF, FGF1)
Fibroblast growth factor-2 (basic FGF, FGF2)
Follistatin
Granulocyte-colony-stimulating factor (G-CSF)
Hepatocyte growth factor/scatter factor (HGF/SF)
Interleukin-3 (IL-3)
Interleukin-8 (IL-8)
Intermedin
Keratinocyte growth factor (FGF-7)
Leptin
Midkine
Neuregulin
Osteogenic protein-1
Placental growth factor (PlGF)
Platelet-derived endothelial-cell growth factor (PD-ECGF)
Platelet-derived growth factor (PDGF)
Pleiotrophin
Progranulin
Proliferin
Transforming growth factor-α (TGFα)
Transforming growth factor-β (TGFβ)
Tumor necrosis factor-α (TNFα)
Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF)

A separate and distinct class of inhibitors is comprised of proteolytic fragments derived from cleaved larger molecules. Angiostatin is an internal fragment of plasminogen and specifically inhibits endothelial cell proliferation [64]. Enzymes such as macrophage-derived elastase and serine proteases generate angiostatin or angiostatin-like fragments [65, 66]. Endostatin, a 20-kDa fragment of collagen XVIII, is a specific angiogenesis inhibitor that induces endothelial apoptosis [67, 68]. Both angiostatin and endostatin were discovered in the serum of tumor-bearing experimental mice, suggesting that tumor-associated protease activity generates these inhibitors. Removal of the primary tumor led to a marked decline in serum angiostatin and endostatin, followed by rapid angiogenic growth of metastatic lesions [9, 69]. Endostatin is present at a low circulating level in normal subjects [70]. Collectively, these endogenous angiogenesis inhibitors play a dominant role in suppressing angiogenesis in health and contribute to tumor dormancy (Table 2).

2.3. Balance and Imbalance of Angiogenesis. Vascular growth is physiologically governed by a homeostatic balance between

Table 2: Endogenous inhibitors of angiogenesis.

Angiopoietin-2 (in the absence of VEGF)
Angiostatin
Antithrombin III fragment
Arresten
Canstatin
Chondromodulin I
Connective tissue growth factor (CTGF)
Decorin
Endorepellin
Endostatin
Fibronectin 20-kDa fragment
Interferons-α, β, and γ
Interleukin-4 (IL-4)
Interleukin-10 (IL-10)
Interleukin-12 (IL-12)
Interferon-inducible protein-10 (IP-10)
Kringle 5
Metastatin
METH-1
METH-2
2-Methoxyestradiol
Osteopontin cleavage product
PEX
Pigment epithelium-derived factor (PEDF)
Plasminogen activator inhibitor (PAI)
Platelet factor-4
Prolactin 16-KDa fragment
Proliferin-related protein
Prothrombin kringle 2
Maspin
Restin
Soluble fms-like tyrosine kinase-1 (S-Flt-1)
SPARC cleavage product
Tetrahydrocortisol-S
Tissue inhibitors of matrix metalloproteinases (TIMPs)
Thrombospondin-1 and -2
Transforming growth factor-β (TGF-β) (*activated form)*
Troponin-1
Tumstatin
Vascular endothelial growth inhibitor (VEGI)
Vasostatin

Table 3: Genetic control of angiogenesis.

Id1	p53
Id3	Rb
HIF-1a	VHL
K-*ras*	PTEN
N-*myc*	*trk* B
c-*myc*	p16INK4a
c-*fos*	
c-*src*	
c-*myb*	
c-*jun*	
HER2/*neu*	
EGFT	
Raf	
Mek	
p73	
Del-1	
FzD	
Bcl2	
MDNM2	
PML-RAR	
ElF-4E	

positive and negative angiogenesis regulators, so that neovascularization is normally suppressed [71]. Vascular proliferation occurs when angiogenic growth factor production is upregulated, or when expression of endogenous inhibitors is downregulated, or when both events occur simultaneously [72–74]. The genetic regulators of angiogenesis are closely related to tumor growth promotion and suppression (Table 3). Gene knockout studies in mice have shown that Id1 and Id3, peptides that control cell differentiation by interfering with DNA binding of transcription factors, are required for normal vascular formation and induction of angiogenesis in tumor-bearing animals [75]. The activated forms of the oncogenes H-*ras*, v-*raf*, c-*myc*, c-*src*, Her-2/neu, and p73 are associated with cellular production of VEGF as well as tumorigenesis [76–83]. Several tumor-suppressor genes regulate angiogenesis inhibition, including p53, Rb, vHL, phosphatase and tensin homolog (PTEN), and *trk* B [84] Wild-type p53 controls expression of the angiogenesis inhibitor thrombospondin and decreases tumor neovascularization; mutant p53 leads to the opposite effect [73]. The retinoblastoma (Rb) gene and the von Hippel-Lindau (vHL) gene both downregulate VEGF expression; their mutation leads to VEGF production, angiogenesis, and tumor growth [85, 86].

2.4. The Avascular Dormant Phase of Cancer. Microscopic cancer cells are commonly present in the healthy adult, the result of errors during replication of 60–90 trillion cells. To acquire sustenance, the incipient tumors (60–80 cells) may migrate toward existing host vessels, a process known as vessel cooption, but their growth remains limited [87–89]. Tumors are capable of growth to approx 0.5 mm^3 in diameter (10,000,000 cells) before reaching a steady state of growth. Beyond this size, their metabolic demands exceed the supply of oxygen and nutrients obtained by passive diffusion from nearby blood vessels. This state corresponds to carcinoma *in situ*, and the rate of tumor cell proliferation is balanced by apoptosis [90]. Such microscopic tumors may exist for years without clinical detection.

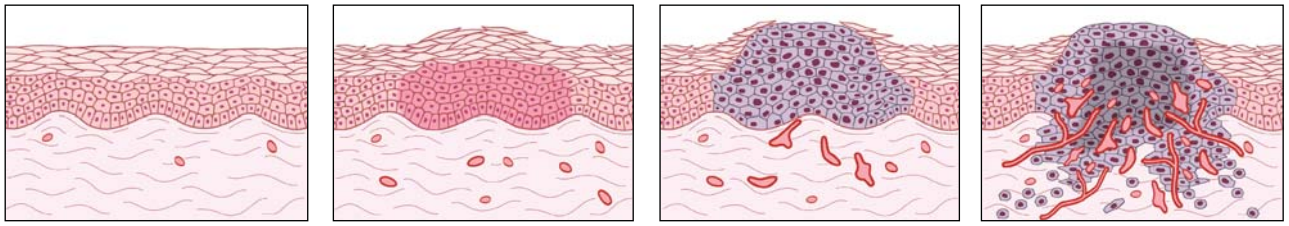

Figure 1: The switch to the angiogenic phenotype occurs during multistage tumorigenesis. As malignancy develops, cells progress from a prevascular stage (normal to early hyperplasia) to a vascular stage (late hyperplasia to dysplasia to invasive carcinoma). Angiogenesis becomes clearly evident during dysplasia and is critical for further growth. Targeting tumor angiogenesis may be a novel strategy for preventing cancer. (Reprinted by permission from the Angiogenesis Foundation. Copyright 2011 by The Angiogenesis Foundation. All rights reserved).

Autopsy studies have shown that these microscopic cancers are present in the breasts of up to 40% of women between the ages of 40 and 50 years, and in 50% of prostates in men between 50 and 60 years. By age 70, microscopic cancers are detected in the thyroids of virtually everyone. Most of these tumors never become clinically significant, leading to the concept of "cancer without disease" as a normal state during aging. Physiological angiogenesis inhibition is regarded as one of the mechanisms that prevent microscopic cancers from converting to a malignant phenotype.

2.5. The "Switch" to the Angiogenic Phenotype during Multistep Tumorigenesis. To expand beyond the limits of the preexisting vascular supply, tumors recruit new blood vessels from surrounding vessels, an event known as the "switch" to the angiogenic phenotype [91] (see Figure 1). Three classic studies employing transgenic mice have delineated this switch as normal cells undergo the transition from normalcy to hyperplasia to dysplasia, and finally to frank carcinoma.

In a model of spontaneous β-islet cell tumor formation, Rip1-Tag2 transgenic mice selectively express the SV40 T-antigen oncogene in their insulin producing β cells and undergo a predictable sequence of multistep tumorigenesis [71, 92, 93]. The transformed β-cells are localized to approx 400 islets in the pancreas, of which 100% express the oncogene. Over time, 50–70% of these islets become hyperplastic nodules. A distinct angiogenic stage occurs at 6-7 wk of age between the hyperplastic stage and the time at which subset islets become invasive carcinomas at 12–16 weeks. The angiogenic capacity of these lesions is observed as visible intense tumor vascularization, accompanied by induction of capillary sprouting, endothelial proliferation, and a starburst-like convergence of capillaries when islets are harvested *in vivo* and cocultured with endothelial cells *in vitro*. Importantly, nonangiogenic islets are unable to grow beyond 0.6–0.8 mm^3 in size, whereas the small subset of angiogenic islets can expand into a lethal tumor burden [94].

A second study utilized the bovine papillomavirus oncogene in a transgenic mouse model of dermal fibrosarcoma [95, 96]. Distinct stages of tumorigenesis are observed, from normal cells to a proliferative hyperplastic stage (mild and aggressive fibromas) to neoplasia (fibrosarcoma). The preneoplastic fibromas grow horizontally within the dermis as thin avascular lesions, and the fibrosarcomas are expansile and densely vascularized. Angiogenesis is first observed during the late preneoplastic stage (aggressive fibroma) and sustained until death of the animals by fibrosarcoma. Aggressive fibromas and fibrosarcomas secrete bFGF. By contrast, bFGF is not secreted by normal cells or by mild fibromas.

A third study involved K14-HPV16 transgenic mice in which the human papillomavirus (HPV) type 16 oncogene is targeted to expression in basal cells of the epidermis by regulatory elements of the human keratin-14 promoter [97]. These basal keratinocytes undergo sequential changes from normal cells (no vascularization) to hyperplasia (mild vascularization from the underlying dermis) to dysplasia (abundant vessels under the basement membrane in close apposition to aberrant keratinocytes) to squamous cell carcinoma (intense angiogenesis breaching the basement membrane into the tumor). In hyperplasia, dysplasia, and at the invading cancer front, angiogenesis is associated with mast cell infiltration and degranulation [98]. Mast cells contain numerous angiogenic stimulators in their secretory granules, such as the serine protease MCP-4, VEGF, bFGF, TGFβ, TNFα, and IL-8 [99, 100]. In dysplasia and carcinoma, tissue expression of VEGF was increased, correlating to increased tumor vessel density [101].

Together, these data demonstrate that angiogenesis is a discrete, genetically regulated and rate-limiting step during multistep tumorigenesis, that the transition from prevascular to vascular phase is accompanied by the production and release of one or more angiogenic growth factors, and that host inflammatory cells may amplify the angiogenic switch by contributing additional stimuli.

2.6. The Vascular Phase of Cancer. The onset of angiogenesis precedes an exponential phase of tumor growth accompanied by local organ invasion. The velocity of angiogenic capillary growth ranges from 0.223 to 0.8 mm/day [102–104]. Studies of avascular tumor explants placed in the anterior chamber of the eye show that once new vessels reach the explant, tumors can expand 16,000-fold in size in 2 wk [105]. During this expansion, cancer cells grow as a cuff around each new microvessel with a thickness of 50–200 μm. In this configuration, one endothelial cell supports the metabolic needs of 5–100 cancer cells [106, 107]. Eventually, invading blood vessels occupy 1.5% of the tumor volume [108]. Tumor angiogenesis also facilitates cancer metastases

Table 4: Approved antiangiogenic agents and cancer indications.

Bevacizumab (Genentech/Roche)	*Colon, Lung, Breast, Brain, Kidney*
Cetuximab (Bristol-Myers Squibb/Imclone)	*Colon, Head and Neck*
Endostatin (Simcere)†	*Lung*
Erlotinib (Genentech/Roche/OSI)	*Lung, Pancreatic*
Everolimus (Novartis)	*Kidney, Pancreatic/NET*, Brain/SEGA***
Imiquimod (Graceway/3M)	*Actinic keratosis, Basal cell carcinoma*
Interferon alfa (Roche/Schering)	*Melanoma, Kaposi's sarcoma*
Lenalidomide (Celgene)	*Myleodysplastic syndrome, Multiple myeloma*
Pazopanib (GlaxoSmithKline)	*Kidney*
Sorafenib (Bayer/Onyx)	*Kidney, Liver*
Sunitinib (Pfizer)	*Kidney, GIST, Pancreatic/NET**
Temsirolimus (Wyeth)	*Kidney, Lymphoma*
Thalidomide (Celgene)	*Multiple myeloma*
Vandetanib (AstraZeneca)	*Thyroid*

† Available only in China.
* Neuroendocrine tumor.
** Subependymal giant cell astrocytoma, associated with tuberous sclerosis.
Source: Angiotracker, The Angiogenesis Foundation (http://www.angio.org/).

by allowing cells to exit through the neovascular network into the systemic circulation [109]. Elegant studies of mammary carcinomas in mice have shown that a 1 cm tumor sheds up to $4 \times 10 - 6$ malignant cells into the circulation every 24 h [110].

3. Targeting Tumor Angiogenesis for Cancer Prevention

The concept of "antiangiogenesis" was first proposed in 1971 by Judah Folkman, who hypothesized that inhibition of neovascularization at an early stage of cancer development could prevent tumor growth and metastases and maintain tumor dormancy [7]. A vast literature establishes that angiogenesis inhibition is an effective strategy to restrict cancer growth in animal models bearing a wide variety of cancers [8, 111]. To date, more than 300 angiogenesis-inhibitory molecules have been identified as potential drug candidates, including many natural and synthetic chemical entities (reviewed in *55*). Selective targeting of angiogenic blood vessels is possible as a result of differential proliferation rates between normal and tumor-associated endothelium. The normal vasculature is highly quiescent, with only one in every 10,000 endothelial cells dividing at any given time, and a physiological doubling time ranging from 47 to 20,000 days [112–114]. In contrast, the doubling rate for tumor endothelium is 2–13 days. Thus, antiangiogenic agents are selective in inhibiting proliferating tumor vasculature, but do not affect normal blood vessels.

3.1. Targets of Tumor Angiogenesis. Specific molecular and cellular targets have been identified for tumor angiogenesis. These include targets present during the orderly events characterizing new blood vessel growth [115]. These include (1) angiogenic growth factor production, release, and receptor activation, (2) degradation of vascular basement membrane, (3) endothelial proliferation, migration, and survival, (4) blood vessel sprouting and invasion, (5) tubular morphogenesis, (6) arterial-venous patterning, (7) vascular maturation, and (8) recruitment of endothelial stem cells.

3.2. Clinical Principles of Antiangiogenic Therapy. The clinical development of antiangiogenic therapy began in the late 1980s. The first successful treatment of a vascular tumor (pulmonary hemangiomatosis) occurred in 1989 using interferon-α 2a as an antiendothelial agent [116]. The first drug to enter formal clinical trials as an angiogenesis inhibitor was TNP-470 in 1992 [117]. Since 2004, more than 14 different antiangiogenic agents have been demonstrated to be efficacious in treating solid and hematogenous cancers, primarily in the setting of advanced disease (see Table 4). To date, most agents that are specific or selective for angiogenesis are well tolerated in humans, with fewer serious (Grade 3 and 4) toxicities observed in their clinical trials when compared to cytotoxic chemotherapy drugs [118–120]. Because only proliferating endothelium is targeted, the traditional side effects of chemotherapy, such as leukopenia, alopecia, and mucositis, are rarely observed. With some antiangiogenic agents, a maximum tolerated dose (MTD) cannot be determined [70]. This has led some clinical investigations to incorporate pharmacodynamic techniques for determining the optimal biological dose (OBD) of agents in clinical trials.

Collectively, translational research studies have shown that overall disease burden is important to consider in the evaluation of the clinical effects of any antiangiogenic agent. Advanced cancers contain well-established, extensive vascular networks that may respond minimally to angiogenesis inhibitors. Vascular destructive agents, also known as vascular targeting agents, may be required to achieve a clinically significant effect on tumor perfusion [121–123]. Indeed, most preclinical studies of angiogenesis inhibitors

demonstrate drug efficacy in the setting of incipient disease (prevention) or small tumors (early intervention). Clinical trials of the same agents, however, have often enrolled patients with advanced, metastastic, and heavily pretreated disease, perhaps explaining differences between mice studies and the results of human trials [124]. Antiangiogenic therapy in the adjuvant setting to suppress minimal residual disease, or as an intervention for early-stage disease or cancer prevention, has been proposed as the scenario of the greatest clinical benefit using angiogenesis inhibitors [125].

The remainder of this paper is devoted to discussing the potential for angiogenesis inhibitors for prevention of cancer.

4. Early Intervention and Cancer Prevention

4.1. Early Intervention. Angiogenesis inhibition offers an opportunity to interrupt an early, rate-limiting step in tumorigenesis [126, 127]. Suppression of pathological blood-vessel growth prevents early tumors from progressing to the malignant phenotype. Clinical correlates to preinvasive angiogenic lesions are commonly encountered in breast (ductal carcinoma *in situ*—DCIS), cervix (cervical intraepithelial neoplasia—CIN), skin (actinic keratosis), oropharynx (late Barrett's esophagus), lung (squamous metaplasia with dysplasia in bronchial mucosa), colon (premalignant adenoma), and prostate (high-grade prostate intraepithelial neoplasia, HGPIN) [128–133]. Microscopic metastases are also present in many cancer patients who are undergoing tumor resection with curative intent. For example, 25% of colon cancer patients eventually develop hepatic metastases after primary tumor resection emerging from preangiogenic lesions that were present at the time of surgery [134–136]. By suppressing tumor neovascularization at subclinical stages, tumor progression and metastatic growth may be halted.

4.2. Superiority of Early Therapy and Sustained Angiogenesis Suppression. Animal studies have demonstrated that early administration of angiogenesis inhibitors is highly efficacious. The drug TNP-470 (*O*-[chloroacetyl-carbonyl] fumagillol) is a potent antiangiogenic analog of the antibiotic fumagillin [124, 137]. A rat model of liver metastasis using K12/TRb rat colon adenocarcinoma cells was employed to study the differential efficacy of early, early prolonged, or delayed administration of TNP-470 (15 mg/kg) on metastatic burden and survival [134]. Treatment initiated at d1 after tumor inoculation (early intervention) and maintained for 28d (prolonged therapy) led to a 46% reduction in liver metastases and improved survival time compared to controls ($P = 0.011$). Another study showed a superior reduction in metastases with early (d 0–6) compared to delayed (d 7–13) TNP-470 treatment in rabbits bearing VX2 carcinoma [138].

The effect of TNP-470 on subclinical disease has also been elegantly studied by Shusterman and colleagues [139]. In the first study, xenografts of human neuroblastoma-derived CHP-134 were implanted into athymic (*nu/nu*) mice, with initiation of antiangiogenic treatment 12 h following grafting (early primary tumor model). Treated tumors were reduced by 90%, compared to control animals. The second study involved administration of TNP-470 12 h following tail vein injection of CHP-134 cells into SCID/Beige mice (metastatic model). Autopsy of saline-treated control mice showed neuroblastoma deposits in the kidney, liver, adrenal gland, and ovaries in 75% of subjects, whereas TNP-470-treated mice showed no evidence of metastases. A third study evaluated TNP-470 effects in mice whose tumors were initially 0.35 mm^3, but then became difficult to palpate following 10 d of cyclophosphamide treatment (minimal residual disease model). TNP-470 was then administered subcutaneously. Tumor growth was suppressed in the TNP-470-treated group by 82%, compared to saline-treated controls. Histopathological analyses showed increased apoptosis by TdT-mediated nick-end labeling (TUNEL) assay in treated animals, but no with difference in tumor cell proliferation by Ki-67 assessment [90, 139]. These data demonstrate the importance of timing in antiangiogenic therapy and its efficacy in subclinical disease.

Table 5: Chemopreventive agents that possess antiangiogenic properties.

Alpha-difluoromethylornithine (DFMO)
Aspirin
Brassinin
Celecoxib
Curcumin
1 α,25-dihydroxyvitamin D3
Ellagic acid
Epigallocatechin 3-gallate
Finisteride
Genistein
N-acetylcysteine (NAC)
Naringenin
Oltipraz
Resveratrol
Retinoids
Selenium
Silymarin
Statins
Sulindac
Tamoxifen

Source: Angiotracker, The Angiogenesis Foundation (http://www.angio.org/).

4.3. Evidence for Antiangiogenic Cancer Prevention. Cancer chemoprevention is defined as the use of pharmacological, natural, or dietary agents to inhibit the development of invasive cancer by blocking DNA damage caused by carcinogens or by arresting the progression of premalignant cells after damage has already occurred [4]. Angiogenesis inhibition blocks carcinogenesis by preventing progression to the invasive phenotype [111, 140, 141]. A number of well-known chemopreventive agents have antiangiogenic properties *in vivo* and *in vitro* (see Table 5). These include retinoids, vitamin D3, tamoxifen, oltipraz, curcumin, linoleic acid, ellagic

acid, selenium, α-difluoromethylornithine (DFMO), *N*-acetyl-*l*-cysteine (NAC), catechins, and celecoxib [142–151]. Classical angiogenesis assay systems, such as the chorioallantoic membrane assay (CAM), the corneal micropocket assay, and modified rat aortic ring assay, have been used to screen for biological activity of established chemopreventive agents [152, 153]. Known angiogenesis inhibitors such as endostatin have also been shown to suppress carcinogen-induced tumor development in rodent models [154]. The antiangiogenic properties of select chemopreventive molecules shall be discussed.

4.4. Antiangiogenic Factors in Dietary Sources

4.4.1. Green Tea Catechins. After water, tea is the second most popular liquid in the world, and its consumption is linked with a decreased risk of colon, prostate, lung, esophageal, and other cancers [155–157]. Laboratory studies have demonstrated that green tea and its catechins prevent mutagenesis, tumorigenesis, cancer invasion and metastases, and angiogenesis [158–163]. Polyphenol catechins in tea, predominantly flavanols, possess chemopreventive and antiangiogenic activity. Epigallocatechin-3-gallate (EGCG) is a potent tea flavonoid that specifically inhibits endothelial cell proliferation stimulated by bFGF and induces avascular zones in the chick CAM assay. Mice that consume 1.25% green tea (human equivalent of drinking 2-3 cups of tea/day) show inhibition of VEGF-stimulated corneal neovascularization by as much as 70% and reduction of tumor cell invasion by 50% [163]. Green tea solution 0.6% administered to mice as the sole source of drinking fluid results in less tissue VEGF expression seen by immunostaining and lower microvessel density in lung adenomas, as well as significantly fewer tumors induced by the NNK carcinogen [162].

The molecular mechanism of EGCG is the result of its inhibition of urokinase and two gelatinases (MMP-2 and -9) involved in vascular as well as tumor invasion [164, 165]. The MMP inhibitory activity is independent of zinc or calcium binding by EGCG. High doses of EGCG induce apoptosis when topically applied to SKH-1 hairless mice bearing UVB-induced squamous cell carcinomas [166]. Clinical trials are underway in Western and Asian nations to study the chemopreventive potential of green tea for oral, prostate, skin, and other cancers.

Preliminary data from these trials suggest clinical efficacy. An Italian study involving men with high-grade prostate intraepithelial neoplasia (HGPIN) demonstrated a protective effect from consuming daily green tea catechins over the course of a single year [167]. The double-blind, placebo-control study randomized sixty men with HGPIN into a treatment arm receiving 600 mg of purified green tea catechins (equivalent to 2-3 cups of tea/day) and a placebo arm. The men were followed for one year, at which point they underwent prostate mapping via core needle biopsies. Based on those biopsy results, 30% of the placebo group progressed to prostate cancer, while the treatment arm demonstrated an impressively low progression rate of 3%. Similarly, a Japanese interventional study of patients with recently removed colonic adenomas demonstrated that participants drinking an average of 12 cups equivalent of green tea polyphenols, obtained through both purified extracts and whole tea, had a 50% reduction in risk of adenoma recurrence compared to patients who drank an average of six cups of green tea [168]. Chemopreventive effects in humans have also been observed through experimental trials involving the treatment of precancerous oral and cervical lesions [169, 170].

Polyphenon E, an extract from green tea leaves containing a defined mixture of polyphenolic catechins, is in clinical trials for prostate, bladder, esophageal, lung, head and neck cancers, and leukemia. In topical form, Polyphenon E 15% ointment was approved by the U.S. FDA in 2006 as a treatment for external genital warts, which is considered an angiogenic neoplasm and a precursor to cervical cancer [171].

4.4.2. Genistein. Genistein (4′,5,7-trihydroxyisoflavone), an isoflavonoid found in soybeans, has both chemopreventive and antiangiogenic activity. It suppresses carcinogenesis in a variety of animal models of mammary and prostate carcinoma following oral and parenteral administration [172–174]. Multiple antitumor mechanisms of action have been identified, including angiogenesis inhibition, induction of apoptosis, G2 cell cycle arrest, inhibition of c-*fos* expression and NF-κB activation, modulation of sex steroid receptors and growth factor signaling pathways [175–177]. Genistein inhibits angiogenesis by the following mechanisms: inhibition of bFGF- and VEGF-driven endothelial cell proliferation, migration, and tube formation; inhibition of extracellular matrix degradation by suppression of bFGF-induced endothelial production of plasminogen activator (PA) and plasminogen activator inhibitor (PAI); and suppression of receptor tyrosine kinase activity for VEGF, EGF, and PDGF [178, 179].

The antiangiogenic activity of genistein was initially detected in a study of healthy Japanese individuals who consumed a traditional soy-rich Japanese diet [180]. Urine from these subjects was collected, fractionated, and examined for activity to inhibit bFGF-stimulated endothelial cell proliferation. Of two fractions with antiendothelial activity, one contained genistein, daidzein, and *O*-desmethylangolensin. The impact of dietary soy intake was significant. In men who consumed a Japanese versus Western diet, urinary genistein was 7052 nmol/day compared to 184.4 nmol/day, respectively [181]. Soy intake has been shown to be inversely associated with cancer risk. Historically, breast cancer incidence rates have been 4 to 7 times higher among white women in the US compared to in women in China or Japan. However, when Asian women migrate to the US, their breast cancer risk rises over several generations to reach that of US white women, suggesting that modifiable factors, such as diet, rather than genetics, are responsible for the international differences. A study of over 73,000 women in China showed that daily intake of soy products such as soy milk, tofu, and fresh soybeans decreased premenopausal breast cancer risk [182]. Likewise, American women of Asian descent who consume a traditional soy-based diet have a low incidence of breast

cancer [178, 183]. Early soy intake (>1.5 times per week) during childhood was found to reduce later breast cancer risk by 58% in a study of Asian women in California and Hawaii [184]. Similarly, Japanese men in Hawaii who consume a high soy diet have low mortality from prostate cancer, although the incidence at autopsy of *in situ* prostate neoplasia is similar to men in Western societies [185]. Based on laboratory findings and epidemiological data, genistein and a manufactured derivative known as genistein-concentrated polysaccharide (GCP), are being evaluated in prevention trials for prostate cancer [186].

Such clinical studies have helped dispel the theoretical concerns that soy intake may worsen breast cancer or interact with tamoxifen treatment due to the fact that genistein is a phytoestrogen. In fact, among women with breast cancer, soy food consumption has now been shown in numerous, large-scale studies to be significantly associated with decreased risk of death and recurrence, regardless of estrogen receptor status or tamoxifen use [187].

4.4.3. Resveratrol. Resveratrol (3,4′,5-trihydroxystilbene) is a natural phytoalexin and polyphenol found in more than 72 plant species, such as mulberries, peanuts, grapes, and grape products, including red and rose wine. Fresh grape skins contain 50–100 μg resveratrol per g and yield a concentration in Italian red wine of 1.5–3 mg/L [188]. White wine contains minimal levels of resveratrol. Resveratrol inhibits angiogenesis in the chick CAM assay, suppresses VEGF- and bFGF-induced corneal neovascularization (at 3-4 mg, equivalent to 3-4 glasses red wine/day), and inhibits tumor vascularization in T241 fibrosarcoma growing in mice [189]. Resveratrol also inhibits chemically induced mammary carcinogenesis, skin cancer tumorigenesis, and tumor growth and metastasis in mice bearing Lewis lung carcinoma [188, 189]. In mice with full thickness skin wounds, resveratrol delays wound healing angiogenesis and the time required for complete wound closure [190]. A number of antiangiogenic mechanisms have been identified, including suppression of capillary tube formation inhibition of endothelial cell DNA synthesis and binding of VEGF to human endothelial cells reduction of vascular cell adhesion molecule-1 (VCAM-1) interference with phosphorylation of endothelial mitogen-activated kinases; suppression of COX-2 enzyme and inhibition of MMP-9 expression [189, 191–193].

Red wine in particular has been repeatedly shown to have protective effects in large population studies. The California Men's Health study of over 84,000 men demonstrated an inverse relationship between red wine consumption and lung cancer incidence. Notably, there was a 61% risk reduction for men drinking at least one glass of red wine per day [194]. This protective benefit for lung cancer with greater than one glass per day was replicated in a Spanish population, using a case-control methodology involving hospitalized lung cancer patients [195]. Additionally, the Health Professionals Follow-up Study identified a protective effect for prostate cancer with a 36% risk reduction in men drinking 2–4 glasses of red wine per week [196].

4.4.4. Lycopene. Lycopene, a type of natural pigment in the carotenoid family, gives tomatoes and other fruits such as watermelon and papayas their bright red color. Lycopene is an angiogenesis inhibitor which suppresses signaling by PDGF and Platelet Activation Factor *in vitro* [197]. In animal studies, lycopene suppresses spontaneous mammary tumors, hepatocarcinogenesis, colonic crypt foci, prostate cancer, and hepatoma metastases [198].

Two interventional studies have also demonstrated potent biologic effects of lycopene in the setting of malignancy. In one study, 32 men who were recently diagnosed with early-stage prostate cancer were instructed to eat one meal per day incorporating commercially made tomato sauce containing 30 mg of lycopene for 3 weeks. They then underwent curative prostatectomy; pathologic examination of the resected tissue showed a 3-fold increase of lycopene concentration in the prostate tissue, along with a slight decrease in blood prostate-specific antigen (PSA) level [199]. In a separate study, 41 men with recurrent prostate cancer were asked to consume a tomato-rich diet to achieve a minimum lycopene intake of 25 mg/day along with 40 g/day of soy protein for a total of 8 weeks. While the study design made it difficult to separate the dietary effects of lycopene from soy, it is notable that the mean serum VEGF levels of all the subjects was reduced from 87 to 51 ng/mL in a statistically significant way, and that 34% of the men experienced reduced PSA levels [200].

In the Health Professionals Follow-Up Study of over 51,000 men, the highest quintile of lycopene consumption was found to have a 15% risk reduction for developing prostate cancer compared to men in the lowest quintile [201]. The risk reduction was even greater if tomato sauce was ingested; men consuming more than 2 servings/week had a nearly 23% risk reduction compared to men consuming less than 1 serving/month.

4.4.5. Omega-3 Polyunsaturated Fatty Acids (PUFAs). Omega-3 fatty acids are unsaturated fatty acids that are vital for normal metabolism but cannot be synthesized by the human body. The best known sources of the long-chain omega-3 fatty acids—docosahexaenoic acid (DHA) and eicosapentaenoic (EPA)—are cold water oily fish such as salmon, herring, mackerel, anchovies, sardines, and trout. Both preclinical and epidemiological studies suggest that omega-3 PUFAs are effective cancer preventative agents. Omega-3 PUFAs inhibit angiogenesis by downregulating angiopoietin-2 and may competitively inhibit the bioconversion of omega-6 PUFA's into their angiogenesis-promoting derivatives such as prostaglandins and arachidonic acid [202]. In contrast, omega-6 PUFAs, present at high levels in sunflower oil, peanut oil, and corn oils, have been shown *in vitro* to stimulate endothelial migration and tube formation. Furthermore, omega-3 PUFAs have been shown to suppress Akt/m-TOR signaling pathway [203]. In animal models, Omega-3 fatty acids have been shown to suppress a variety of tumors and to prevent osteolytic metastastic lesions in bone from breast cancer [204].

Intake of omega-3 PUFAs from seafood has been associated with a decreased risk for certain cancers, including pancreatic, colon, breast, and prostate cancer. In a case-control study of 532 people diagnosed with pancreatic adenocarcinoma, consumption of omega-3 fatty acid of at least 0.85 g/day was associated with a decreased risk of pancreatic cancer; those with the highest intake had a 30% risk reduction [205]. A meta-analysis of fish intake and prostate cancer in case-control and cohort studies revealed no association between fish consumption and prostate cancer incidence, but showed a significant 63% reduction in prostate cancer mortality [206].

4.4.6. Glucosinolates, Isothiocyanates, and Indole-3-carbinol. Cruciferous vegetables—which include cabbage, broccoli, cauliflower, collard greens, mustard greens, radishes, Brussel sprouts, bok choy, and kale—are rich in glucosinolates, a mustard oil glycoside that imparts a spicy, bitter flavor. The enzyme myrosinase—stored in a separate compartment of the plant cell and liberated when the plant is crushed—converts glucosinolate to the bioactive molecules isothiocyanate and indole-3-carbinol, both of which are antiangiogenic. Sulforaphane, a type of isothiocyanate, acts through inactivation of hypoxia inducible factor-1 alpha, activation of FOXO transcription factors, and promotion of endothelial cell apoptosis [207–209]. Indole-3-carbinol inhibits endothelial cell proliferation, tube formation and induces apoptosis [210, 211].

Epidemiological evidence suggests that regular dietary intake of cruciferous vegetables may lower the risk of developing several cancers. A major prospective dietary study, the European Prospective Investigation into Cancer and Nutrition (EPIC), followed the dietary habits and health of 521,468 subjects in 10 European countries between 1991–2000 [212]. After an average followup of 8.7 years, 1,830 people were diagnosed with lung cancer. Regular consumption of cauliflower and cabbage by current smokers was associated with a 23% reduction in the risk for squamous cells carcinoma of the lung. The study also found an almost 50% reduced risk of cancer of the upper digestive tract (oral cavity, pharynx, larynx, and esophagus) among people who ate the most cauliflower and cabbage (34 g/day) compared with those who ate the least (3 g/day) [213].

Another large prospective study of more than 35,000 women living in Iowa and followed for 20 years found a 18% reduced risk for non-Hodgkin's lymphoma (NHL) among women who had the highest consumption of cruciferous vegetables; in particular, consumption of at least 4 servings/month or broccoli was associated with a 28% risk reduction for non-Hodgkin's lymphoma [214]. In separate study of nearly 67,000 women in the Nurses' Health Study, more frequent dietary intake of broccoli (at least 2 servings/week) was associated with a 33% risk reduction for ovarian cancer [215].

4.4.7. Flavonoids. Flavonoids are a family of polyphenols that serve as important plant pigments. Their natural roles may include acting as photoprotectants, antimicrobials, deterrents against herbivores, as well as attractants to pollinators and seed dispersant animals. They are subcategorized by chemical structure into flavones, flavonols (such as quercetin), anthocyanidins, proanthocyanidins, ellagic acid, ellagitannins, and isoflavones (such as genistein mentioned earlier), among others. In contrast, the term flav**a**nol specifically refers to the catechins, mentioned earlier.

Flavonoids in fruits and vegetables include quercetin, anthocyanidins, proanthocyanidins, ellagitannins, among others. Flavonoids are antiangiogenic through a variety of mechanisms; they inhibit VEGF expression, inhibit endothelial cell migration, and decrease matrix metalloproteinases MMP-2 and MMP-9 [216–220]. The U.S. Department of Agriculture identifies spinach, onions, parsley, beets, and thyme among high flavonoid-containing vegetables and herbs [221]. Fresh salad greens such as lettuce, chicory, arugula, and red lettuce are also rich in polyphenolic flavonoids [222].

Quercetin. Quercetin is a flavonoid found in numerous types of fruits and vegetables. Its antiangiogenic properties include inhibition of MMP-2 and MMP-9 secretion from tumor cells and inhibition of endothelial cell proliferation and migration [219]. For example, quercetin has been shown to reduce *in vitro* tube formation of VEGF-stimulated human umbilical vein endothelial cells (HUVECs) grown on a 3-dimensional matrix by as much as 40% [220]. With regards to epidemiologic data, a large prospective study of 41,000 women living in Iowa between the ages of 55 and 69 found a number of flavonoid-containing leafy greens, which are abundant in quercetin, was associated with a significant risk reduction for lung cancer [223]. Women who reported eating more than six servings of leafy greens per week were nearly half as likely to develop lung cancer during the four-year follow-up period, when compared to women who ate the least greens. This benefit extended to both smokers and nonsmokers alike.

One of the richest sources of dietary flavonoids is red onion, which has particularly high levels of quercetin. Case control studies from Italian and Swiss populations have shown that moderate (1–7 servings/week) to high frequency (>7 servings/week) of onion consumption protects against a variety of cancer including colorectal, prostate, ovarian, and laryngeal cancers [224]. For example, there was a nearly 73% risk reduction for ovarian cancer in the population when comparing those who frequently consumed onion (>7 servings/week) compared to those with less frequent consumption (<1 serving/week). Similarly, there was an 88% risk reduction for esophageal cancer in the group with highest versus lowest onion intake. In a separate Dutch cohort study, consumption of at least half an onion/day was associated with 50% risk reduction in gastric cancer [225]. Finally, researchers using data from the large-scale EPIC study showed that high onion consumption was associated with a 21% risk reduction for ovarian cancer [226].

Anthocyanins. Anthocyanins are pigments that are present in many types of berries and grapes as well as red wine.

They exhibit a purple color at neutral pH, red in acidic, and blue in alkaline conditions. Anthocyanins are end-products of the flavonoid pathway, while anthocyanidins are their aglycone precursors. Their natural function may be to attract pollinators and animals to eat the fruit or plant and disperse their seeds.

They have been shown to inhibit angiogenesis and tumor growth in experimental animals injected subcutaneously with N-nitrosomethylbenzylamine (NMBA), an esophageal carcinogen [227]. Rats that were fed an anthocyanin-rich freeze-fried extract (5% in diet) obtained from black raspberries (BRB), blackberries, or strawberries showed a reduced number of esophageal tumors as compared to controls –41% less by BRB, 46% less by blackberries, and 24% less by strawberries [228]. In another study of esophageal papillomas in rats, animals fed BRB had fewer papillomas that were of smaller volume, with reduced cell proliferation and suppression of VEGF and HIF-1alpha expression seen by immunohistochemistry as compared to the non-BRB treated mice [229, 230]. DNA microarray studies of rat esophageal carcinogenesis have shown that dietary black raspberries modulate the expression of genes associated with angiogenesis, including the cyclooxygenase and lipoxygenase pathways of arachadonic acid metabolism, as well as MMP-10 expression; CD34 staining as a marker for microvessel density was also found to be significantly reduced in the BRB diet animals [228, 229]. Berries are also rich in other natural polyphenols such as ellagic acid, which contributes to bioactivity.

An extract from black raspberries has been studied in human subjects diagnosed clinically with oral intraepithelial neoplasia. BRB 10% was applied in a gel form to the oral mucosa four times daily. After 6 weeks, there was reduced histological grade of dysplastic lesions in 50% of treated subjects, and reduced levels of COX-2 and iNOS in the lesions [231].

Proanthocyanidins. Proanthocyanidins are a type of tannin—large polymeric chains of flavonoids—found in many plants and fruits, notably cacao, cinnamon, cranberry, apples, grapes, black current, chokeberry, and persimmon. Proanthocyanidins are thought to be the major source of flavonoids ingested in the Western diet [232]. Historically tannins from wood bark were used as tanning agents to turn animal hide into leather due to their ability to precipitate proteins. They are also responsible for the astringent taste of certain foods and beverages [233]. Among their natural roles is protection against predation [234]. Anthocyanins and proanthocyanidins share steps in the flavonoid biosynthetic pathway. Specifically, proanthocyanidins are generated from the polymerization of flavonoid monomers, which are products of a branch pathway of anthocyanin biosynthesis.

Proanthocyanidins constitute 60% of the polyphenol content in cacao, the source of chocolate [235]. It is in the yeast-based fermentation of the raw cacao bean where the characteristic flavor precursors develop and polyphenols complex into procyanidin polymers with a core structure of (–)-epicatechin, similar to green tea [236]. Pentameric and octameric procyanidins isolated from cacao beans have been shown to inhibit the growth of cultured human aortic endothelial cells and human dermal microvascular endothelial cells (HDMECs) following angiogenic stimulation with low-level H2O2 [237]. The cacao pentameric fraction downregulates the expression of ErbB2 tyrosine kinase. Cocoa powder extract inhibits TNF-alpha-induced VEGF secretion *in vitro*. When JB6 mouse epidermal cells were pretreated with a polyphenol-rich cocoa powder extract, then exposed to TNF-alpha, there was a dose-dependent inhibition of TNF-alpha-induced VEGF expression compared to untreated controls [238]. At the highest but noncytotoxic concentration of cacao extract, VEGF expression was reduced more than twofold compared to controls.

There is evidence that cacao consumption can affect human vasculature and health. After ingestion, cacao flavonoids are stable in the gastric environment and can be detected in human plasma [239]. In studies of human volunteers, angiotensin-converting enzyme (ACE) activity is inhibited by 18% only three hours after a single intake of 75 grams of dark (72% cocoa) chocolate [240]. In a study of 4,849 individuals in Italy, those who ate one 20 gram serving of dark chocolate every 3 days had serum C-reactive protein (CRP) significantly lower than those who did not [241]. Notably, the Kuna Indians living on the San Blas Island of Panama—who have a ten-fold higher dietary intake of cacao compared to Panamanian mainlanders—have lower blood pressure, live longer, and have lower incidence of cancer compared to mainlanders [242]. Consistent with their cacao consumption, the Kuna have 6-times higher urinary excretion of cacao procyanidin metabolites than mainlanders [243].

Procyanidins are also found in cinnamon, which have been found to inhibit angiogenesis and tumor growth. Cinnamon extract inhibits VEGF receptor-2 on endothelial cells and suppresses endothelial cell proliferation, migration, and tube formation *in vitro* [244]. A water-soluble extract from dried cinnamon bark, orally administered every day or every other day for 20 days to mice bearing experimental melanomas suppresses melanoma growth [245]. The cinnamon extract suppressed tumor microvessel density, and as shown by quantitative RT-PCR, the expression of angiogenic factors VEGF, FGF, and TGF-β as well as COX-2 and HIF-1alpha, which promote angiogenesis. In addition, the cinnamon extract suppresses melanoma metastasis as measured by the size and weight of the spleen and draining lymph nodes of mice bearing melanoma *in vivo* [245].

Many edible berries also contain proanthocyanidins, including the American cranberry, black currants, and grapes. Chokeberries, named because of their extreme astringency, contain among the highest levels of proanthocyanidins detected [246]. Mixtures of berry extracts inhibit tube formation of endothelial cells in Matrigel [247]. Extracts from blueberries, bilberries, raspberries, and strawberries suppress VEGF expression by immortalized human keratinocytes (HaCaT cells) *in vitro* and this VEGF inhibition appears to be independent of the antioxidant property of the extracts [247]. Experimental liver cancer in rodents induced by the carcinogen DENA are suppressed in

number and size by an extract from black currants added to the animal's diet (equivalent to 500 mg/kg body weight) [248]. Procyanidins in cranberries may have multiple health benefits including both chemopreventive properties as well as maintaining bladder health due to inhibiting *E. Coli* adherence to uroepithelium [249]. A cranberry extract tested on DU145 human prostate cancer cells significantly inhibited expression of MMP-2 and MMP-9 and increased expression of TIMP-2 *in vitro*, consistent with mechanisms that suppress angiogenesis [250]. Cranberry juice, given as a 20% solution, reduces the incidence of azoxymethane-induced colonic aberrant crypts in rats by 77% versus drinking water controls when supplemented for three weeks before and ten weeks after carcinogen exposure [251].

Both apples and apple juice are rich sources of procyanidins in addition to other polyphenols previously described such as quercetin and catechins. Based on the USDA Continuing Survey of Food Intakes by Individuals (CSFII), apples are a major source of proanthocyanidins in the U.S. diet [232]. In a recent study, scientists examined the specific chemopreventive properties of a polyphenol extract of apple juice. Apple procyanidins were found to inhibit Cox-1 [252]. Several studies have shown that cloudy apple juices, such as apple cider, contain much higher concentrations of procyanidins than clear apple juices. Specifically, one study determined that the suspended particles in cloudy apple juice contain up to 60% of apple procyanidins in the juice.

Epidemiological studies have provided accumulating evidence that apples have cancer-preventive properties, particularly against lung and colorectal cancers. In the Nurses Health Study involving 77,000 women, a statistically significant 37% risk reduction for lung cancer was observed among women for increases of 1 serving per day of apples or pears [253]. Similar results were obtained from a Finnish cohort study involving 10,000 men and women [254]. The results of a case-control study conducted in Hawaii with 528 lung cancer cases and 528 controls found a statistically significant decrease in lung cancer risk with increased consumption of apples [255]. An analysis of case-control studies conducted in Italy found that people who consumed at least one apple per day had a significantly reduced risk of colorectal cancer and cancers of the oral cavity, larynx, breast, and ovary relative to those who ate less than an apple a day [256]. In a prospective cohort of 35,159 Iowa women aged 55–69 years, intake of apple juice or cider was associated with lower risk of developing non-Hodgkin's lymphoma [214].

Ellagitannins. Ellagitannins are glycosides of the flavonoid ellagic acid and fall under the category of hydrolyzable tannins. They can be found in numerous types of fruits and nuts including pomegranate, strawberries, blackberries, raspberries, muscadine grapes, walnuts, and pecans [257]. Upon consumption, ellagitannins are hydrolyzed to ellagic acid which is antiangiogenic. Colonic microbiota further convert ellagic acid to urolithin A which is also bioactive [258].

All parts of the pomegranate contain high levels of ellagitannins, of which the primary type is punicalagin. Bioactive polyphenols are found not only in the edible aril (pulp and seed) which is popularly consumed, but also concentrated in the peel of the fruit, which is not usually consumed but may be part of the juice extraction by some processors. Indeed, polyphenol extraction increases 6.5-fold when the whole fruit is processed compared to juice from arils alone [259]. Ellagitannins have been shown to inhibit the growth of prostate cancer in both *in vitro* and *in vivo* laboratory experiments, and studies have suggested that the compound's antiangiogenic properties play a role in this inhibition. Interestingly, pure pomegranate juice has been found to be more potent than its separated individual polyphenol components, likely due to synergistic effects [260]. Effects of pomegranate extract (POMx)—derived from the skin and seeds of the pomegranate fruit—have been shown on endothelial cells (HUVECs) and human prostate cancer cells (LNCaP line) *in vitro* [261]. Pomegranate extract inhibited the proliferation of both cell types and suppressed the secretion of VEGF and HIF-1alpha. Mice implanted with human prostate tumors derived from the LAPC4 tumor line were administered the human equivalent of 1.7 cups (8 oz) of pomegranate juice per day. Pomegranate juice significantly decreased tumor microvessel density, as well as tumor size, compared to the control animals [261]. Pomegranate juice, when given as a 20% solution, reduces the incidence of azoxymethane-induced colonic aberrant crypts in rats [251]. In a study of men with recurrent prostate cancer who drank 1 cup of pomegranate juice per day, their prostate-specific antigen (PSA) doubling time was extended from 15 to 54 months [262].

4.4.8. Menaquinone. A form of vitamin K, menaquinone (vitamin K2), found in certain food sources is antiangiogenic and associated with a reduced risk for developing several forms of cancer. Menaquinone is distinct from the phylloquinone (Vitamin K1) present in dark leafy vegetables. Instead, menaquinone is a fat—soluble vitamin formed naturally by bacteria in fermented dairy products, including cheese and yogurt, in fermented soy such as natto, and also present in dark meat. Certain cheeses, such as Dutch Gouda, Swiss Emmental, and Norwegian Jarlsberg, have particularly high concentrations of menaquinone. In laboratory studies, menaquinones suppress angiogenesis, enhance tumor apoptosis, and inhibit the proliferation of cancer cells [263].

A subpopulation of the European Prospective Investigation into Cancer and Nutrition (EPIC) study, called the Heidelberg cohort, followed the diet and health status of more than 24,300 participants for at least ten years, starting in 1994. The participants answered detailed questionnaires at regular intervals about their diet and general health. During the follow-up period, 1,775 cancer cases were diagnosed, of which 458 were fatal. Among the foods documented, participants who consumed the most cheese (at least 41 g/day) had a significantly reduced risk of dying from cancer compared with those who consumed the least (less than 14 g/day) [264]. Cheese consumption contributed to about 45% of total menaquinone intake. In terms of specific cancers, higher consumption food containing menaquinone was associated with significantly lower

incidences of lung cancer and prostate cancer. Participants with the highest levels of menaquinone in their diets had a 62% reduced risk of lung cancer compared with those with the least, and a similar reduced risk of dying from lung cancer.

Prospective clinical studies of vitamin K2 have shown chemopreventive activity in patients. In a study of 40 women in Osaka diagnosed with viral liver cirrhosis, individuals were assigned randomly to an intervention or control group. During more than 7 years of followup, the cumulative proportion of people who developed hepatocellular carcinoma (HCC) was significantly smaller in the group that received vitamin K2 (45 mg/day) [265]. Vitamin K2 decreased the risk of HCC to 20% compared to that of the control group. On an annual incidence basis, HCC developed in only 1.6% in the treatment group compared with 8.8% in the control group and 7.9% in the general cirrhotic population.

4.4.9. Curcumin. Curcumin (diferuloylmethane), a flavonoid derived from the plant *Curcuma longa*, is present in tumeric spice. It has chemopreventive and antiangiogenic activity and inhibits carcinogenesis in skin, stomach, intestines, and liver. Dietary ingestion of curcumin has been shown to prevent the formation of colon polyps, suppress proliferation of colon cancer and prostate cancer cells, and decrease intratumoral microvessel density [266–268]. Studies of endothelial cells exposed *in vitro* to curcumin show induction of apoptosis; downregulation of gene transcripts for VEGF, bFGF, and MMP-2; COX-2 inhibition; upregulation of TIMP; disruption of vascular tube formation; and inhibition of endothelial cell motility by interfering with the Ras-mediated c-Jun N-terminal kinase (JNK) pathway [269–273].

A Phase I prevention trial of curcumin (500–800 mg/day) showed histological improvement of lesions in patients with various malignant and premalignant lesions, including recently resected bladder cancer, oral leukoplakia, intestinal metaplasia, CIN, and Bowen's disease [274]. In a Phase II study, dietary curcumin was given for 30 days to 44 subjects with aberrant crypt foci (ACF), a premalignant marker for colorectal cancer. Those who consumed 4 g/day of curcumin had a 40% reduction in the number of rectal ACF lesions [275].

4.4.10. Beta-cryptoxanthin. Beta-cryptoxanthin is a natural carotenoid pigment present in brightly colored orange, red, or yellow foods. Structurally it is related to beta-carotene, and is ultimately metabolized to vitamin A in the body and can be found circulating in the blood after consuming carotenoid-rich foods. Vitamin A and retinoic acid analogs are antiangiogenic and synthetic derivatives have been investigated as chemopreventive agents [276–280].

Papaya is a rich source of carotenoids, with the same lycopene content as tomatoes, but twice the beta-cryptoxanthin content. Studies in tropical populations have shown that increased daily consumption of papayas results in higher levels of beta-cryptoxanthin in the blood [281]. Consumption of such beta-cryptoxanthin-rich foods may reduce cancer rates in high-risk populations. For example, higher papaya consumption has been shown to be inversely associated with the risk of developing high-grade cervical lesions. A nested case control study involving a population of HPV-positive women in Brazil showed that consuming one or more servings of papaya per week cut the risk of developing a high grade cervical lesion by 81% [282].

Other studies have shown a risk reduction with the consumption of papaya and carotenoid-rich fruits for other cancers, including lung and gall bladder cancers [283, 284]. In the Singapore Chinese Health Study, 63,257 Chinese men and women ages 45–74 participated in a prospective study of diet and cancer [284]. Using a food composition database, an estimate of their carotenoid intake, including beta-cryptoxanthin was quantified. In the first 8 years of follow-up, 482 lung cancer cases occurred in the cohort. A high level of dietary beta-cryptoxanthin was associated with reduced risk of lung cancer. Comparing the highest to lowest quartile, there was a 27% risk reduction among all subjects, and a 37% risk reduction among current smokers.

In a study of women, diet, and breast cancer risk, 403 breast cancer cases and 602 controls from the Nurses' Health Study were examined for high breast densities, a strong predictor for breast cancer risk [285]. Overall, circulating total carotenoids were inversely associated with breast cancer risk. Among women in the highest tertile of mammographic density, total carotenoids were associated with a 50% reduction in breast cancer risk.

4.4.11. Other Novel Dietary Inhibitors. We have identified antiangiogenic activity in a number of other dietary-derived chemopreventive molecules. These include brassinin, a phytoalexin found in Chinese cabbage; the citrus-derived bioflavonoids hesperidin and naringenin; ellagic acid from berries, pomegranate, and grapes; silymarin from milk thistle and artichoke; and the organosulfur allyl disulfide derived from garlic [286–291]. Further studies are underway to define their molecular targets in angiogenesis, their optimal biological doses, and efficacy in inhibiting tumor vascularity.

The diverse natural sources of these and other antiangiogenic chemopreventive molecules raise the possibility of designing scientific diets for patients at high risk for cancer, or for those with known disease to chronically suppress angiogenesis and tumorigenesis.

5. Conclusion and Future Directions

Angiogenesis is a critical, rate-limiting step in the development of all known cancers, and its inhibition suppresses tumor growth, progression, and metastases. Antiangiogenic therapy represents a new approach to the early intervention and prevention of malignant disease. During the next two decades, the total yearly number of newly diagnosed cases of cancer is projected to rise from 12.4 million new cases per year in 2008 to 26.4 million in 2030, and the number of annual deaths is projected to increase 170%, to 17 million [292, 293]. According to the World Health Organization

and International Agency for Research on Cancer, cancer is the leading cause of death worldwide as of 2010 [293]. The implementation of effective chemoprevention strategies based on angiogenesis inhibition attained through dietary sources may decrease these numbers in a cost-effective and quality of life enhancing manner. Dietary effects are already thought to underlie many of the large international differences in incidence seen for most cancers [294]. Indeed, nutritional factors have been estimated to contribute to 20–60% of cancers worldwide and to approximately one-third of deaths from cancer in Western countries [295].

The identification of dietary sources of antiangiogenic molecules has been aided tremendously through observational epidemiologic studies, which have the ability to identify specific foods associated with reduced cancer risk. These studies generally fall under the categories of prospective cohort and retrospective case-control studies, and more of both of these types of studies will be necessary to identify additional sources of antiangiogenic compounds and to help confirm the chemopreventive properties of previously identified foods and their constituent bioactive molecules.

The dietary prospective cohort study allows thorough assessment of dietary exposures with a reduced risk of recall bias as patients are initially cancer-free when enrolled. As study participants are followed into the future and observed for the development of cancer and other cancer-related endpoints, investigators can identify specific foods present in participants' diets that are associated with cancer risk reduction. With the evolving understanding of the underlying molecular basis of various naturally antiangiogenic foods, an important consideration for future cancer prevention cohort studies will be to ensure accurate assessment of participants' dietary intake of bioactive compounds. This will include not only comprehensively querying participants about all potential dietary sources of the bioactive compounds of interest, but also obtaining more detailed information about the preparation of foods, as this can significantly impact the amount of antiangiogenic molecules obtained through diet. Furthermore, the subtypes of foods consumed, such as the specific variety of apple or tomato eaten by a participant, and the way the food is processed or cooked prior to consumption, can strongly affect the amount of consumed bioactive molecules and would be important information to collect. Thus, while the food frequency questionnaires (FFQs) used in past cohort studies have attempted to quantify the intake of various foods, future studies focused on antiangiogenesis may be able to make a more detailed and accurate assessment of this particular aspect of dietary intake.

Case-control studies can also be valuable as a method for expediently identifying promising dietary exposures without the need for the long timeframes and massive study populations that typically characterize dietary cohort studies. While case-control studies are nearly always vulnerable to recall bias on the part of the cancer patients who compose the case populations, they are a valuable starting point in identifying promising risk-reducing foods that can be further explored through prospective and, more recently, interventional dietary studies. The key to these studies will be the use of well-designed dietary survey instruments and methods to allow data capture on short time horizons that can accurately gauge the intake of antiangiogenic and chemopreventive biomolecules. Finally, interventional dietary studies are a particularly promising methodology with which to confirm the antiangiogenic and chemopreventive properties of specific foods and bioactive molecules. In particular, recently diagnosed cancer patients or those with pre-invasive angiogenesis-dependent lesions such as colonic adenomas, prostatic intraepithelial neoplasia, cervical intraepithelial neoplasia, and actinic keratoses could potentially show significant benefit from short-term antiangiogenic dietary interventions with regards to progression, metastasis, or recurrence of their lesions [167]. Investigators with access to serum or biopsy samples could also follow changes to angiogenesis biomarkers, tumor biomarkers, or tumor characteristics [262, 296]. Because of their experimental nature, randomized interventional dietary studies have the potential to become a valuable method for validating the antiangiogenic nature of bioactive molecules and foods.

The United States Department of Agriculture's update to its longstanding *Food Pyramid* dietary recommendations—namely, its replacement with the new *MyPlate* initiative that pushes for an increase in the amount of whole fruits and vegetables consumed at each meal—does appear to indirectly promote increased consumption of certain foods that contain natural sources of antiangiogenic molecules [297]. For example, specific recommendations of the initiative include glucosinolate-rich cruciferous vegetables, lycopene-containing tomatoes, resveratrol-bearing grape products, and beta-cryptoxanthin-abundant orange and yellow vegetables.

Data and conclusions from rigorous dietary cancer prevention studies should be implemented into public health policy. Optimal health outcomes result not just from high quality medical care, but also from diet and lifestyle patterns that can intercept disease at the earliest microscopic stages. Investments in cancer prevention, guided by the biological principles of antiangiogenic therapy, will expand on the substantial clinical applications already established by the biopharmaceutical industry. Cancer prevention using antiangiogenic factors present in widely available foods further offers an egalitarian strategy for large populations in societies that increasingly recognize the value of health-promoting dietary choices. Many dietary sources of angiogenesis inhibitors are common ingredients in the world's most popular culinary traditions (i.e., Asian, Mediterranean, etc.). We propose that an antiangiogenic diet is a practical and cost-effective method to reduce the risk of cancer and other diseases and to enhance quality of life.

In summary, tumor angiogenesis is a critical target for cancer prevention. Natural antiangiogenic molecules are present in numerous dietary sources and represent a wide spectrum of mechanisms that can suppress the growth of microscopic tumors. The control of blood vessel growth through dietary antiangiogenesis promises to redefine cancer as a disease that can be suppressed throughout an individual's lifetime, from infancy through adulthood.

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Review Article

The Amazing Power of Cancer Cells to Recapitulate Extraembryonic Functions: The Cuckoo's Tricks

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Inflammation is implicated in tumor development, invasion, and metastasis. Hence, it has been suggested that common cellular and molecular mechanisms are activated in wound repair and in cancer development. In addition, it has been previously proposed that the inflammatory response, which is associated with the wound healing process, could recapitulate ontogeny through the reexpression of the extraembryonic, that is, amniotic and vitelline, functions in the interstitial space of the injured tissue. If so, the use of inflammation by the cancer-initiating cell can also be supported in the ability to reacquire extraembryonic functional axes for tumor development, invasion, and metastasis. Thus, the diverse components of the tumor microenvironment could represent the overlapping reexpression of amniotic and vitelline functions. These functions would favor a gastrulation-like process, that is, the creation of a reactive stroma in which fibrogenesis and angiogenesis stand out.

1. Introduction

In the nineteenth century, Rudolf Virchow postulated a link between cancer and inflammation on the basis of observations that tumors often arose at sites of chronic inflammation, and the inflammatory cells were present in biopsied samples from tumors [1]. Twenty-five years ago, Dvorak recognized that the composition of tumor stroma is very similar to that of granulation tissue of healing skin wounds. He therefore suggested that "tumors are wounds that do not heal" [2]. Since that time, inflammation has been implicated in tumor development, invasion, and metastasis and in the development of clinical features as fever and cachexia [3]. These observations suggested that common cellular and molecular mechanisms are active in wounds and in cancer tissue [4].

2. Wound Repair Phenotypes: Gastrulation Revisited

The inflammatory response that is induced in the injured skin could be described as a succession of three overlapping phases during which the phenotypes of metabolic progressive complexity in using oxygen are expressed. Each one of these phases emphasizes the trophic role of the mechanisms developed in the damaged tissue. Hence, nutrition by diffusion predominates in the first phase; trophism is mediated by inflammatory cells in the second phase and finally blood circulation and oxidative metabolism play the most significant nutritive role in the third phase [5].

In the first or immediate phase of the inflammatory response, interstitial hydroelectrolytic alterations stand out. The vasomotor response, with vasoconstriction and

Table 1: Phenotypes expressed in the acute inflammatory wound healing response.

(i) *Ischemia-reperfusion phenotype*
(a) Oxidative and nitrosative stress
(b) Interstitial hydroelectrolytic alterations
(c) Increased lymphatic circulation (circulatory switch)
(ii) *Leukocytic phenotype*
(a) Infiltration by granulocytes and agranulocytes (macrophages-lymphocytes)
(b) Lymphangiogenesis
(c) Lymph node reaction
(d) Wound immunotolerance
(e) Resolution
(iii) *Angiogenic phenotype*
(a) Endothelial cell proliferation
(b) Fibrogenesis
(c) New vascular network
(d) Epithelial regeneration

vasodilation, is responsible for the ischemia-reperfusion phenomenon, which in turn causes oxidative and nitrosative stress in the injured tissue. In this phase, during the progression of the interstitial edema, the lymphatic circulation is simultaneously activated. In the following intermediate phase of the inflammatory response, the tissues are infiltrated by inflammatory blood-born cells, particularly leukocytes. Symbiosis of the leukocytes and bacteria for extracellular digestion by enzyme release, that is, phagocytosis, produces enzymatic stress. Furthermore, macrophages and dendritic cells take advantage of the lymphatic circulation activation and migrate through it until reaching the lymph nodes, where they activate lymphocytes [5, 6].

During the third phase of the inflammatory response, angiogenesis permits numerous substances, including hormones, to be transported by the blood circulation. Although the final objective of angiogenesis is to form new mature vessels for oxygen, substrates, and blood cell transport, other functions could be carried out before the new mature vessels are formed. Thus, angiogenesis could have antioxidant and antienzymatic properties, favoring therefore the resolution of the inflammation, as well as wound repair by epithelial regeneration and scarring. Consequently, in this phase the new formed tissue is structured, specialized, and matured by remodeling [5–7] (Table 1).

It has been previously proposed that the inflammatory response associated with the wound healing process in the skin could recapitulate ontogeny through the reexpression of two hypothetical embryonic trophic axes, that is, amniotic and yolk sac or vitelline, in the interstitial space of the injured tissue. If so, inflammation could represent the debut during postnatal life of ancestral biochemical mechanisms that were used for normal embryonic development. The re-expression of these ancient mechanisms, with a prenatal solvent path, is perhaps inappropriate and hard to recognize since they are anachronistic during postnatal life and because they are established in a different environmental medium [8].

The molecular and cellular contribution made by the two extraembryonic tissues that surround the fetus, the amnion and yolk sac, to the interstitial space located between them, namely, the mesoderm, are essential for organogenesis [9]. It could be assumed that both cavities are controlled by an array of inductive and inhibitory signals originating from the adjacent extraembryonic mesenchyma [8] (Figure 1).

The amniotic axis could play a leading role in primitive interstitial hydroelectrolytic changes. The early mammalian embryo already has the ability to manage fluids in the interstitial space. Body fluid is distributed among three major fluid spaces, that is, intracellular fluid, interstitial fluid, and plasma. Nevertheless, the fluid distribution in each of these compartments is dramatically different in the fetus compared to the adult. Particularly, the amniotic fluid that surrounds the fetus may be considered an extension of the extracellular space of the fetus. Thus, the lymphatic system plays an essential role in the regulation of fluid distribution between the plasma and the interstitial fluid and, probably, with the amniotic fluid [10]. In addition to the rich amino acid content, amniotic fluid contains abundant peptides, carbohydrates, lipids, hormones, and electrolytes with water [11]. The amniotic fluid has antimicrobial properties, and this may be part of the innate immune system. A reduced volume of amniotic fluid may decrease the natural host defense conferred by this fluid and predispose it toward intrauterine infections [12]. Finally, it has been shown that amniotic-fluid-derived stem cells are able to differentiate into neurogenic, mesodermal, and endodermal lineages [13].

The vitelline axis, represented by the yolk sac, is the final destination of migrating visceral endoderm cells. The visceral yolk sac expands, and blood islands, structures consisting of hematopoietic progenitors surrounded by a loose network of endothelial cells, appear [14]. Endothelial cell precursors associated with blood islands differentiate and coalesce to form a primitive circulatory bed, which later connects to the embryo via the vitelline vessels [15]. Also, a major function of the yolk sac is the accumulation of carbohydrates, proteins, and lipids (*vitellum*) for embryo nutrition [16, 17]. Particularly, the yolk sac plays a vital role in providing lipids and lipid-soluble nutrients to embryos during the early phases of development [16, 18]. The yolk sac uses high-density lipoproteins (HDLs) and very-low-density lipoproteins (VLDL) as carriers to incorporate cholesterol from the maternal circulation and to transfer it to the embryonic side [16]. In turn, the interstitial lipid accumulation of cholesterol, a precursor molecule of many hormones like aldosterone, corticoids, androgens, strogens, and progesterone, may favor fluid infiltration and cell migration, proliferation, and differentiation during embryo development [19]. The ability to transport fat in the form of lipoprotein through the circulatory system by eukaryotes is one of their most significant functions right from the beginning of existence [20].

It could be accepted that these primitive functions are internalized during gastrulation to create the intraembryonic mesoderm. Thus, this germ layer would integrate the amnion- and yolk-sac-related functions [8]. Fibroblasts are

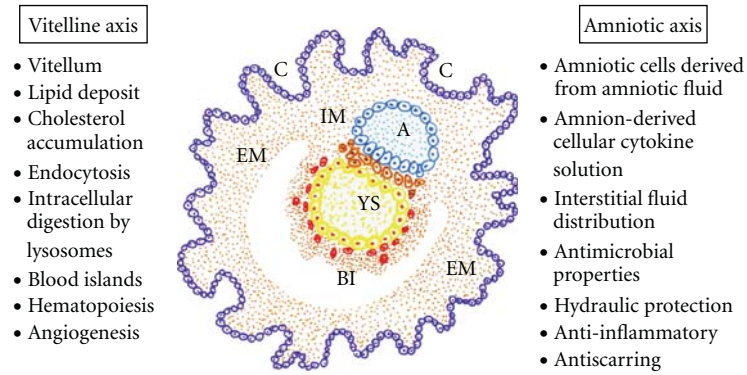

Figure 1: *Representative drawing of the early mammalian embryo during gastrulation.* The internalization of the extraembryonic mesoderm (EM) during gastrulation allows for the creation of the intraembryonic mesoderm (IM) that could thus join functional amniotic and vitelline properties from the amnion (A) and yolk sac (YS), respectively. BI: blood islands; C: chorionic vellosities.

mesodermal derived cells, and perhaps this embryonic origin could justify their great postnatal plasticity. The mesodermal cells of the embryo participate in the extraembryonic structures, including the chorion, the amnion, and the yolk sac [9]. In the human body, the fibroblasts form a heterogeneous collection of mesenchymal cells and they are the principal cellular constituents of connective tissues [21]. The major role of the mesodermal cells and their ability to differentiate from the first stages of embryonic development allow for considering them as the cell prototype that should be resorted to when the repair of any tissue in the body is needed [9]. And for this reason, perhaps the posttraumatic inflammatory response after a skin wound has the same intention, namely, to use the embryonic mesodermal phenotype with a therapeutic objective [8].

Local fibroblasts residing in the skin are considered the most prominent source of myofibroblasts. However, a variety of other precursor cells contribute to the myofibroblast population, depending on the nature of the injured tissue and the particular microenvironment [22]. Activated myofibroblasts are generated from a variety of sources, including resident mesenchymal cells, epithelial and endothelial cells, via the epithelial/endothelial mesenchymal transition as well as from circulating fibroblast-like cells called fibrocytes, derived from bone marrow stem cells [21, 23]. In addition, circulating monocytes have the capacity to differentiate into nonphagocytes, that is, mesenchymal cells and endothelial cells [24].

The reason why the myofibroblasts are attractive to a broad scientific and clinical audience is due to the large panel of cells that can develop this phenotype upon activation. It appears that myofibroblasts can be recruited from any local cell type and they are suitable for rapidly repairing injured tissue [22]. Fibroblasts can be induced to acquire the myofibroblast phenotype during wound repair. Several days after injury, a subset of wound fibroblasts can differentiate into myofibroblasts, which are responsible for repopulating the wounded area in parallel to angiogenesis, thus forming the granulation tissue [6, 7]. Granulation tissue is then repopulated with fibroblasts to produce a more densely collagenous extracellular matrix which is more akin to the matrix found in interstitial stroma [25].

Tissue remodeling requires the removal of granulation tissue, and maturation of collagen is oxygen dependent [6]. Indeed increasing wound oxygenation results in increased collagen deposition and tensile strength [26]. However, hypoxia benefits the expansion, differentiation, adhesion, growth factor secretion, and regenerative potential of mesenchymal stem cells derived from subcutaneous adipose tissue [27]. Finally, the phenotypic changes suffered by the keratinocytes during reepithelization suggest a partial epithelial-mesenchymal transition. Following the completion of wound repair, keratinocytes revert from their mesenchymal-like phenotype to an epithelial phenotype [28, 29].

In the adult organism, many pathways that play an essential role during embryo development are inactivated later in life although some of them may be transiently expressed during adult repair [30]. That is why we have considered that wound repair would require the upregulation of signaling pathways characteristic of the extraembryonic functions, that is, amniotic and vitelline, during the embryo development. If so, emulation by the wound tissue of these extraembryonic functions perhaps requires retracing the mechanisms that produce and distribute the extracellular fluid and substrates; activate the migration and invasion of stem or progenitor cells and hematopoietic-derived cells, and induce the establishment of a complex vascular network by lymphangiogenesis and angiogenesis; finally all of them are required to mediate normal new tissue growth and development [8]. The above-mentioned extraembryonic mechanisms seem to be aimed at favoring the grafting of new repaired tissue. Thus, through the successive expression of functions related to natural immunity and later with the acquired immunity, the neoformed tissue is accepted by the host.

This transient recapitulation of embryonic mechanisms through the hypothetical succession of overlapped amniotic-like and vitelline-like functions would achieve the cellular and metabolic diversity necessary for repairing the injured adult tissue with a graft (Table 1).

3. The Inflammatory Cancer Cell

Nowadays, the causal relationship between inflammation and cancer is widely accepted [1, 3, 4, 31–37]. Inflammation has long been thought to have contributed to the development of cancer [1, 35, 37]. And consequently, chronic inflammation is a major cause of cancer [32, 34, 36].

The understanding of the pathogenesis and progression of cancer requires the establishment of the altered genetic/metabolic factors that are essential to the development, growth, and proliferation of malignant cells [38]. This new frontier of cancer research requires the appropriate marriage of genetic/proteomic studies or the geneticist approach to the biochemical/metabolic cellular studies or the biochemical approach [38].

With regards to the geneticist approach, in many cancers a stem cell tumor model probably takes place [39]. Although a stem cell may sustain the first oncogenic hit, subsequent alterations required for the genesis of a cancer stem cell can occur in descendent cells [40]. This mechanism could explain the marked tumoral heterogeneity, either interturmoral, that is, variability between tumors arising in the same organ, or intratumoral, that is, variability within individual tumors [40].

With regards to the biochemist approach, metabolic transformation of malignant cells is essential to the development and progression of all cancers [38, 41–43]. Cancer cells, similar to normal cells, live in niches and microenvironments that are heterogeneous. Specifically, intratumoral gradients of nutrients and oxygen could play a profound role in modulating tumor cell metabolism [42].

The comparison of tumors with wounds that do not heal [2] suggests that during the host invasion, the malignant tumor cells could express the previously proposed inflammatory phenotypes during wound repair [44]. Due to the plasticity of cancer stem cells, it should be kept in mind that while a malignant tumor develops, it can express phenotypes that also share the inflammatory response such as an ischemic phenotype (hypoxic) with edema and lymphangiogenesis (circulatory switch) a leukocytic phenotype being adapted, with migration to the regional lymph nodes and development of cachexia, and an angiogenic phenotype with the supply of nutrients and oxygen and tumoral mass growth [44–46] (Figure 2).

It has already been proposed that these phenotypes represent the expression of trophic functional systems of increasing metabolic complexity in the wound inflammatory response [5, 6]. Their expression by cancer cells could have a similar significance [46]. In this hypothetical circumstance, malignant tumor cells could adopt an inflammatory-like phenotype that evolves in three hypothetical functional phases of increasing metabolic complexity and which would also have a trophic significance [44–46].

Moreover, acute wounds are initially hypoxic, and chronic ischemic wounds are essentially hypoxic [47, 48]. However, in the wounded tissue molecular and functional heterogeneity could be related to the heterogeneous distribution of oxygen with hypothetical pockets of graded levels of hypoxia [48].

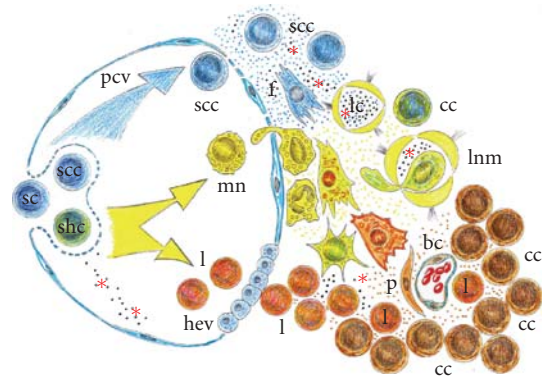

Figure 2: *Evolutive phases of the inflammatory cancer cell.* Cancer cells can adopt an inflammatory phenotype to invade neighboring tissues and survive in these ectopic sites. In the successive phases of tumorigenesis, the cancer cells invade the host by expressing natural and adaptive immune-related mechanisms. sc: stem cell; scc: stem cancer cell; shc: stem hematopoietic cell; f: fibroblast; mn: monocyte; mf: myofibroblast; g: granulocyte; mØ: macrophage; lc: lymphatic capillary; cc: cancer cell; lnm: lymph node metastasis; hev: high endothelial venule; l: lymphocytes; pcv: postcapillary venule; bc: blood capillary; p: pericyte; *tumoral antigen.

The hypothesized similarity of the inflammatory response in wounds and tumors, based on the molecular-related, that is, genetic and metabolic, pathways is an interesting proposal because it could allow for translational inflammatory research between tissue repair and cancer. Moreover, the cancer cell also successively uses the natural and acquired immunity to be grafted into the host. The need to be recognized by the host, by both the cells that make the wound repair and by the cells that cause tumorigenesis to develop, would indicate that in the adult organism the creation of a new tissue without a previous immunologic acceptation could not be possible. Regardless of whether the neoformed tissue is normal, like during embrionary development and wound repair, or pathological, like in cancer, the expression of extraembryonic functions would be essential for homing in the host. Therefore, maybe the different inflammatory-related extraembryonic ways that converge in the embryo, in the wound repair process and in tumor growth, have the final aim of getting their immunological acceptation.

4. The Evolutive Phases of the Inflammatory Cancer Cell

It has been proposed that the relation between inflammation and tumor development depends on whether the tumor is benign or malignant [44]. The benign tumor cells seem to be able to induce the inflammatory response in the host. Therefore, it is possible that the host participates in establishing the tumor through a process called desmoplasia, which consists of fibroblastic cells and the extracellular matrix, a leukocytic response represented by lymphocytes, macrophages, and dendritic cells, and lymphangiogenesis and angiogenesis [49]. Essentially, all the elements that constitute the inflammatory response participate in the "host reaction,"

which could, therefore, have a trophic purpose for the tumor cells [44, 46]. Consequently, this inflammatory response of the host would develop in the interstitial space of the tumor, which invades in order to be more efficient, trophically [46].

However, when tumor cells reach higher grades of malignancy, their invasive capacity reflected in the classic metastatic cascade, that is, primary tumor (T), lymph node invasion (N), and distant metastases (M), seems to reflect a new capacity to express the inflammatory response more than to induce it in the host [45]. In this hypothetical circumstance the inflammatory phenotypes would be expressed by tumor cells to invade the host.

Therefore, it should be kept in mind that while a malignant tumor develops it can express phenotypes that are common to the inflammatory response, including a hypoxic phenotype with edema and lymphangiogenesis, a leukocytic phenotype with migration to the regional lymph nodes and distant metastasis, and an angiogenic phenotype with granulation tissue development and tumor and metastasis growth.

4.1. The Hypoxic Phenotype and the Interstitial-Lymphatic Tumoral Axis. Hypoxia and inflammation meet at several points in the setting of cancer [50]. During the initial avascular stages of tumor growth, which is when the tumor mass measures less than 0.5 cm [51], the cells seem to adopt an anoxic-hypoxic phenotype [52]. When tumor cells come into contact with oxygen, they could undergo a process of reoxygenation, with oxidative stress and edema [44].

The experimental evidence indicates that the cell mechanism for adapting to hypoxia is the prolyl-hydroxylase-(PHD)-hypoxia-inducible transcription factor (HIF) system [50]. The distribution of PHD-HIF system within the inflamed tumor is involved in both its growth and vascularization [50]. In this way, cancer cells could adopt a hypoxic metabolism to survive. Most cancer cells rely on aerobic glycolysis, a phenomenon termed "the Warburg effect." Thus, cancer cells can convert glucose and glutamine into biomass most efficiently and will proliferate faster [53]. In addition, glutamine metabolism has important "nonanabolic" functions, including the regulation of oxidative stress, signal transduction, and autophagy [43].

Cancer cells at this stage can overexpress matrix metalloproteinases (MMPs). In many instances, therefore, the extensive alterations produced by MMPs in the stromal microenvironment could promote tumor progression [54, 55]. Thus, during the earlier phase of tumor progression, the metabolic autonomy and invasive capacity of the tumor cells would induce their premature migration to the peripheral tissue [56].

Metabolically active cancer cells could induce interstitial edema [57]. There is increasing evidence that conditions characterized by an intense inflammatory response are associated with alterations in cellular membrane potential, with subsequent depolarization and abnormal ion transport. Moreover, disturbances in ion transport are associated with intracellular as well as interstitial edema [58]. Interstitial fluid flow is elevated in tumors, thus favoring the diffusion of solutes and proteins and inducing a substantial influence on cancer cells [59, 60]. The tumor interstitial fluid is absorbed by lymphatic capillaries and drains through lymph nodes in the thoracic duct, where it reaches the blood via the great veins of the neck [60]. Tumor interstitial fluid is suggested to be a rich sample for discovering biomarkers [61].

Compared with nonneoplastic tissue, the tumor stroma contains increased amounts of collagens, proteoglycans, and glycosaminoglycans [61, 62]. The accumulation of glycosaminoglycan fragments especially has been proposed as an important mechanism for edema formation because of its hydrophilic properties [63]. Glycosaminoglycans, that is, hyaluronan, are long unbranched polysaccharide chains which tend to adopt highly extended random-coil conformations and occupy a huge volume for their mass [63]. They attract and entrap water and ions, thereby forming hydrated gels, while permitting the flow of cellular nutrients [63]. Thus, interstitial edema could favor nutrition by diffusion through the malignant tumor. Under inflammatory conditions, hyaluronan is more polydisperse with a preponderance of lower-molecular forms and favors edematous infiltration of the tissues as well as the interstitial fluid flow and the tissue lymph pressure gradient [54, 63, 64].

Tumor interstitial fluid represents the early microenvironment of the tumor cells [57, 59]. One may therefore envisage that access to tumor interstitial fluid bathing the cancer cells is considerably important in order to understand how tumors develop and progress [57, 59]. It could be suspected that during this early phase of the inflammatory cancer cell response, while edema progresses, the lymphatic circulation is simultaneously activated and this circulatory switch establishes an interstitial-lymphatic tumoral axis by which lymph can reach the systemic blood circulation [60]. In this way, lymphatic tumoral vessels are transformed in routes for trafficking through the body, exploited not only by immune cells but also by cancer cells [65]. Nevertheless, the interstitial tumoral fluid flow associated with edema can have important effects on tumoral tissue morphogenesis and function, cancer cell migration, and differentiation, and matrix remodeling, among other processes [66] (Figure 3).

A number of studies in animal tumor models have established the concept that tumors, rather than just accidentally invading preexisting lymphatic vessels in their vicinity, can actively induce tumor-associated lymphangiogenesis by secreting appropriate growth factors, such as vascular endothelial growth factor (VEGF)-C, VEGF-D, or VEGF-A [65]. The formation of new lymphatic vessels, termed lymphangiogenesis, is often observed around or within the tumors [67]. With tumor progression, cancer cells secrete lymphangiogenic cytokines and growth factors which result in the formation of lymphatic vessels [68]. Tumor-associated lymphangiogenesis leads to a more extensive drainage network to capture the increased interstitial fluid flowing from the tumor cells and through the microenvironment [60]. Lymphatic vessels are also active modulators of immunity. The tumor associated with the lymphatic system may affect not only the local microenvironment, but also the host immune response against the tumor [69]. Recent findings raise the possibility that the tumor-associated lymphatic vessel and draining lymph nodes may be important in tumor immunity,

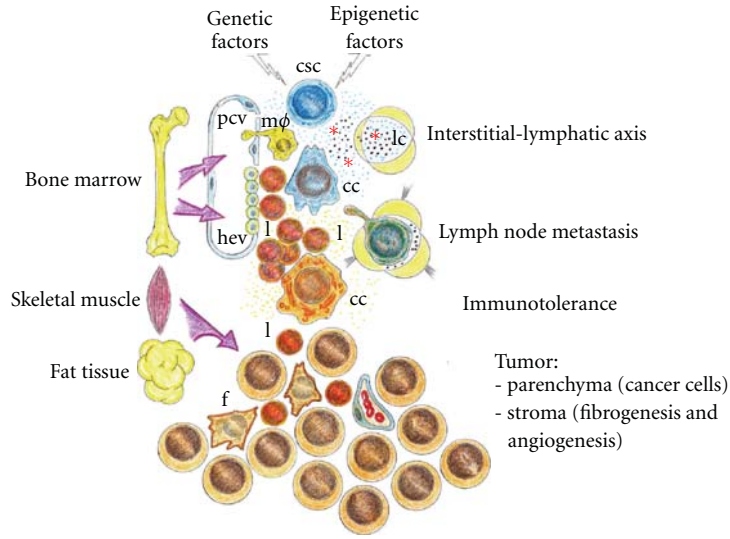

Figure 3: *Successive and overlapped stages of tumorigenesis.* Genetic and epigenetic factors stimulate the formation of a cancer stem cell that invades the interstitial space favored by the inflammatory interstitial-lymphatic axis, which stands out the tissue circulation of fluid and the cellular migration. The tumor cell, by means of using the natural and adaptive immune mechanisms, becomes immunotolerant, which favors the following phases of tumor development. Then, the cancer cell induces the creation of a stroma formed by a special type of granulation tissue, and this allows for the creation of a tumoral parenchyma provided with functional heterogeneity. Finally, this heterogeneous tumor mass plunders the trophic stores of the host inducing cachexia.

which in turn govern metastasis. Peritumoral lymphangiogenesis associated with increased drainage to the lymph nodes could activate and maintain tumor tolerance by the host [69]. Hence, targeting lymphangiogenesis by developing antilymphangiogenesis agents might constitute a novel way to prevent lymphatic progression in some tumors [70].

It has been shown that tumoral lymphatic vessels could display a retrograde draining pattern. Today, the mechanisms of tumoral lymphatic dysfunction are not entirely known [68]. It is hypothesized that the rapid growth of tumor results in tissue edema, which generates mechanical forces to compress the lymphatics, or tumor cells can destroy the intratumoral lymphatic structure [68]. However, the high colloid osmotic pressure and protein concentration of the tumoral interstitial fluid [59] associated with the tumoral lymphatic dysfunction could favor cancer cell trophism by diffusion, modulate their metabolic microenvironment, and, therefore, play a key role in tumor growth and metastasis.

4.2. The Adoption of a Leukocytic Phenotype by Cancer Cells. The cancer cell could express a leukocytic phenotype with anaerobic glycolysis as the main source of energy, which permits lymphatic migration and invasion of the host [44–46].

Hypoxic tumor cells may suffer oxidative and nitrosative stress, like what occurs to leukocytes in the chronic inflammatory response by the generation of reactive oxygen and nitrogen species. Increased oxidative damage levels in malignant cells could result from more reactive species formation with unaltered antioxidant defenses, unaltered reactive species formation with decreases in antioxidant defenses, failure to repair oxidative damage, so that levels rise, or any combination of the above [71]. Overall, evidence supports the view that at least some malignant cells produce more reactive species for its own benefit, and the antioxidant defense and repair activities may sometimes rise, but not enough to cope with the extrareactive species [71].

In most inflammatory responses, the actions of reactive species are mediated by the IκB kinase/nuclear factor (NF)-κB (IKK/NF-κB) system, and in turn, this system can be regulated by hypoxia and/or reoxygenation [52, 72]. More specifically, the expression of inducible genes leading to the synthesis of cytokines, chemokines, chemokine receptors, adhesion molecules, and autacoids relies on transcription factors, and among the primary transcription factors, NF-κB plays a main role in the regulation of inflammatory mediators [73]. In addition, reactive oxygen and nitrogen species generated by both oncogene-expressing cells and inflammatory cells could cause oxidative damage to host DNA resulting in activation of oncogenes and/or inactivation of tumor suppressor genes and various epigenetic changes that favor tumor progression [72].

The activated IKK/NF-κB pathway may play a tumor-promoting role by protecting tumor cells from death, that is, antiapoptotic, or enhancing their proliferation [72]. However, although much attention regarding NF-κB has focused on inflammatory responses and tumor development, the current upsurge of interest in stem cell biology has led to studies of the function of NF-κB in stem cells of the bone marrow. NF-κB has been suggested to regulate secretion of growth factors and cytokines in adult and neonatal stem cells from the bone marrow environment [74].

Tumors in their development share an array of inflammatory activation mechanisms with leukocytes, including toll-like receptors (TLRs) [32, 75], prostaglandin and leukotrienes' metabolism [76], mitogen-activated protein kinases

(MAPKs), that is, extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal kinases (JNKs) and p38 isoforms (p38s) [77], deregulation of phosphoinositide 3-kinase (PCI3K)/phosphatase and tensin homolog (PTEN)/Akt pathway [78], infiltrating tumor-associated macrophages (TAMs) [31] and lymphocytes T and B [34] and microRNA expression [36].

The activation of a leukocytic phenotype by the tumor cells could imply their transition to a more elaborated inflammatory response. Hence, tumor cells may usurp key mechanisms by which inflammation interfaces with cancer to further their colonization of the host [46].

The association between inflammation and cancer can be established by studying the influence of inflammatory cells in tumor progression, including neutrophils, eosinophils, macrophages, myeloid-derived suppressor cells, mast cells, and lymphocytes [1, 31, 35]. However, opposing effects of inflammation on cancer have been described, and, at the risk of oversimplifying for the sake of clarity, it can be said that acute inflammation counteracts while chronic inflammation promotes cancer development [79, 80]. Nevertheless, such a link may not be as simple as the one currently portrayed because certain types of inflammatory processes in skin, such as psoriasis or atopic dermatitis, and possibly other tissues as well, may also serve as a tumor suppressor function [81].

Chronic inflammation is involved in shaping the tumor microenvironment and has been referred to as "host reaction," although it might be more appropriate to think of it as "tumor promoting" reaction [31]. In this way, cancer cells could reconvert the inflammatory host response to obtain an immunosuppressive inflammatory microenvironment and thus may escape from the host aggression [1, 31, 82]. An upregulation of immune tolerance by cancer cell could be induced using adaptive immunity [31, 34, 82, 83]. On the contrary, an inflammatory host response that switches the nature of the tumor promoting an immunosuppressive microenvironment could kill tumor cells and produce cancer regression [31, 82, 84].

Independently of the action of host leukocytes, and owing to the great plasticity of malignant cells, we could suspect that among the wide variety of phenotypes that they could express, it has been shown that tumor cells co-opt some of the signaling molecules of the host innate immune system, like chemokines, selectins, and their receptors, for invasion, migration, and metastasis [84]. Inflammation-associated products can also be secreted by the tumor cells themselves, giving rise to tumor microenvironment, which is overloaded and enriched with inflammatory factors with marked immunosuppressive abilities [80, 85]. In addition, immune cells in the tumor microenvironment not only fail to exercise antitumor effector functions, but also are co-opted to promote tumor growth and metastasis [31, 86] (Figure 3).

The hypothetical activation by chemokines of the leukocytic phenotype in the tumor cells could permit these cells to fulfill functions characteristic of activated inflammatory cells. For example, functions associated with neutrophils, such as the hyperproduction of extracellular proteases, including MMPs and other protease enzymes that carry out a true extracellular digestion of the basement membrane and the extracellular matrix, also aid invasiveness in the early stages of the disease [55, 87]. The extracellular matrix is a reservoir for many molecules, including growth factors, and cytokines, which are only released upon their dissolution [88]. Also, cancer cells can present pseudopodia formation and directional migration [89]. Other functions seem to correspond to a monocyte-macrophage phenotype in the sense that tumor cells migrate to the regional lymph nodes through the lymphatic capillaries [69, 90]. Moreover, the so-called fusion theory explains that the acquisition of the metastatic phenotype occurs when a healthy migratory leukocyte fuses with a primary tumor cell. And interestingly enough, the fusion of genetic and cytoplasmic material between cells of different origins is an important physiological process during development [91].

Cancer cells dissociated from the primary tumor could change their phenotype to become more autonomous, express specific adhesion molecules, produce lymphangiogenic factors and eventually evade the host defense [68]. The hypothetical adoption of a leukocytic phenotype by most tumor cells would also imply the acquisition of a similar metabolism. In this situation, like the activated leukocytes, cancer cells function would require glycolysis as the main source to obtain substrates and energy [92]. In this sense, the metabolic regulation of cancer metastatic cells could be closely related to a specific mutational activation of oncogenes and inactivation of tumor suppressor genes [93]. In contrast to normal cells, cancer cells tend to convert glucose into lactate even in the presence of sufficient oxygen, a term called "the Warburg effect" or aerobic glycolysis [94]. Glutamine is also a major cancer cell energy and anabolic substrate [93]. The oncogenes, Akt, Myc, and Ras, MAP-kinase, PI3K, and mammalian target of rapamycin (mTOR) pathways, and HIF can stimulate the transcription of a number of genes that encode the proteins that mediate the glycolysis and glutaminolysis pathways [93, 94]. This metabolic characteristic of the tumor cell in this stage would explain why tumors grow in the lymph node, regardless of their angiogenic ability [95]. In turn, nutrition of cancer cells during the expression of a leukocytic phenotype could also be based on extracellular, that is, proteolytic enzymes, and intracellular, that is, phagocytic, functions including autophagy and digestion [44, 45].

During the adoption of a leukocytic phenotype, soluble factors could push tumor cells towards premature migration to peripheral tissue [45]. Considerable progress has been made in recent years regarding how tumor cells circulating in the blood and lymphatic system interact with and extravasate into secondary sites and what determines whether these disseminated tumor cells survive, remain dormant, or go on to form macrometastases [96]. Hence, the leukocytic phenotype would favor the homing of metastatic tumor cells to specific organs, especially those where populations of resident macrophages are abundant, that is, lung (alveolar macrophages), liver (Kupffer cells), brain (glia), and bone (osteoclasts) [44]. Particularly, specific chemokines in distant organs and chemokine receptors on the tumor cells dictate a metastasis pattern according to the cancer cells [97].

Tumor-cell-derived factors, which could also be partially contributed by tumor-infiltrating macrophages [31], have been found to induce the expansion of the lymphatic network in the lymph nodes, even before the arrival of the metastatic cancer cells, as if to create a favorable environment for the future metastasis [98, 99]. Also, disseminated tumor cells in bone marrow can be detected in 20–40% of cancer patients without any clinical or histopathological signs of metastasis [100]. The particular bone marrow environment may induce these cells to survive and disseminate later into other distant organs. This "dormant stage" of disseminated tumor cells may explain why these cells are relatively resistant to chemotherapy [101].

The adoption of a leukocytic phenotype by cancerous cells could be associated with an increased degree of lymphangiogenesis. In animal models, a strong relationship between lymphangiogenesis and survival has been shown [67]. Lymphangiogenesis at the site of the primary tumor as well as in the draining lymph node actively contributes to metastatic cancer spread, and its inhibition might be of interest for preventing tumor metastasis [67, 98, 102]. Lymphangiogenesis not only is crucial for cancer cells to metastasize, but also offers the tumor the possibility to disseminate inflammatory mediators in the host, which would produce a systemic inflammatory response syndrome (SIRS) [45, 103]. The SIRS, mediated in part by proinflammatory mediators including cytokines, plays a role in the genesis of cachexia associated with both critical illness and chronic inflammatory diseases [104]. The systemic inflammatory and immune response to the presence of a tumor is manifested in different ways, according to the type and extent of the tumor. Fever, night sweats, weight loss, fatigue and malnutrition may all be attributable to tumor-induced inflammation [3]. Cancer cachexia is a continuum with three stages of clinical relevance: precachexia, cachexia, and refractory cachexia, but not all patients traverse the entire spectrum [105]. Additionally, although an acute phase protein response is usually produced, the C-reactive protein in serum is the most widely accepted index of systemic inflammation, and cachexia can be present in the absence of overt systemic inflammation [105]. Thus, the host, as a yolk-sac-like structure, becomes a vitellum supplier for the tumor growth.

The tumor cell, through the lymphatic system, can also modulate the host immunity, using its own antigen production. Tumoral antigens in the interstitial fluid are collected into open-ended lymphatic capillaries, which can form a mesh-like network by lymphangiogenesis, which could then be transported to the lymph nodes [106]. However, tumoral antigens in prenodal lymph, before entering the node and once inside, could be filtered by dendritic cells and nodal antigen-presenting cells, respectively [106]. Thus, lymph-carried tumoral antigens might have particular relevance for the induction and maintenance of peripheral tolerance to cancer cells.

The functional plasticity of dendritic cells allows for adapting the immune system to mount functionally distinct types of responses, including the immunologic tolerance that could contribute to tumor development [82]. In this way, it is accepted that dendritic cell vaccines will be offered to patients either with early- or late-stage disease to elicit a strong and long-lived antigen-specific T-cell immunity [82].

Additionally, lymphatic vessel density can increase in the chronic inflamed tumor tissue, although without reestablishing an efficient lymphatic connection with the draining lymph nodes [107]. This defective lymphatic drainage, associated with the enduring local tumoral antigen stimulation, could be a crucial trigger of the cascade of events leading to lymphoid neogenesis [108]. De novo lymphoid tissue formation or tertiary lymphoid organs occur during states of chronic inflammation [69]. Tertiary lymphoid organs may accumulate tumor antigens and tumor antigen-presenting cells, bypassing the lymph node function and therefore circumventing their tolerance-maintaining function [69, 108]. In addition, myeloid-derived suppressor cells, a heterogenic population of immature myeloid cells that consists of myeloid progenitors and precursors of macrophages, granulocytes, and dendritic cells, use a number of mechanisms to suppress various T-cell functions as well as to induct regulatory T-cells [109].

4.3. Granulation-Tissue-Related Cancer Cells. Cancer cells, to grow inside the host, need to induce the formation of a stroma to change in an organ. Therefore, the comparison of tumors with wounds that do not heal includes, among other characteristics, the development of a stroma in which the formation of fibroblasts, through a process named fibroplasia, mainly takes place [6]. This phase of skin wound healing, also called granulation phase, is basically characterized by active fibroplasia and angiogenesis [110] (Figure 3).

The role of fibroblasts in wound healing has been extensively studied over the past years. Fibroblasts and myofibroblasts mainly produce the new extracellular matrix necessary for supporting cells and blood vessels, which provides nutrients and oxygen needed for cell growth and proliferation [110]. This new matrix consists of collagen, proteoglycans, and fibronectins produced by the fibroblasts. Fibroblast activity is predominantly regulated by platelet-derived growth factor (PDGF) and transforming growth factor β (TGFβ) [111]. Growth factors involved in fibrogenesis also include fibroblast growth factor-2 (FGF-2) and hepatocyte growth factor (HGF), which are also chemotactic factors for mesenchymal stem cells. Complementary to this, it has been demonstrated that keloid-derived fibroblasts induce higher mesenchymal stem cell migration toward themselves than normal fibroblasts [112]. Also, it must be noted that keloids are locally aggressive scars that typically invade healthy surrounding tissues [112]. Recent studies indicate that mesenchymal stem cells from the bone marrow provide "fibroblasts" to the skin in adults and are thought to enhance skin repair/regeneration [33, 113].

In recent years, the tumor stroma has become the focus of intense research. In particular, fibroblasts, a heterogeneous collection of mesenchymal cells, are among the most abundant cell types in the microenvironment of solid tumors [37]. Cancer-associated fibroblasts, through the release of cytokines and growth factors, could modulate the cancer stem cell phenotype and could also lead to enhanced

angiogenesis [33, 37]. It has been suggested that cancer-associated fibroblasts may be derived from tumor cells that undergo epithelial-mesenchymal transition [114]. One characteristic of the cancer-associated fibroblasts is their heterogeneity, which is also expressed through the formation in solid tumors of a very different profile of extracellular matrix proteins [37]. Mesenchymal stromal cells also can suppress immune responses, favoring tumor cells' escape from the host immune response [115].

Angiogenesis characterizes a phase of cancer evolution that permits numerous substances, including hormones, to be transported by the blood. Angiogenesis requires migration of endothelial cells into the interstitial space with the subsequent proliferation and differentiation into capillaries [44]. Tumors induce angiogenesis by activating tumor stromal cells. The release of angiogenic factors from the extracellular matrix through new formed epitopes promotes angiogenesis. Thus, the neoplastic cells switch to an angiogenic phenotype [116]. However, tumor angiogenesis produces a tumor-associated vasculature that is chaotic, both in structure and function. Although angiogenesis supplies a growing tumor with nutrients and oxygen, the neo-vasculature is poorly formed often with leaky blood vessels that do not link the arterial to the venous circulation but is rather dead-end [42, 117]. This characteristic impairs tumor blood flow and the delivering of oxygen [118], but they favor its growth since without angiogenesis tumors rarely grow to larger than 2 to 3 mm [119]. However, the tumor during its growth seems to prioritize the venous-lymphatic circulation in detriment of the arteriovenous circulation, which is characteristic of the specialized tissues. This circulatory switch perhaps could offer some metabolic advantages to the tumor biology; for instance, it would favor an environment poor in oxygen, strengthening therefore an efficient anaerobic metabolism that forms biomass, and to the blood endothelium with an inflammatory phenotype; it would give more permeability to molecules and cells and it would also enrich the composition of the interstitial space. In essence, a "reactive stroma" would be created [59, 120]. The normal stroma in most organs contains a minimal number of fibroblasts, whereas a reactive tumor stroma could be associated with an increased number of cells, including fibroblasts, enhanced vessel density, and protein deposition [59].

Cancer cells are also associated with high endothelial venule development. Lymphocytes get into lymph nodes from the blood through high endothelial venules. T and B cells subsequently move into the T zone and B-cell follicles, respectively, and then migrate in a stromal-guided random walk [121]. In contrast to the flat endothelial cells that line other types of blood vessels, high endothelial venule endothelial cells are almost cuboidal, and they selectively express certain tissue-specific adhesion molecules and chemokines [122]. High endothelial venules are mainly found in the paracortical and interfollicular areas of the lymph node and are surrounded by an intricate stromal network consisting of fibroblastic reticular cells. However, high-endothelial venule-like structures are also associated with tertiary lymphoid organs, which represent highly organized lymphoid tissues induced by inflammation [122]. In addition, recruitment of lymphocytes and dendritic cells to inflamed lymph nodes by trans-high-endothelial venule migration involves the mediation of heparan sulfate [123].

Tumors build their blood vessels by mechanisms that involve endothelial cell response, including circulating endothelial precursors and circulating endothelial cells, to tumor signaling [97]. Recent works provide evidence indicating that the state of endothelial cell activation in the tumoral niche, rather than angiogenesis itself, may dictate tumor dormancy or escape. In essence, the crosstalk between tumor cells and stromal cells, including endothelial cells within the tumor microenvironment, mediates tumor evolution [97, 124].

Within the factors that play a role in initiating tumoral neovascularization, the "classical," that is, VEGF, fibroblast growth factor-2 (FGF-2), also termed basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factors (TGFs), and angiopoietins (Angs), and the "nonclassical" mediators of angiogenesis, that is, erythropoietin, angiotensin II, endothelins, and thyroid hormones, stand out [125].

However, as it has been previously explained, tumor vasculature is typically aberrant [117] with a reduced pericyte coverage, which in turn destabilizes vascular integrity and function [126]. Additionally, vascular heterogeneity could be associated with subpopulations of cancer cells that differ in their energy-generating pathways, macromolecular biosynthesis, and redox control [42, 43, 126, 127]. These populations may function symbiotically since the cells proximal to a better-oxygenated tumoral tissue consume oxygen with glucose and glutamine, serving as substrates and secreting lactate that can be used by another subpopulation of hypoxic cancer cells as their main energy source [42, 126]. In a similar compensatory way, the activation of HIF in the hypoxic cancer cell subpopulation or in stromal cells within the tumor could augment their vascularization and oxygenation. This change could be associated in turn with tumoral blood flow redistribution, rendering other areas of the tumor ischemic, and consequently with a hypoxic metabolism that induces an increase in tumor invasiveness and risk of metastasis [50]. Thus, it can be suspected that the metabolic and functional heterogeneity of the tumors, which is imposed by their stroma, that is, granulation tissue-like, is dynamic. This tumoral dynamic heterogeneity could constitute a favoring factor of chronicity.

5. Inflammatory Cancer Phenotypes and Related Metabolisms: Regarding the Need for a Metabolic Staging of Cancer

Given the hypothetical plasticity of malignant tumor cells, the above-mentioned inflammatory phenotypes would need the corresponding metabolic plasticity. Hence, the mechanisms that govern tumoral evolution could be based on the increasing metabolic capacity of the tumor cell to use oxygen over the inflammatory phenotypes that supposedly drive the successive phases of release, migration, and proliferation [44–46]. Since it has been proposed that these phases of

tumoral evolution go from hypoxia to the progressive development of an oxidative metabolism, it has been speculated on whether the tumor cell reproduces most of the successive stages by which life passes [44] from its origin without oxygen until it develops an effective, although costly, system for the use of oxygen [128]. If so, in the successive metabolic switches that cancer undergoes, it acquires an increasing ability to both invade the host and use its sources of substrates until its metabolic reserves are all used up [44, 45].

The ability of cancer cells to change their metabolism and, therefore, their inflammatory phenotypes is one of the fundamental reasons why metabonomics offers a platform for biomarker development in the field of oncology [129]. Yet cancer metabolism represents an ideal field for metabolic profiling because of the way its metabolism differs metabolism from normal cells, especially that of the glucose and phospholipid [130]. Tumoral metabolic profiling, not only could allow us to study each tumoral inflammatory phenotype but would also offer the chance of correlating tumoral metabolism with its invasiveness to the host.

The hypoxic phenotype would characterize the initial step of tumoral evolution. In this early phase of the cancer cell response, it could be considered that hypometabolism, anaerobic glycolysis with lactate production, and a low energy expenditure [131] could be related to a primitive cellular trophic mechanism, like diffusion. [44, 45]. In this supposed state of cancer cell stunning, complex or specialized functions could not be expressed. It is conceivable that near-anoxic cancer cells initiate a transcriptional response that compensates the low metabolic demand with reduced oxygen availability. The key player of this adaptive response is HIF [132–134]. However, the HIF complex modulates signaling by Notch, a critical regulator of undifferentiated stem and progenitor cells [134].

The Notch signal pathway is involved in cell fate decisions during normal development but also in the genesis of several cancers [135, 136]. Activation of Notch leads to proteolytic cleavage of the intracellular domain of Notch. Thus, translocation of Notch intracellular domain into the nucleus induces the transcriptional activation of Notch target genes [135, 137]. Notch target genes include proteins and factors involved in the control of the cell cycle and survival processes [137, 138]. In human cancers, activation of Notch signaling also can establish crosstalks with many oncogenic signaling pathways, such as developmental signals, for example, Wnt and Hedgehog signaling [138]. In particular Hedgehog plays a key role in a variety of processes such as inflammation, carcinogenesis, and embryogenesis [137, 138].The signaling pathways that control self-renewal of stem cells are an essential element for tumor survival. That is why cancer stem cells use many of the above-mentioned signaling pathways that are found in normal stem cells, such as Wnt, Notch, and Hedgehog [137, 139].

Tumor metabolism related to the leukocytic phenotype could be able to manage the increased oxidative and nitrosative stress and hypermetabolism imposed by reoxygenation. Recently, the presence and generation of reactive oxygen species attracted increased interest as a microenvironmental factor as part of reoxygenation affecting the survival of tumor cells [140]. In particular, upon reoxygenation, hypoxic cells could experience apoptosis, but there is also evidence that malignant cells can increase their tolerance in response to adverse metabolic conditions [127, 140]. In turn, metabolic stress situations, such as oxidative stress, low pH, or low glucose, are likely to be major determinants of the metabolic phenotype. The regulation of this "metabolic flexibility" is poorly understood and will require a much greater degree of understanding if effective therapeutic strategies targeting metabolism are to be developed and effectively deployed [127].

The pentose phosphate cycle is composed of two branches: the irreversible oxidative pentose pathway that converts glucose-6-phosphate to ribose phosphates thereby yielding two moles, NADPH H+, per mole glucose, and the nonoxidative pentose phosphate pathway that reversibly converts three pentose phosphates into two hexose phosphates, for example, fructose-6-phosphate, and one triosephosphate [131, 141]. The normal cells produce most of the ribose-5-phosphate for nucleotide synthesis through the oxidative pentose pathway; however, in tumor cells, the nonoxidative pentose phosphate pathway is the main source for ribose-5-phosphate synthesis. In turn, it is accepted that there are major differences in the relative share of these two pathways in the delivery of pentose phosphates when comparing slow and fast-growing carcinoma [131].

In particular, in response to oxidative-stress, central carbohydrate metabolism could be reconfigured, so that the metabolic flux reroutes from glycolysis into the pentose phosphate pathway, which allows cells to mount an effective response to this cellular stress [142, 143]. Activation of the pentose pathway also could contribute to many of the unique metabolic requirements of tumor cells. Through its generation of NADPH, the oxidative arm of the pentose phosphate pathway provides reducing power to drive anabolic metabolism [143]. If so, deficiency of the pentose phosphate pathway could influence the development of a diverse variety of oxidative stress-associated human diseases, ranging from autoimmune diseases to carcinogenesis [144].

The mammalian target of rapamycin (mTOR) is an evolutionary conserved Ser/Thr kinase, which could play a major role in the metabolic reprogramming of tumor cells [143, 145]. In addition to its well-known roles in promoting protein synthesis and inhibiting autophagy [43], mTORC1 has been found to stimulate glucose uptake, its conversion to glucose 6-phosphate, and metabolic flux through both glycolysis and the oxidative arm of the pentose phosphate pathway [143]. As mTORC1 signaling is aberrantly elevated in the majority of genetic tumor syndromes and sporadic cancers, this pathway is poised to be a major driver of this metabolic conversion of tumor cells [146].

Notch-mediated signals can upregulate several factors that in turn transmit bidirectional signals among cancer cells expressing both ligands and receptors. Therefore, it is not surprising that Notch signal crosstalks with many oncogenic signaling pathways, such as developmental signals including Wnt and Hegdehog, as well as transcriptional factors, for example, NF-κB [138]. In particular, IL-1 activates Notch signaling pathways probably through NF-κB pathway. Notch

pathway is also a critical downstream target of IL-6 [138]. It has been accepted that the family of NF-κB transcription factors is involved in the expression of genes related to innate and adaptive immunity, which suggests that this signaling pathway could also favor the expression of the leukocytic phenotype in cancer cells. Therefore, Notch signaling could also be required to convert the hypoxic stimulus into epithelial-mesenchymal transition favoring the motility and invasiveness of cancer cells [140, 147].

Finally, sonic hedgehog (Shh), a member of the Hedgehog family, is involved in numerous aspects of embryonic development including angiogenesis and lymphangiogenesis [148]. Shh is an established morphogen critical to the development of the vascular system, but in adult ischemic pathologies, it stimulates the production of angiogenic factors, including VEGF-A and angiopoietin-1. It also promotes endothelial cell chemotaxis [148, 149]. In the ischemic cancer cell, Shh acting concomitantly with the Notch signaling pathway could also stimulate the production of angiogenic factors [135, 137, 149]. These newly characterized pathways have been functionally implicated in the development and tumor-associated angiogenesis. They also illustrate the complex regulation of endothelial cell phenotypes [126]. As a result, a broader concept of the tumoral angiogenic process is needed for its better study and for a better comprehension of the involved mechanisms since both blood and lymphatic vessels are major vascular components of the tumor. This is the reason why the tumor-associated blood/lymph angiogenesis is accepted as a process induced by complicated cytokine networks, mediated by the paracrine and autocrine interactions between tumor cells and stromal cells [150].

Thus, tumor nutrition mediated by blood and lymphatic capillaries could be established thanks to angiogenesis. The new functional properties of tumor microcirculation could include the exchange of oxygen, nutrients, and waste products [44, 45] and favor tumor growth, invasion, and metastasis [151, 152]. Recent information on mitochondrial metabolism in malignant neoplasia emphasizes that, although tumor cells maintain a high glycolytic rate, the principal ATP production may be derived from active oxidative phosphorylation [153]. The result of these metabolic characteristics is that tumors burn glucose while consuming muscle protein and lipid stores of the organism. As a result, tumor metabolism gives them a selective advantage over normal cells [154].

6. Cancer Cell Meets Amniotic and Vitelline Functional Axes

The concept that cancer and embryonic cells have much in common is an old idea. The morphological resemblance between cancer cells and the cells of fetal tissues has been repeatedly discussed in current biomedical literature [155] (Table 3). In the mid-19th century, upon observing cancer tissue under the microscope, the forefathers in pathology noticed the similarities between embryonic tissue and cancer and suggested that tumors arise from embryo-like cells [156]. More recently, it became clear that neoplastic cells possess a more embryonic phenotype than their tissue of origin and that this involves the expression (or reexpression) of embryonic genes [157]. Both embryos and tumors display similar antigens, elaborate angiogenic growth factors, and subvert apoptotic cell death. Furthermore, they may both escape immune destruction by similar mechanisms [157].

Recent studies have shown that it is possible to reprogram the melanoma tumorigenic phenotype by exposing melanoma cells to factors present in the embryonic microenvironment [158]. This suggests that melanoma cells may share some characteristics with stem cells that allow them to respond to the ones from the embryonic microenvironment [159]. It became clear that neoplastic cells posses a more embryonic phenotype than their tissue of origin and that this involves the re-expression of embryonic genes [157, 159]. The convergence of embryonic and tumorigenic mechanisms has indeed allowed suggesting the use of embryonic vaccines against cancer in the past [157].

Tissue interstitium under conditions of long-lasting inflammation is associated with oxidative stress, edema, enzymatic stress, persistent leukocyte stimulation, lymphangiogenesis, angiogenesis, and fibrosis [160, 161]. It has been proposed that if the insult is sustained, for example, chronic inflammatory response, additional proinflammatory mediators can activate a wide variety of leukocytes including macrophages and lymphocytes, which can contributes to further tissue destruction and inflammation [162]. However, prolonged inflammation in wounds contribute to the development of fibroproliferatiove scarring, in other words, keloids and hypertrophic scars [163]. Moreover, in autoimmune diseases, persistent antigenic stimulation recruits endogenous mesenchymal stem cells to the site of the lesion that contributes to the fibrotic evolution [164].

Transforming growth factor (TGF)-β signaling in stromal cells, for example, fibroblasts, also exerts significant effects on tumor development and growth. It has been shown that TGF-β, an important tumor suppressor, also regulates infiltration of immune cells as well as fibroblasts in the tumor microenvironment and promotes tumor progression [165]. Fibroblasts could also produce c-Kit ligand, the most important mast cell growth factor, while mast cells' proteases released from activated mast cells have an important effect on fibroblasts [166]. There is evidence that cancer-associated fibroblasts have a cancer-promoting phenotype [167]. Consequently, cancer-associated fibroblasts could produce significant extracellular matrix remodeling during tumor progression mediated by tumor-specific extracellular matrix proteins and matrix metalloproteinases isoforms [37].

Adult mesenchymal stem cells can be defined as multipotent cells able to differentiate into various types including specialized mesenchymal cells. The behavior of mesenchymal stem cells towards the immune system is context sensitive. Although the antifibrotic effects of mesenchymal stem cells have been demonstrated, the molecular mechanisms behind this effect are not yet fully understood. However, the angiogenic support provided by mesenchymal stem cells is considered the more supportive effect, because reestablishment of blood supply is fundamental for the recovery of damaged patients [168].

In contrast to adult wound healing, the early gestation fetus has the remarkable ability to heal skin wounds without scarring [169, 170]. There are numerous intrinsic and outrinsic differences between the fetus and adult that may influence wound healing. The fetal wound is continuously bathed in amniotic fluid rich in growth factors and extracellular matrix components such as hyaluronic acid, type III collagen, and matrix metalloproteinases. In addition, fetal wound healing is characterized by a rapid upregulation in progenitor cells of genes involved in cell growth and proliferation, with decreased platelet aggregation and degranulation, compared to adult wound healing [170].

One intrinsic difference also includes fetal tissue oxygenation. The fetus has a very low pO2 since there is a large transplacental oxygen gradient between maternal arterial and umbilical venous blood. Consequently, the fetus can heal in a relatively hypoxemic environment [169].

Epithelial to mesenchymal transitions (EMTs) are transdifferentiation programs that are also required for tissue morphogenesis both during embryonic and cancer development [171, 172]. The conversion of epithelial cells to mesenchymal cells is fundamental for embryonic development and involves profound phenotypic changes, including the loss of cell-cell adhesion and the acquisition of migratory and invasive properties [114, 171]. Recent evidence suggests that normal stem cells and cancer stem cells share a mesenchymal phenotype that enhances their ability to preserve stemness, to retain migratory properties, and to respond to different stimuli during expansion and differentiation [171]. Thus, EMT induction in cancer cells results in the acquisition of invasive and metastatic properties [171, 172]. Interestingly enough, these invasive cells, with both a stem-cell-like and mesenchymal phenotype, can generate an epithelial-like structure by mesenchymal to epithelial transition (MET) and, therefore, could be involved in the formation of macrometastasis [171]. Furthermore, it is accepted today that metastasis progression should be considered an independent and parallel process in tumorigenesis governed by the EMT that occurs among tumor cells [91, 114, 172]. In addition, although the majority of tumors are epithelial, they also exert mesenchymal characteristics [91, 171, 172].

An EMT process also occurs in mammalian embryos during gastrulation [114]. Gastrulation is a developmental phase that delineates the three embryogenic germ layers, named ectoderm, endoderm, and mesoderm. Haeckel coined the term gastrulation derived from the Greek word *"gaste,"* meaning stomach or gut, that transforms the rather unstructured early embryo into a gastrula with several specific characteristics: the three primary germ layers are formed; the basic body plan is established, including the construction of the rudimentary body axes; the cells assume new positions, allowing them to interact with cells that were initially not close to them [173]. The nascent mesoderm generated during gastrulation could involve an internalization process of extraembryonic phenotypes [114]. In essence, gastrulation could be represented as the creation of an interstitial space in which extraembryonic, that is, amniotic and vitelline, functions are expressed using mesenchymal cells. If so, the mesoderm would represent the vehicle or the mediator for the internalization of the extraembryonic functions into the embryos [8] (Figure 1).

The mesenchymal state is associated with the capacity of cells to migrate to distant organs and maintain stemness allowing their subsequent differentiation into multiple cell types during development and the initiation of metastasis [114, 171]. In amniotes, members of the TGF-β superfamily induce gastrulation and Nodal signaling together with fibroblast growth factor (FGF) and control the specification of the mesendoderm in all vertebrates [171]. Mesenchymal stem cells are a heterogeneous population with several subgroups of cells with different proliferative and differentiation potentials [9]. Mesenchymal stem cells support hematopoiesis and are able to differentiate towards the mesodermal lineage to generate smooth muscle cells, fibroblasts, pericytes, myofibroblasts, osteoblasts, chondrocytes, and adipocytes [9].

Hypoxia is commonly associated with conditions such as tissue ischemia, inflammation, and solid tumors. However, hypoxic niches in the developing embryo are associated with regulation of cellular differentiation [174]. In this sense, mammalian development occurs in a relatively oxygen-poor environment and before the circulatory system is established. Therefore, it would seem logical that blood vessel patterning could be fine-tuned by local hypoxic microenvironments that are encountered during embryogenesis, organogenesis, and tumorigenesis [174]. This is why it could be considered that hypoxia could influence the behavior of cancer stem cells and their progeny promoting a defective tumor angiogenesis with vascular-like networks [159].

Fibroblasts are among the most abundant cell types in the microenvironment of solid tumors. Carcinoma-associated fibroblasts promote tumor growth and invasion and stimulate angiogenesis [37]. However, the altered phenotype of carcinoma-associated fibroblasts with the production of an impaired extracellular matrix and favoring the interstitial infiltration by inflammatory cells [37] could be key factors for inhibiting the differentiation of the tumoral microcirculation. In turn, this immature microcirculation would be responsible for the tumor-uncoupled metabolic functions regarding the normal microcirculation, as well as its proliferative ability and invasiveness of the host.

The vast arrangement of the mesenchyma around and between the developing amniotic and yolk sac cavities suggests an important role of the mesenchyma in orchestrating embryo development. Mesenchyma isolated specifically from the amniotic membrane could differentiate into neuronal-like cells which are identified to secrete dopamine [175]. Cells derived from amniotic fluid also have a neuronal, dopaminergic phenotype [176]. These results allow for considering the amnion as an embryonic functional axis with strong neural potential [175, 176]. In addition, experimental and clinical studies have demonstrated that amniotic membrane transplantation has important biological properties, including anti-inflammatory, antimicrobial, antifibrosis, and antiscarring, as well as low immunogenicity [177, 178]. Amnion-derived multipotent progenitor cells secrete a unique combination of cytokines and growth factors, known as "amnion-derived cellular cytokine solution," which

establish a communication network between mesenchymal and epithelial cells during embryo development. That is why using the amnion to accelerate wound healing through its functions has been proposed, which regulates migration, proliferation, and differentiation of fibroblasts as well as of keratinocytes [179].

In turn, the extraembryonic visceral yolk sac in mammals is composed of two layers, that is, the visceral endoderm, which is active in endocytosis/digestion and has large lysosomes, and the underlying mesoderm layer [180]. In the embryonic mesoderm layer, "blood islands" develop supporting hematopoiesis and angiogenesis [181]. While formation of the various primitive hematopoietic populations is restricted to the yolk sac, progenitors of definitive hematopoietic cells that arise in the yolk sac may contribute to hematopoiesis in the embryo proper [181]. Also, a major function of the yolk sac is associated with the accumulation of carbohydrates, proteins, and lipids for embryo nutrition (*vitellum*) [16]. Particularly, the yolk sac plays a vital role in providing lipids and lipid-soluble nutrients to embryos during the early phases of development [16]. Interstitial lipid accumulation of cholesterol, a precursor molecule of many hormones, like aldosterone, corticoids, androgens, strogens, and progesterone, may favor fluid infiltration and cell migration, proliferation, and differentiation during embryo development [19].

Dissecting out the possible contribution of extraembryonic lineages to the embryo proper has been difficult, in large part because of the inaccessibility of the mammalian embryo within the uterus [181]. In addition, the term "extraembryonic" is somewhat a misnomer, as there is no clear anatomical or molecular demarcation to separate the embryonic from the extraembryonic tissues during the early steps of the development. Also, at later stages both are integral components of the developing embryo [182]. However, recent studies suggest that extraembryonically derived functions and cells make an increasingly significant and possibly exclusive contribution to the embryonic development [44, 181]. Thus, the molecular and cellular contribution made by both extraembryonic structures, that is, the amnion and the yolk sac, to the interstitial space located between them, namely, the mesoderm, are essential for organogenesis. Particularly, both in the amnion axis and in the yolk sac axis, the extraembryonic mesenchyma plays an important role [9].

The internalization of extraembryonic functions, that is, amniotic-like and vitelline-like, by mesenchymal cells not only could be a key process of the embryonic development, but also could be used by the postnatal organism when it suffers an injury and, therefore, needs to be repaired. Thus, an acute or chronic injury could induce a dedifferentiation process with the expression of different and overlapping inflammatory phenotypes that resemble similar phenotypes expressed during embryo development. Particularly, molecular and cellular amniotic and vitelline mechanisms involved in gastrulation would return [8].

Moreover, the relationship between inflammation and cancer also could be based on the orchestration of extraembryonic functions. If so, tumorigenic cancer cells could successively induce the expression of overlapping amniotic and vitelline-like phenotypes that promote the invasion, control, and remodeling of the interstitium. The amniotic-like phenotype could offer the cancer cells an interstitial-lymphatic axis [59], favoring transport, nutrition by diffusion, excretion, and bacteriostatic and anti-inflammatory protection [179]. In turn, the vitelline phenotype could favor the regulation of lipid metabolism genes, including cholesterol and eicosanoid homeostasis [76], hematopoietic/bone marrow control [113], and the induction of an "angiogenic switch" [117, 126] to permit tumor and metastatic growth. The integration of both extraembryonic phenotypes by the cancer cell would support the functional and metabolic heterogeneity needed to successively modulate their microenvironment during their development in the host (Table 2).

Table 2: Hypothesized cancer cell extraembryonic (amniotic and vitelline) and embryonic (gastrulation) phenotypes.

(i) *Amniotic-like phenotype*
(a) Abnormal ion transport
(b) Extracellular matrix permeability
(c) Diffusion
(d) Increased interstitial fluid (cytokines-substrates)
(e) Interstitial-lymphatic axis
(f) Circulatory switch
(g) Stem cancer cell
(ii) *Vitelline-like phenotype*
(a) Fat transport
(1) Lipoproteins
(2) Cholesterol
(b) Hematopoiesis
(1) Bone marrow control
(2) Platelets
(3) Neutrophils
(4) Mast cells
(5) Tumor-associated macrophages
(6) Lymphocytes
(7) Dendritic cells
(c) Lymphatic metastasis
(d) Immunotolerance
(e) Angiogenic switch
(f) Cachexia
(1) Using the host as vitellum
(iii) *Gastrulation–like phenotype*
(a) Tumoral stroma
(1) Epithelial-mesenchymal transition
(2) Granulation tissue
(b) Tumoral parenchyma
(1) Mesenchymal epithelial transition
(2) Tumor growth
(c) Tumoral organ
(1) Tumoral tissue heterogeneity

Table 3: Common metabolic and functional characteristics of the embryonic and the cancerous axis under hypoxia.

(i) Avascular stage of development
(ii) Notch-signaling pathway
(iii) Hedgehog-signaling pathway
(iv) Mammalian target of rapamycin (mTOR)
(v) Prolyl hydroxylase (PHD)-hypoxia-inducible transcription factor (HIF) system
(vi) Aerobic glycolysis ("Warburg effect")
(vii) Oxidative and nitrosative stress
(viii) Antioxidant defenses: glutamine metabolism, oxidative and nonoxidative pentose phosphate pathways
(ix) Matrix metalloproteinases
(x) IκB kinase/nuclear factor (NF)-κB (IKK/NF-κB) system
(xi) Chemokines and adhesion molecules
(xii) Toll-like receptors
(xiii) Lymphangiogenesis
(xiv) Prostaglandins and leukotrienes active metabolism
(xv) Mitogen-activated protein kinases (MAPKs)
(xvi) Tumoral/embryonic antigens
(xvii) Lymphoid neogenesis
(xviii) Epithelial mesenchymal transition
(xix) Mesenchymal stem cells/fibroblasts
(xx) Growth factors and chemotactic factors for mesenchymal stem cells
(a) Platelet-derived growth factor (PDGF)
(b) Transforming growth factor β (TGF-β)
(c) Fibroblast growth factor-2 (FGF-2)
(d) Hepatocyte growth factor (HGF)
(xxi) Angiogenesis ("angiogenic switch")
(a) Vascular endothelial growth factor A (VEGF-A)
(b) Angiopoietin-1
(c) Endothelial cell chemotaxis
(xxii) Selective metabolic advantage

The new point of view proposed in the current review would be based on considering the extraembryonic mechanisms as the expression of an ancient type of inflammatory response. Ancestral mechanisms of natural and acquired immunity immersed into the amniotic and vitelline axes would allow the acceptation of new and foreign tissues by the host. Therefore, the neoformed tissues, either physiological, that is, embryo and repair tissue, or pathological, that is, cancer, both of them being alien to the host organism, would need to use similar extraembryonic mechanisms to be grafted successfully.

The involvement of these extraembryonic mechanisms, which are individualized in each patient in tumorigenesis, would be a key inducing factor of the vast heterogeneity of cancer [183, 184]. Moreover, cancer cells could be modified through epigenetic modifications that alter gene-expression patterns. Like all the cells that constitute the human body, a cancer cell is a direct descendent of the fertilized egg from which the cancer patient developed. Compared with the fertilized egg, the cancer genome will also have acquired epigenetic changes with an altered chromatin structure and gene expression [185].

7. Conclusion

It could be concluded that cancer cells acquire the ability to invade the host organism through the recapitulation of extraembryonic, amniotic, and vitelline functions. In this way, through the hypothesized overlapping expression of these two extraembryonic functional axes, cancer cells would adopt the different phenotypes that they need to develop. Maybe this is the reason why pluripotent cancer-initiating cells would acquire the nature of embryonic cells. Thus, the cancer-initiating cells would have the amazing ability of inducing, for their own benefit, the expression in the host of functional extraembryonic mechanisms.

The immunological properties of the extraembryonic functions would have the aim of inducing the acceptance of the embryo by the maternal immune system. In addition, the adoption during the postnatal life of the above-mentioned extraembryonic functions for developing new tissues during wound repair and tumorigenesis could have the same objective. Therefore, an appropriate immunological and trophic microenvironment would be created for coexisting with the host.

Since the tumor tissue is what induces the expression of extraembryonic functions in the host that favor its development, it would become an autonomous organism that progressively takes over its nutritional stores. This behavior could be compared with how the cuckoo adapts for reproduction. This bird uses foreign nests to lay its eggs, which will be incubated by other birds. After hitching, the newborn cuckoos throw out all potential competitors from the nest and thus monopolize the nutritional support of the deceived parents. In the same way, tumor cells act selfishly, compared to the altruistic behavior characteristic of the normal cells of the host. Hence, in cancer, new immunotolerant tissues monopolize the nutritional resources of the host. This behavior could represent a last attempt to survive this indomitable eukaryotic cell even at the expense of "murdering" its supporter.

Abbreviations

Angs:	Angiopoietins
bFGF:	Basic fibroblast growth factor
EMT:	Epithelial to mesenchymal transitions
ERKs:	Extracellular signal-regulated kinases
FGF-2:	Fibroblast growth factor-2
HDL:	High-density lipoproteins
HGF:	Hepatocyte growth factor
HIF:	Hypoxia-inducible transcription factorsystem
IKK:	IκB kinase
IKK/NF-κB:	IκB kinase/Nuclear factor kappa B system
JNKs:	c-Jun NH2-terminal kinases

MAPKs: Mitogen activated protein kinases
MET: Mesenchymal to epithelial transition
mTOR: Mammalian target of rapamycin
NF-κB: Nuclear factor kappa B
PCI3K: Phosphoinositide 3-kinase
PDGF: Platelet-derived growth factor
PHDs: Prolyl hydroxylases
PTEN: Phosphatase and tensin homolog
Shh: Sonic hedgehog
SIRS: Systemic inflammatory response syndrome
TAMs: Infiltrating tumor associated macrophages
TGF-β: Transforming growth factor β
TLR: Toll-like receptors
VEGF-A: Vascular endothelial growth factor A
VLDL: Very- low-density lipoproteins.

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Review Article

Utility of Vascular Endothelial Growth Factor Inhibitors in the Treatment of Ovarian Cancer: From Concept to Application

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Despite recent advances in the management of ovarian cancer, it remains the most lethal gynecologic malignancy. Vascular endothelial growth factor (VEGF) has been shown to play a pivotal role in the progression of ovarian cancer leading to the eventual development of malignant ascites. On this basis, agents rendering VEGF ineffective by neutralizing VEGF (bevacizumab), blocking its receptors (aflibercept), or interfering with the postreceptor signaling pathways (sunitinib) provide us with the rational treatment options. These agents are generally used in combination with the standard chemotherapeutic drugs. Here, we discuss the basis of and the logic behind the use of these agents in the treatment of epithelial ovarian cancer, as well as their evaluation in different preclinical and clinical studies.

1. Introduction

Ovarian cancer is the sixth leading cancer diagnosed among women in the world and the second most common gynecologic cancer, comprising nearly 4% of all female cancers [1, 2]. Due to lacking early warning signs and effective screening tools, approximately 75% of patients present with late stage disease [3].

Conventional cytotoxic chemotherapy induces a large proportion of responses in the first-line treatment of advanced ovarian cancer with response rates of 60–80%, but the subsequent relapse and death will develop in the majority of patients [4]. Most cases of advanced ovarian cancer will show resistance to chemotherapy in spite of intensive primary interventions. Targeting tumor vasculature has been theoretically a focus of great interest in this regard due to not only its vital role of conducting tumor blood supply, but also its epithelial cells being more genetically stable than tumor cells [5].

Reviewing the role of vascular endothelial growth factor in ovarian cancer and the feasibility and possible role of VEGF-targeted strategies in ovarian cancer treatment as well as their promises and challenges is the aim of this article.

2. The Role of Angiogenesis in Ovarian Physiology

Female reproductive cycle is intricately connected to the coordinated action of angiogenic factors and steroid hormones. Dominant follicles starting maturation in ovulatory cycle are those with higher vascularity that eventually synthesize the steroid hormones required for endometrial development by turning into corpus luteum. This increased vascularity continues during luteal phase to supply nutrients and steroid precursors and helps to make active steroid hormones accessible to the endometrium. Cyclical changes in the level of vascular growth factors in different stages of menstrual cycle indicate the importance of angiogenesis in ovarian physiology. Increased intrafollicular levels of VEGF have been shown during the initial part of the ovulatory cycle, with peak concentrations just before the start of the luteal phase [6].

3. Tumor Angiogenesis and Its Role in Ovarian Cancer

Like their normal counterparts, tumor cells are in crucial need of a vascular system to satisfy their own requirements of having access to oxygen and nutrients supply and waste removal. Tumor angiogenesis is the mechanism required for fulfilling these requirements, without which tumors fail to grow beyond 1-2 mm and may remain dormant [7].

Tumor-induced blood vessels possess ultrastructural abnormalities, including lack of functional pericytes, dilation and convolution, exceptional permeability, and vascular walls being infiltrated by tumor cells [8].

In ovarian cancer, an imbalance between tumor levels of pro- and antiangiogenic factors in favor of angiogenesis activation occurs. It is indicated as increased proangiogenic factors, including VEGF, fibroblast growth factor (FGF), platelet-derived growth factors (PDGFs), tumor necrosis factor-alpha (TNF-α), angiopoietins and interleukins (IL-6, IL-8), and decreased antiangiogenic factors, including angiostatins and endostatins [9]. It has been indicated that the modulation of angiogenic pathways in ovarian surface epithelium may alter its tumorigenicity [10]. Although microvessel density (MVD) in ovarian cancer has correlated with extent of disease and, inversely, with overall survival (OS) or progression free survival (PFS) [11], different studies have reported contradictory data regarding a convincing correlation between MVD and ovarian cancer prognosis [12–14].

4. The Role of Vascular Endothelial Growth Factor in Ovarian Cancer

VEGF promotes proliferation, migration, stabilization, and survival of endothelial cells and mobilization of endothelial progenitor cells from bone marrow and yields a direct effect on tumor cell proliferation and invasiveness as well [9, 15].

VEGF, formerly known as vascular permeability factor (VPF), has also a key role in enhancing vascular permeability [9]. In advanced ovarian cancer, VEGF-induced hyperpermeability of peritoneal blood vessels and subsequent intraperitoneal hyperosmolarity caused by leaked plasma proteins will lead to malignant ascites, a prevalent, debilitating manifestation of the late-stage disease indicating disease progression and treatment failure [16]. Moreover, some leaked proteins such as plasminogen activator, matrix metalloproteinases, interstitial collagenases, and gelatinase-A provide space for new cell growth through degrading extracellular matrix while fibrinogen facilitates microvascular growth [15].

VEGF induces tumor angiogenesis in a way very similar to how it promotes physiological angiogenesis [17]. As a mitogen for vascular endothelial cells, VEGF promotes new blood vessels formation, and as a survival factor, it stabilizes new, poorly formed tumor vasculature and inhibits endothelial cell apoptosis, resulting in sustained tumor growth [15]. It has been demonstrated that VEGF inhibition normalizes tumor vessels and enhances oxygen and chemotherapeutics delivery to the tumoral tissue [6].

Investigations show that VEGF also contributes to tumor metastasis by inducing the formation of structurally abnormal blood vessels that can be easily penetrated by neoplastic cells [17]. Moreover, increased expression of matrix metalloproteinase-2 by VEGF can enhance the invasiveness of tumor cells [9].

In addition to its paracrine effect on tumor vascular endothelial cells, VEGF has been found to have an autocrine effect by interesting discovery of VEGF receptors on tumor cells [18, 19]. However, the functional significance of this finding and the underlying cellular processes of VEGF/VEGFR autocrine loop are being studied. A functional role for VEGFR-2 and a distinct VEGFR-2-mediated pathway promoting tumor growth in ovarian cancer have been demonstrated [20].

VEGF expression in ovarian cancer has been evaluated in several studies. Some degree of VEGF expression in all examined ovarian cancer specimens as well as significantly higher levels of VEGF expression in tumor specimens compared to benign ovarian tissue have been reported [21]. A correlation between increased titers of VEGF in cytosolic fractions from tumor specimens and increased stage and decreased survival was found as well. In early stage ovarian cancers, increased VEGF expression has been shown to correlate with worse disease-free survival (DFS) and poor OS [22]. In addition, higher serum levels of VEGF associated with ovarian cancer were considered as an independent risk factor and a prognostic parameter for ascites, more metastasis, advanced-stage disease, and decreased survival [23, 24]. VEGF upregulation enhanced the invasiveness of ovarian cancer cells in vitro [25] and VEGF blockade in animal models of ovarian cancer inhibited ascites formation and slowed the tumor growth [26]. Several retrospective clinical studies in ovarian cancer have also demonstrated that intratumoral VEGF and VEGFR-2 expression and VEGF gene polymorphisms are independent poor prognostic factors [27–29]. Overexpression of neuropilin, a coreceptor enhancing VEGF signaling, has also been found in ovarian cancer [30, 31].

5. Anti-VEGF Agents in Ovarian Cancer: Previous and Current Studies

Known to play a key role in normal ovarian physiology and in ovarian cancer, VEGF signaling axis has been an attractive target in antiangiogenic approaches. Agents that target this pathway are currently in clinical development for ovarian cancer (Table 1).

5.1. VEGF Ligand Binders

5.1.1. Bevacizumab. Bevacizumab is a recombinant humanized VEGF monoclonal antibody derived from its murine equivalent A4.6.1. It directs against all active isoforms of VEGF and prevents them from binding to VEGFR [32]. Known as the first anti-VEGF agent approved by the Food and Drug Administration (FDA) in 2004 for clinical use in colorectal cancer, bevacizumab has also been the first anti-VEGF agent to be evaluated in the treatment of ovarian cancer [4, 33].

Table 1: Anti-VEGF agents in clinical development for ovarian cancer.

Mechanism(s) of action	Drug	Molecular target(s)
VEGF ligand binders	Bevacizumab	VEGF A (all isoforms)
	Aflibercept (VEGF trap)	VEGF A and B, PlGF
VEGF receptor tyrosine kinase inhibitors	Ramucirumab	VEGFR2
	Cediranib	VEGFR1-3, c-Kit, PDGFR-β
	Semaxanib	VEGFR2
Multiple-receptor tyrosine kinase inhibitors	Sunitinib	VEGFR1-3, Flt-3, PDGFR-α, PDGFR-β, c-Kit, CSF-1R, RET
	Sorafenib	VEGFR1-3, PDGFR-β, Flt-3, c-Kit, Raf-1
	Vatalanib	VEGFR1-3, PDGFRβ, c-Kit, c-Fms
	Intedanib (BIBF1120)	VEGFR1-3, PDGFR-α, PDGFR-β, FGFR1-3
	Pazopanib	VEGFR1-2, PDGFR-β, c-Kit
	Motesanib	VEGFR1-3, PDGFR, c-Kit
	Vandetanib	VEGFR2-3, EGFR
	AEE788	VEGFR, EGFR

VEGF: vascular endothelial growth factor, PlGF: placenta growth factor, VEGFR: vascular endothelial growth factor receptor, PDGFR: platelet-derived growth factor receptor, CSF-1R: colony stimulating factor 1 receptor, EGFR: epidermal growth factor receptor.

Table 2: Clinical trials of single-agent therapy with bevacizumab in ovarian cancer.

Trial number	Phase	Stage of the disease	Number of patients	CR	PR	MPFS (m)	MOS (m)
Completed							
NCT00022659 (GOG170D) [37]	II	Persistent or recurrent EOC or PPC	62	3%	18%	4.7	17
NCT00097019 (AVF2949g) [38]	II	Recurrent EOC or PSC	44	0	15.9%	4.4	10.7
Ongoing							
NCT00866723(08-323)*	II	Relapse after bevacizumab maintenance therapy	32	N/A	N/A	N/A	N/A

EOC: epithelial ovarian cancer, PPC: primary peritoneal cancer, PSC: peritoneal serous carcinoma, CR: complete response, PR: partial response, MPFS: median progression-free survival, MOS: median overall survival, m: months, N/A: nonaccessible.
*Accessed from http://www.clinicaltrials.gov/ on April 18, 2011.

Preclinical Data. Several experiments have shown that neutralizing VEGF by bevacizumab has marked antitumor effects. In 1998, Mesiano et al. examined the role of bevacizumab in immunodeficient mice with ovarian cancer and demonstrated that bevacizumab significantly inhibited subcutaneous tumor growth, partially inhibited its intraperitoneal growth, and completely prevented ascites production [34]. Hu et al. reported additive or synergistic effects of this antibody in combination with paclitaxel in ovarian tumor xenograft studies including enhanced sensitivity to paclitaxel and marked reduction of tumor growth and ascites formation [35]. Those effects have been confirmed by Mabuchi et al. using bevacizumab in combination with cisplatin. Moreover, they showed that maintenance treatment with bevacizumab could inhibit recurrence and significantly prolong survival in vivo [36].

Clinical Data

(i) As a Single Agent. The initial clinical evidence regarding the activity of bevacizumab for ovarian cancer, mainly in recurrent, heavily pretreated patients, was first reported by Monk et al. in 2005 [42]. Since then, bevacizumab has been examined in two prospective phase II trials as a single agent for patients with recurrent ovarian cancer, predominantly platinum-resistant disease as shown in Table 2 [37, 38].

(ii) Combined with Chemotherapy

Retrospective Studies. In 2006, Wright et al. reported a partial response (PR) of 35% and a median PFS of 5.6 months in a retrospective analysis of 23 patients with recurrent platinum-refractory epithelial ovarian cancer treated with a combination of bevacizumab with some cytotoxic agents including cyclophosphamide, 5-fluorouracil, docetaxel, and gemcitabine and liposomal doxorubicin [43]. In another study on 35 patients with recurrent ovarian cancer who received a combination of gemcitabine, platinum, and bevacizumab, Richardson et al. reported an overall response rate (ORR) of 78%, a complete response (CR) of 48%, and a median PFS of 12 months [44]. In a study by Chura et al. on 15 heavily pretreated patients with recurrent ovarian cancer, a combination of bevacizumab and metronomic oral cyclophosphamide resulted in a CR of 13.3% and a PR of 40% [45]. O'Malley and colleagues have recently reported the results of a study on two groups of heavily pretreated

Table 3: Completed clinical trials of bevacizumab combined with chemotherapy in ovarian cancer.

Trial number	Phase	Chemotherapy	Stage of the disease	Number of patients	Outcomes
NCT00127920 (AV53206s) [39]	II	Carboplatin + paclitaxel	Newly diagnosed stage III/IV	20	CR: 30% PR: 50%
NCT00072566 (NCI-5789) [40]	II	Metronomic cyclophosphamide	Platinum-sensitive recurrent	70	CR: 0 PR: 24% MPFS: 7.2 (m) MOS: 16.9 (m)
NCT00129727 (OVCA) [41]	II	Carboplatin + paclitaxel + bevacizumab + maintenance bevacizumab	Newly diagnosed stage ≥ IC	62	CT: CR: 56% PR: 22% Ca-125: CR: 89% PR: 7%
NCT00343044 (3040200, AVF3648s)*	II	Topotecan	Platinum-resistant recurrent EOC, PPC, FTC	N/A	N/A

CR: complete response, PR: partial response, MPFS: median progression-free survival, m: months, MOS: median overall survival (months), EOC: epithelial ovarian cancer, PPC: primary peritoneal cancer, FTC: fallopian tube cancer.
*Accessed from http://www.clinicaltrials.gov/ on April 18, 2011.

patients with recurrent ovarian cancer: 29 patients treated with weekly paclitaxel as compared to 41 patients treated with weekly paclitaxel and biweekly bevacizumab. They have indicated that addition of bevacizumab to weekly paclitaxel has resulted in a significant increase in PFS with a trend towards improved OS [46].

Clinical Trials. To date, three clinical trials on bevacizumab combined with chemotherapy have been published. A summary of the outcome from these studies is listed in Table 3 [39–41].

Bevacizumab is currently being evaluated in combination with various chemotherapy regimens briefly described in Table 4.

The results from GOG218 (NCT00262847) have been reported at the 2010 American Society of Clinical Oncology (ASCO) annual meeting. Based on their report, there was a 3.8-month improvement in PFS (14.1 months for the maintenance regimen of BEV as opposed to 10.3 months for standard chemotherapy) [47]. Initial results of ICON7 (NCT00483782) trial presented in October 2010 showed that the addition of bevacizumab to standard chemotherapy resulted in 15% improvement in PFS at 12 months, 1.7-month improvement in median PFS, and 1.5-month overall improvement in PFS. Treatment effect is numerically greater in advanced-stage patients with no new side effects [48].

Side Effects. Being generally well tolerated, the most common bevacizumab-attributable side effect, hypertension, can be medically controlled. However, gastrointestinal perforation and thromboembolic disease were documented as two major complications [33], and the overall rate of bowel perforation in ovarian cancer seems to be higher than other solid tumors. Other common adverse events include proteinuria, bleeding, and wound-healing complications [9]. Preliminary analysis of a prospective study evaluating the safety and efficacy of bevacizumab in cancer patients has demonstrated severe pulmonary and nonpulmonary hemorrhage as an associated risk with a rate of 0.5% and 1.2%, respectively [49]. Also, a meta-analysis of randomized controlled trials has recently reported that bevacizumab may significantly increase the risk of serious hemorrhage in cancer patients [50]. Reversible posterior leukoencephalopathy syndrome (RPLS), tracheoesophageal fistulae [6], spontaneous nasal septal perforation, and erosive osteoarthritis are significant, but rare, complications [9]. In a recent meta-analysis of published randomized controlled trials, bevacizumab in combination with chemotherapy or biological therapy, compared with chemotherapy alone, was associated with increased treatment-related mortality [51].

5.1.2. Aflibercept (VEGF Trap). Aflibercept is a soluble decoy receptor based on VEGF receptor-1 and VEGF receptor-2 fused to the Fc portion of human immunoglobulin G1 that binds and inactivates some members of VEGF family, including VEGF-A, VEGF-B, and placental growth factor (PlGF) [4].

Preclinical Data. In 2003, Byrne et al. reported that single-agent aflibercept significantly reduced both ascites and tumor burden in experimental ovarian cancer models [26]. This preclinical efficacy was further validated by Hu et al. who showed a 98% reduction in tumor burden, inhibition of ascites, and prolonged survival in a mouse model of human ovarian cancer treated by aflibercept plus paclitaxel [52].

Clinical Data. Aflibercept has been investigated in some clinical studies described in Table 5.

Side Effects. Based on the initial results of their study, Tew et al. reported that the most common grade 3-4 side effects of

Table 4: Ongoing clinical trials of bevacizumab combined with chemotherapy in ovarian cancer.

Trial number	Phase	Chemotherapy	Stage of the disease	Number of patients (estimated)
NCT00127920 (AV53206s)	II	Carboplatin + paclitaxel + bevacizumab	Newly diagnosed stage III/IV	20
NCT00296816 (TEACO)	II	Oxaliplatin + docetaxel + bevacizumab	Newly diagnosed stage IB-IV	145
NCT00511992 (AVF3953)	II	Paclitaxel + cisplatin + bevacizumab followed by bevacizumab	Newly diagnosed stage II-III	20
NCT00588237 (06-064)	II	Paclitaxel + cisplatin + bevacizumab	Initial treatment of optimal stage II or III (adjuvant)	42
NCT00267696 (2005CO073)	II	Gemcitabine + carboplatin + bevacizumab	Platinum-sensitive recurrent	45
NCT00698451 (CR015094)	II	Carboplatin + liposomal doxorubicin + bevacizumab	Platinum-sensitive recurrent	54
NCT00418093 (04-356)	II	Oxaliplatin + gemcitabine + bevacizumab	Platinum-sensitive recurrent	40
NCT00868192 (08-0508)	II	Pemetrexed + bevacizumab	Recurrent having failed platinum- and taxane-based regimens	25
NCT00504257 (MCC-14920, MCC-105366c)	II	Docetaxel + bevacizumab	Platinum-resistant recurrent	44
NCT00744718 (2008-000878-20, S-20080033)	II	Carboplatin + bevacizumab	Platinum-resistant recurrent	30
NCT00846612 (06-948, AVF3910s)	II	Liposomal doxorubicin + bevacizumab	Platinum-resistant recurrent	48
NCT00856180 (08-148)	II	Cyclophosphamide + bevacizumab	Platinum-resistant recurrent	20
NCT00407563 (ALSSOPR0501)	II	Abraxane (protein-bound paclitaxel) + bevacizumab	Platinum-resistant recurrent	48
NCT00937560 (MO22225)	II	Carboplatin + paclitaxel + bevacizumab	Previously untreated, but initial surgery	188
NCT00583622 (2007-0368)	II	Gemcitabine + docetaxel + melphalan + carboplatin + bevacizumab	Second or later complete remission, or untreated or refractory relapse to platinum treatment or lack of response to salvage treatment	40
NCT00483782 (ICON7)	III	Carboplatin + paclitaxel ± bevacizumab	Newly diagnosed	1520
NCT00262847 (GOG218)	III	Carboplatin + paclitaxel versus carboplatin + paclitaxel + bevacizumab ± maintenance bevacizumab	Newly diagnosed, previously untreated stage III or IV	2000
NCT00565851 (GOG213)	III	Carboplatin + paclitaxel ± bevacizumab followed by bevacizumab and secondary cytoreduction surgery	Platinum-sensitive recurrent	660
NCT00434642 (OCEANS, AVF4095g)	III	Carboplatin + gemcitabine ± bevacizumab	Platinum-sensitive recurrent	487
NCT00652119 (2007-0223)	N/A	Carboplatin + paclitaxel + bevacizumab	Newly diagnosed stage III/IV	46

* Accessed from http://www.clinicaltrials.gov/ on April 18, 2011.

aflibercept therapy were hypertension (9%) and proteinuria (4%). Other adverse events included headache, fatigue, dysphonia, nausea, asthenia, diarrhea, renal dysfunction, and a remarkably lower incidence of bowel perforation (1%) [53].

5.2. VEGF Receptor Tyrosine Kinase Inhibitors

5.2.1. Ramucirumab (IMC-1121B). Ramucirumab is a fully humanized monoclonal antibody that specifically and potently inhibits VEGFR-2 [9]. On the basis of in vitro studies, ramucirumab has been investigated in mouse xenograft models of human ovarian cancer resulting in reduced tumor growth, increased apoptosis, and decreased tumor microvessel proliferation and density [55]. Being observed in several phase I clinical trials in solid tumors [56], ramucirumab is currently being assessed in a phase II trial as a monotherapy in patients with persistent or recurrent epithelial ovarian cancer [57].

Ramucirumab has shown safety in phase I trials. Two dose-limiting toxicities including hypertension and deep vein thrombosis were noted by Spratlin et al. in a phase I trial [58].

Table 5: Clinical trials for VEGF trap in ovarian cancer.

Trial	Phase	Stage of the disease	Results
Single agent			
NCT00327171 (ARD6122, AVE0005)—Completed	II	Platinum-resistant and topotecan and/or liposomal doxorubicin-resistant advanced ovarian cancer	Preliminary results from 162 randomized patients showed a partial response of 11%. [53]
NCT00396591 (ARD6772)—Completed	II	Platinum-resistant and topotecan and/or liposomal doxorubicin-resistant advanced ovarian cancer with recurrent symptomatic malignant ascites	First results demonstrated the efficacy of two weekly IV aflibercept in prolonging the time to repeat paracentesis in eight out of ten evaluable patients. [54]
NCT00327444 (EFC6125)—Completed	II/III	Platinum-resistant and topotecan and/or liposomal doxorubicin-resistant advanced ovarian cancer with recurrent symptomatic malignant ascites	Results are awaited
Combination with chemotherapy (docetaxel)			
NCT00436501 (MDA-2006-0329)—ongoing	I/II	Recurrent or persistent epithelial ovarian, primary peritoneal, or fallopian tube cancer	Ongoing

IV: Intravenous, VEGF: Vascular endothelial growth factor.
*Accessed from http://www.clinicaltrials.gov/ on April 18, 2011.

5.2.2. Cediranib (AZD2171). This is a novel oral tyrosine kinase inhibitor that selectively blocks VEGFR1-3, PDGFR-β, and c-Kit [4]. Cediranib has been shown to inhibit the growth of human tumor xenografts, including ovarian cancer, in a dose-dependent manner [59]. In 2009, the results of a phase II study of single-agent cediranib for recurrent ovarian, peritoneal, or tubal cancer were reported by Matulonis et al. Overall clinical benefit for the intent-to-treat (ITT) population was 30%; 17% patients achieved a partial response representing the overall response rate. Thirteen percent of patients had stable disease. No patients had a complete response [60]. In another phase II trial by Hirte et al., response rate was 41% and 29% for platinum-sensitive and platinum-resistant disease, respectively [61]. Other phase II and III trials [62–64] are ongoing to investigate its efficacy as single agent or in combination therapy, among which is ICON6, a three-arm randomized placebo-controlled phase III trial, investigating cediranib in combination with platinum-based chemotherapy and as a single-agent maintenance therapy in patients with platinum sensitive relapsed ovarian cancer [64].

In both above-mentioned phase II trials, hypertension and fatigue were the most common grade 3 toxicities. Other reported adverse events were central nervous system hemorrhage, elevated lipase, hypertriglyceridemia, diarrhea, anorexia, vomiting, hyponatremia, oral cavity pain, nausea, constipation, abdominal pain, headache, and hypothyroidism [61, 65].

5.2.3. Semaxanib (SU5416). Potent and selective synthetic inhibitor of VEGFR-2, semaxanib inhibits tyrosine kinase catalysis, tumor vascularization, and growth of different tumor types [66]. In a study by Holtz et al., semaxanib yielded reduced tumor growth and microvessel density in mouse models of ovarian cancer with high VEGF expression. Based on its combination with metronomic paclitaxel, they also provided the first evidence that the interactions between low-dose chemotherapy and antiangiogenic therapy could be affected by tumor VEGF expression as they observed additive effects only in tumors with low VEGF expression [67]. A phase I study of semaxanib in combination with carboplatin in patients with platinum-refractory ovarian cancer has been done [68].

5.3. Multiple-Receptor Tyrosine Kinase Inhibitors

5.3.1. Sunitinib (SU11248). Sunitinib is an orally bioavailable multityrosine kinase inhibitor that blocks VEGFR1-3, Flt-3, PDGFR-α, PDGFR-β, c-Kit, CSF-1R, and RET with proven antitumor activity in renal cell carcinoma and imatinib-resistant or -intolerant gastrointestinal stromal tumor (GIST) [4]. Its effectiveness as a single agent in recurrent or advanced ovarian cancer was proved by Bauerschlag et al. in an ovarian cancer xenograft mouse model, in which the drug significantly suppressed tumor growth and peritoneal metastases, and also remarkably reduced microvessel density count [69].

Having similar gene profile to renal cell carcinoma (RCC), recurrent or refractory ovarian clear cell adenocarcinoma—a biological subtype of epithelial ovarian cancer—may benefit from this agent. In one case report of a 60-year-old woman with ovarian clear cell adenocarcinoma (OCCA), sunitinib resulted in stable disease as fifth-line therapy, decreased CA125 and cystic degeneration of liver metastasis [70]. Based on the promise shown in different phase I, II, and III studies of sunitinib in a number of cancers, four phase II trials of sunitinib as single-agent therapy in ovarian cancer are being pursued [71–74]. One of those

is NCT00388037, first results of which have demonstrated single-agent sunitinib activity and tolerability in advanced ovarian cancer with partial response in 2 and stable disease in 10 among 17 patients [74].

Both on-target and off-target adverse effects including fatigue, diarrhea, dyspepsia, hypertension, hand-foot syndrome, nausea, anorexia, stomatitis, neutropenia, thrombocytopenia, lipase elevations, and hypothyroidism are mentioned as typical side effects of sunitinib in other diseases [9, 75].

5.3.2. Sorafenib (Bay43-9006). Sorafenib is an oral multitargeted tyrosine kinase inhibitor that predominantly inhibits Raf-1—which is vital for cell proliferation—and can block VEGFR1-3, PDGFR-β, Flt-3, and c-Kit [9]. In a study by Matsumura et al., sorafenib was shown to have antitumor effect against ovarian clear cell carcinoma (OCCC) as it inhibited tumor growth in nude mice and significantly reduced tumor size [76]. In a phase I study of sorafenib in patients with ovarian cancer, 50% of them showed evidence of stable disease [77]. In another phase I trial, sorafenib in combination with bevacizumab demonstrated durable partial disease responses in 6 of 13 ovarian cancer patients recruited [6]. In a phase II trial in patients suffering from persistent or recurrent ovarian cancer, partial response was seen in 3% of patient evaluated while 20% had stable disease more than 6 months [78]. Another study of intermittent sorafenib dosing with bevacizumab has promising clinical activity and less sorafenib dose reduction and side effects in advanced ovarian cancer [79]. Six clinical trials of sorafenib combined with other agents are now underway [80–85].

Although diarrhea and hand-foot syndrome are more prevalent than other side effects, alopecia, anorexia, and weight loss also have been reported more common with sorafenib. However, few grade 3 and 4 adverse effects have occurred [75].

5.3.3. Vatalanib (PTK787). Being a multitargeted tyrosine kinase inhibitor, vatalanib blocks VEGFR1-3, PDGFR-β, c-Kit, c-Fms with highest activity against VEGFR-2. In an ovarian cancer mouse model, single-agent vatalanib reduced ascites and tumor growth and yielded increased survival [15]. In a phase I study of vatalanib combined with carboplatin and paclitaxel in advanced ovarian cancer, Schroder et al. showed that vatalanib was feasible and well tolerated [86]. The side effects of vatalanib appear to be similar to those of other VEGF inhibitors. Schroder et al. reported grade 1 and 2 hypertension as the most frequent adverse events in their study.

5.3.4. Vandetanib (ZD6474). Vandetanib is a dual specific inhibitor of VEGFR and EGFR. Monotherapy with vandetanib showed a significant antitumor effect in an ovarian cancer nude mice model [87]. No significant clinical benefit was made by vandetanib monotherapy in patients with recurrent ovarian cancer in a phase II clinical trial [88]. Its efficacy in combination with docetaxel in persistent or recurrent ovarian cancer will be assessed in a phase II clinical trial (NCT00872989) [89]. Common drug-related adverse events include rash, diarrhea, hypertension, fatigue, and asymptomatic QTc prolongation [90].

5.3.5. Intedanib (BIBF1120). Intedanib is a combined inhibitor of VEGFR, PDGFR, and FGFR. Having potential to block proangiogenic signaling pathways in vascular endothelial cells, smooth muscle cells, and pericytes, indetanib can inhibit cell proliferation and apoptosis. In a study by Hilberg et al., indetanib demonstrated high activity at well-tolerated dose as decreased vessel density and vessel integrity, and profound growth inhibition in all tested tumor models [91]. Ledermann et al. tested indetanib as maintenance therapy in a randomized phase II placebo-controlled trial in ovarian cancer patients who had previously responded to chemotherapy and resulted in a 36-week PFS rate of 15.6% and 2.9%, respectively, for indetanib and placebo. Thus, the conclusion that maintenance indetanib could delay disease progression in previously treated ovarian cancer patient was drawn [92]. To investigate its efficacy and safety, indetanib combined with carboplatin and paclitaxel is currently being examined in a randomized, double-blind phase III trial in patients with advanced ovarian cancer [93]. Based on Ledermann and colleagues' study, the rate of gastrointestinal toxicities was slightly higher in the indetanib arm. They also found higher elevation of liver enzymes (43%) compared with placebo (6.3%) [92].

5.3.6. AEE788. AEE788 is a combined VEGFR and EGFR specific inhibitor. Although single-agent AEE788 was effective in reducing tumor weight in a nude mice model of human ovarian cancer, the combination of AEE788 and paclitaxel was superior to the use of either agent alone, inhibiting the progression of intraperitoneal tumor [94]. Encouraging activity was also indicated by metronomic docetaxel chemotherapy combined with AEE788 in orthotopic mouse model resistant to the conventional therapy [6, 95].

5.3.7. Pazopanib. Pazopanib is a potent and selective multitargeted receptor tyrosine kinase inhibitor of VEGFR, PDGFR, and c-Kit. Pazopanib inhibits VEGF-induced endothelial cell proliferation in vitro and angiogenesis in vivo and shows antitumor activity in mouse models [96]. Pazopanib alone and combined with metronomic oral topotecan was tested by Merritt et al. in vitro and in an orthotopic model of ovarian cancer. Pazopanib therapy resulted in reduced murine endothelial cell migration in a dose-dependent manner and decreased tumor cell proliferation. Combination therapy increased tumor cell apoptosis and reduced tumor microvessel density and pericyte coverage [97]. A phase II study reported by Friedlander et al. indicated that pazopanib is active in women with advanced epithelial ovarian cancer with overall response rate of 18% and 21%, respectively, in subjects with and without measurable disease at baseline. Median PFS was 84 days [98]. Several trials of pazopanib are evaluating this agent as monotherapy or combination with other agents [99–103].

5.3.8. Motesanib (AMG706). Motesanib was identified as a potent, well-tolerated inhibitor of VEGFR 1–3 and PDGFR in preclinical models. It also inhibits Kit receptors, thereby directly interfering with signal transduction of the tumor cell [104]. A phase II clinical trial of this agent in persistent or recurrent ovarian cancer is ongoing [105].

6. Other Agents

Some agents from different pharmacological classes have been studied in clinical development for ovarian cancer with possible or indirect effect on VEGF or its pathway.

6.1. Thalidomide. Thalidomide was first introduced as a sedative drug in the late 1950s. In 1961, it was withdrawn from the market due to its teratogenicity [106, 107], but it has been of renewed interest as a potent angiogenesis inhibitor which was approved by FDA [75]. Antiangiogenic and antitumor effects of thalidomide have been demonstrated in preclinical studies [108, 109], although its mechanisms of action are not clearly unveiled. Thalidomide may suppress VEGF, bFGF, and TNF-α. A phase II trial of thalidomide in some solid malignancies, including ovarian cancer, resulted in some responses whereas a prospective randomized trial of thalidomide with topotecan compared to topotecan alone in women with recurrent epithelial ovarian carcinoma reported a remarkable response with combination therapy [5]. A randomized phase III trial (GOG 198) tested tamoxifen versus thalidomide in women having the recurrence of ovarian cancer. Thalidomide was not more effective than tamoxifen in delaying recurrence or death, but was more toxic [110].

6.2. Atrasentan. Atrasentan is a selective antagonist of endothelin type A receptor (ETAR). It reduces microvessel density, expression of VEGF, and matrix metalloproteinase-2, thus increases percentage of apoptotic tumor cells in ovarian cancer xenografts. Combined with paclitaxel, atrasentan has produced additive antitumor, apoptotic, and antiangiogenic effects. Fatigue, edema, and rhinitis are the most common side effects of atrasentan [9].

6.3. Everolimus. Everolimus is an inhibitor of mTOR, a central regulating pathway of cell growth, proliferation, and apoptosis. Inhibition of mTOR can decrease cancer cell proliferation and survival and reduce tumor-secreted VEGF through inhibition of hypoxia-inducible factor-1α (HIF-1α). An in vivo study using xenograft models of ovarian cancer revealed that everolimus inhibited tumor growth, angiogenesis, and production of ascites, suggesting the potential of mTOR inhibitors in the treatment of women with ovarian cancer [9, 98].

6.4. Microtubule Disrupting Agents. Drugs that target tubulin are one of the most effective classes of anticancer agents and are thus a mainstay in the treatment of ovarian cancer [111]. Although the underlying mechanisms for the inhibition of angiogenesis by microtubule disrupting agents (MDAs) are not yet well defined, interference with the HIF-1α/VEGF axis seems to account for at least part, if not all, of the effects observed [112]. While the taxanes are defined as microtubule polymerizing agents, benzimidazole carbamates such as albendazole are known to conversely interfere with the polymerization process. We have recently described how albendazole inhibits VEGF and angiogenesis under in vitro, in vivo, and clinical conditions [113–115]. Follow-up research revealed that the drug interferes with HIF-1α, leading to the suppression of tumoral VEGF mRNA, and VEGF protein [116]. Thus, irrespective of whether a polymerizing or a depolymerising agent, the MDAs interfere with angiogenesis and suppress VEGF production. Treatment of mice bearing advanced intraperitoneal human OVCAR-3 tumors with albendazole, led to suppression of both plasma and ascites VEGF levels, as a consequence of which highly VEGF-dependant malignant ascites formation was completely aborted, leading to extended animal survival [115, 117].

6.5. Celecoxib and Ciglitazone . PGE2 enhances angiogenesis through the induction of VEGF. Celecoxib is a highly selective cyclooxygenase-2 (COX-2) inhibitor and ciglitazone is a peroxysome proliferator-activated receptor γ (PPARγ) ligand, both inhibiting prostaglandin E2 (PGE2) production. In a preclinical ovarian cancer model, celecoxib and ciglitazone reduced tumor growth by downregulating PGE2 synthesis and thus inhibiting VEGF production [9]. In a phase II study in heavily pretreated recurrent ovarian cancer patients, celecoxib in combination with carboplatin was well tolerated and yielded promising activity as salvage treatment [118]. Celecoxib is also being evaluated in an ongoing randomized phase II study testing cyclophosphamide with or without celecoxib [119].

7. Anti-VEGF Therapy Challenges

Besides toxicities and complications, other challenges regarding anti-VEGF therapy are as follows.

7.1. Resistance. Although endothelial cells are genetically stable, emerging evidence indicates that tumor resistance to anti-VEGF agents is common. The hypothetical rationale for the resistance includes epigenic mechanisms of resistance, unresponsiveness of tumor vasculature to anti-VEGF agents [5], upregulation of alternative proangiogenic pathways, enhanced protection by pericytes, increased invasiveness of tumor cells into local tissue normal vasculature, metastatic seeding, and tumor cell growth in lymph nodes and distant organs, as well as failure of anti-VEGF agents in fully blocking all VEGF signaling pathways [120, 121]. Locating additional targets on the tumor endothelium such as non-receptor kinases, and targeting proangiogenic pathways and agents such as HIF1-α are currently being focused to evade the resistance [5].

7.2. Acceleration of Tumor Invasiveness and Metastasis. A perplexing inverse effect resulted from anti-VEGF therapy

has been found in some preclinical studies. Ebos et al. reported:

(i) accelerated experimental metastasis, increased multiorgan metastases, and decreased survival after short-term sunitinib treatment before and after intravenous tumor cells inoculation;

(ii) increased spontaneous metastasis and decreased survival following short-term sunitinib therapy after removal of primary human xenograft tumors.

Acceleration of metastasis observed in mice receiving sunitinib prior to intravenous implantation of tumor cells suggests the possible "metastatic conditioning" effect by the mentioned anti-VEGF agent. It means microenvironmental changes in mouse organs so that they are conditioned to be more permissive to tumor extravasation. This study shows similar findings with additional VEGF receptor tyrosine kinase inhibitors, implicating a class-specific effect for this group of anti-VEGF agents [122]. Also, Paez-Ribes et al. indicated the following:

(i) increased invasiveness and metastasis in tumor-bearing mice treated by the VEGFR2 blocking monoclonal antibody DC101;

(ii) persistent invasive phenotype after cessation of the treatment.

Similar results were reported with sunitinib and SU10944 administration. Paez-Ribes et al. demonstrate that these effects appear to be an adaptive/evasive response by tumor cells themselves involving an augmented invasive phenotype and, in some cases, increased dissemination and the emergence of distant metastasis. They implicate hypoxia in the adaptive response [123].

These studies indicate divergent effects of anti-VEGF agents on primary tumor growth and metastasis and raise the possibility that both induction and suppression of tumor angiogenesis can exert proinvasive/prometastatic effects [122–124].

7.3. Pharmacoeconomics. As health care costs continue to increase, chemotherapy agents, and in particular targeted therapies, have been scrutinized regarding the populations in which they should be used to minimize the societal impact of their utility. Using an intentionally oversimplified cost-effectiveness model comparing the three arms of GOG218 study, Cohn et al. demonstrated that the addition of bevacizumab to the adjuvant management of patients with advanced ovarian cancer is not cost effective and treatment with maintenance bevacizumab, while improving PFS, is associated with both direct and indirect costs [125]. Thus, the optimal duration of maintenance treatment with bevacizumab will also have to be evaluated, and pharmacoeconomic considerations will have to be addressed [9].

7.4. Other Challenges. Despite the advances, some other critical challenges in both clinical-pathologic and preclinical investigations are still ahead [11]. Lack of predictive markers and accurate predictors of therapeutic efficacy seems to be a major challenge of anti-VEGF therapy in ovarian cancer [9, 89, 126]. To date, there are no predictive biomarkers for response to bevacizumab which means that there is no preselection of patients who might benefit from therapy [89]. IL-8 and VEGF polymorphisms have been suggested as potential markers of clinical outcome after bevacizumab-based chemotherapy in refractory ovarian cancer [126]. Retrospective studies will be performed on blood and tumor to study VEGF levels and other angiogenic markers in blood and tumor to try and identify which patients might benefit from bevacizumab [89]. Clinically validated biomarkers by restricting the VEGF-targeted therapy to the selected patients will also reduce the rate of relevant complications, in particular gastrointestinal perforations. Since large placebo-controlled phase III trials are still missing, well-designed trials in which potentially important clinical effects of anti-VEGF agents are not ignored are highly required [9]. These clinical benefits may manifest as prolongation of survival, delay in progression of disease, reduction of tumor burden, alleviation of symptoms associated with the disease, and minimization of toxicities associated with the treatment of the disease [127]. Also, it has been learned that the mere presence of a particular target does not guarantee the therapeutic benefit of the relevant targeted therapy, and, due to the multiplicity and redundancy of the pathways, it is unlikely that inhibition of a single cascade will be highly effective [33, 128]. A better understanding of the relevant signaling pathways, targeting horizontal and vertical pathways, and unveiling the underlying mechanisms of resistance and complications are among the goals of the future studies [11, 98].

8. Conclusion

VEGF is one of the most potent effectors of physiologic and pathologic angiogenesis. The pathophysiology of ovarian cancer is extremely angiogenesis-dependent [9]. Highly expressed in ovarian cancer, VEGF represents an attractive therapeutic target and VEGF inhibitors promise to be of significant value in the treatment of ovarian cancer. Preclinical and clinical studies further support the utility of these approaches. The most promising and widely explored bevacizumab has been used in many clinical studies either as single agent or in combination with chemotherapy in women with resistant or recurrent ovarian cancer. However, lack of accurate predictors of therapeutic efficacy, primary or secondary resistance to the treatment, complications and side effects of the therapy, likely divergent effects of anti-VEGF therapy on primary tumor growth and metastasis, and pharmacoeconomic concerns are the major struggles in the clinical use of VEGF inhibitors in malignancies. Some of the goals to be targeted in future studies include improvement in clinical trial design so that potentially important clinical effects of these agents are not ignored, a more detailed comprehension of VEGF inhibition pathways and discernment of optimal combination therapy. Further investigations are warranted to identify predictive biomarkers required for "individualization" of VEGF-targeted therapy, and to precisely

clarify the mechanisms underlying the complications of the treatment.

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Review Article

Integrin-Mediated Cell-Matrix Interaction in Physiological and Pathological Blood Vessel Formation

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Physiological as well as pathological blood vessel formation are fundamentally dependent on cell-matrix interaction. Integrins, a family of major cell adhesion receptors, play a pivotal role in development, maintenance, and remodeling of the vasculature. Cell migration, invasion, and remodeling of the extracellular matrix (ECM) are integrin-regulated processes, and the expression of certain integrins also correlates with tumor progression. Recent advances in the understanding of how integrins are involved in the regulation of blood vessel formation and remodeling during tumor progression are highlighted. The increasing knowledge of integrin function at the molecular level, together with the growing repertoire of integrin inhibitors which allow their selective pharmacological manipulation, makes integrins suited as potential diagnostic markers and therapeutic targets.

1. Introduction

Invasive cancer is among the leading causes of death worldwide, and rates are still increasing, due to ageing and changes in lifestyle [1]. Cancer is a collective term for many diseases, rather than a single disease, with the common characteristic that tissue growth goes haywire [2]. Patients who have undergone cancer treatment show an increased risk of developing a second tumor, mainly due to the same risk factors that were responsible for the first tumor but also in part due to the treatment of the first tumor with mutagenic chemotherapeutics or radiation [3]. Therefore, new strategies for cancer treatment with as little as possible adverse side effects are needed that effectively eradicate the primary tumor and also do not increase the risk of recurrence.

A tumor initially grows without any connection to the vasculature until it reaches a critical size of about two mm in diameter. Then it remains in a dormant state, in which proliferation and apoptosis due to lack of oxygen, are in a dynamic equilibrium unless it develops in a well-vascularized region or is able to recruit its own vasculature. Hanahan and Weinberg have proposed six hallmarks of cancer, one of them being the induction of angiogenesis [4, 5]. For further growth, the tumor needs to hook up to the vascular system by forming neovessels.

During tumor progression, an angiogenic switch is activated causing a continuous neovessel formation emanating from the normally quiescent vasculature, which sustains tumor growth [6]. This process called tumor angiogenesis is a collective term that is generally used for all types of tumor neovascularization. In addition to vessel co-option and to endothelial cell (EC) sprouting, tumor vessels can also develop by intussusceptive or glomerular angiogenesis, or, in a way of vascular mimicry, even tumor cells themselves can form vessel-like hollow structures. These types of vessel formation can occur in parallel, and also gradual transitions are possible. Vessel formation by the latter types requires less energy than sprouting angiogenesis, is thus carried out faster, and usually can be observed in, for example, gliosarcoma multiforme, melanoma, and breast and colon cancer [7].

For neovessel formation, ECs need to migrate into a previously avascular region and to extensively remodel the extracellular matrix (ECM). In this process, integrins, which are cell adhesion receptors for various ECM proteins and immunoglobulin superfamily molecules, are the most important matrix receptors [8, 9]. Therefore, integrins are

appealing targets for cancer therapy using a variety of integrin-specific antagonists, ranging from endogenous antagonists over humanized or chimeric antibodies to peptides and small nonpeptidic compounds [10–12].

In this paper, based on the general assembly of blood vessels, the specific organization of tumor vasculature will be described, as well as the dynamic sequence of events by which a tumor gains access to the body's vasculature. In this context, the role of integrins and possibilities of their pharmacological manipulation are explored.

2. The Static Picture: The Extracellular Matrix of Blood Vessels

The tissue's ECM is a structure-shaping molecular scaffold and also a repository for cytokines and other growth factors [13]. Cells embedded in this matrix need to be supplied with oxygen and nutrients, signaling molecules need to be received and emitted, and metabolic waste products need to be disposed of. These tasks are optimally fulfilled by the cardiovascular system with its intricate and dynamic network of blood vessels. Depending on their functions, different types of blood vessels show special histological and molecular adaptations. The heart, as a double-acting pump, drives the blood circulation within the vasculature via the aorta through arteries and arterioles into capillaries, from where the blood flows back through venules and veins. Due to the prevailing pressure conditions, the body fluid is forced through the vessel wall to form the lymph, which then is drained by lymph vessels back to the blood circulation. Additionally, the vasculature serves as "highway" system for leukocytes to patrol the body during immunological surveillance and to quickly reach sites of inflammation. The vascular wall is capable of self-sealing upon smaller injuries, and leukocytes are able to penetrate the blood vessel wall in a complex interplay without any obvious vessel leakage. Pathologically, tumor cells capitalize the blood vessel system to disseminate from a primary tumor and to colonize distant organs where they develop metastases.

2.1. General Organization of the Vessel Wall. Histologically, the walls of blood vessels comprise three concentric layers, that is, tunica intima, tunica media, and tunica adventitia [14], which are separated by two sheet-like structures of ECM proteins. The membrana limitans interna and externa establish a border between tunica media and tunica interna and adventitia, respectively. These ECM sheaths tightly connect the cell layers of the vessel wall to form a functional unit, which becomes evident when too weak cell-matrix interactions lead to life-threatening aneurysms.

The tunica intima comprises a single layer of squamous ECs and lines the inner surface of all blood vessels. The tunica media, which is usually the thickest layer in arteries, is composed of mural cells, which are smooth muscle cells in larger blood vessels and pericytes in capillaries. The tunica adventitia finally interconnects the blood vessel with the surrounding connective tissue, and it is usually most prominent in veins.

In different vessel types, that is, arteries, arterioles, capillaries, venules, and veins, this general blueprint is modified corresponding to the respective functional requirements. For example, endothelia, which are continuous in most instances, can become fenestrated, as in exocrine or endocrine gland tissues, or even discontinuous, as in liver, spleen, or bone marrow, in order to facilitate the exchange of hormones or metabolites. Elastic and muscular arteries illustrate other examples for a modification of this general blueprint. In order to even the pulsatile blood flow coming from the heart, the proteins elastin and fibrillin are abundant in the tunica media ECM of elastic arteries, which is the direct cause for the vessel wall's elastic properties. Muscular arteries possess numerous concentric sheaths of smooth muscle cells. By means of vasoconstriction and vasodilation, they can distribute and direct the blood to different organs.

2.2. Extracellular Matrix in the Vessel Wall. The ECM of blood vessels together with their resident cells contributes to essentially all physiological functions of blood vessels and has been reviewed recently [15].

The subendothelial basement membrane (BM) compartmentalizes the vessel's single-layered endothelium from the vascular connective tissue. The molecular architecture of BMs has recently been reviewed [16–18]. Fibronectin, incorporated between endothelial and perivascular cells, is essential for blood vessel morphogenesis [19]. The presence of von Willebrand factor (vWF) is characteristic for the subendothelial BM, where also other BM proteins, such as the network-forming collagens IV and XVIII can be found, together with laminins, nidogens, and perlecan. Thirteen different collagens are present in the vascular wall [20, 21]. The network-forming collagen IV [22] plays a key role for the mechanical stability of the BM [23], which, especially in arterial regions of the circulatory system, has to withstand a considerable blood pressure.

In the tunica media of elastic and muscular arteries, covalently crosslinked supramolecular aggregates of elastin form concentric lamellae and fibers in a proportion of up to 50% of the vessel's dry weight and confer resilience to pulsatile blood flow [24–26]. Regions of the ECM that consist mostly of elastin are confined by EMILINs, that is, homotrimeric elastin microfibril interphase-located proteins [27]. Anchored to microfibrillar bridges of fibrillin-1 and fibulin-5 between these concentric elastin lamellae, vascular smooth muscle cells (VSMCs) are sandwiched in a fishbone-like pattern and thus can effectively regulate the vessel's caliber [25, 28–31]. Dependent on the vessel type, distinct fibulins are involved in the assembly of the ECM. While fibulin-1 is widespread and occurs in the BMs of all blood vessels, heart valves and septa, fibulin-3, and fibulin-4 occur in the walls of capillaries and larger blood vessels [32]. The innermost and outermost elastic lamellae are referred to as membrana limitans interna and membrana limitans externa, respectively. Between the elastic lamellae, type I and III collagens are deposited that bear tensile forces exerted on the vessels and limit their elastic dilatability. In contrast, in the interstitial connective tissue between the subendothelial membrane and the membrana limitans interna, type VI and

type VIII collagens are found [21, 33]. The connection of the membrana limitans interna to the subendothelial BM by type XVIII collagen is assumed [34]. Also type XVI collagen, which is produced by VSMCs and found close to both elastic microfibrils and fibrillar type I and type III collagens, may contribute to the connection between the elastic and collagenous phases of the ECM [35, 36], especially, as type XVI collagen contains a binding site for the major collagen receptor on VSMCs, integrin $\alpha 1\beta 1$ [37, 38].

The ECM of the tunica media is synthesized by VSMCs, which are all encapsulated by an (incomplete) BM containing the usual BM proteins, type IV collagen and laminins [33, 39]. Depending on microenvironmental cues, VSMCs can reversibly acquire distinct phenotypes, which can be characterized as either (i) contractile and differentiated or (ii) secretory, migratory, and less differentiated [37, 39]. Under physiological conditions, the contractile phenotype prevails, at which the VSMCs transduce forces on the pericellular matrix especially by the collagen-binding integrin $\alpha 1\beta 1$, by the laminin-binding integrin $\alpha 7\beta 1$ and by dystroglycan [37]. In contrast, in the secretory, proliferatory, and migratory phenotype, the integrin equipment of the VSMCs predominantly consists of the fibronectin receptor, $\alpha 5\beta 1$, and the integrins $\alpha 4\beta 1$ and $\alpha 9\beta 1$. Consistently, in the proximity of secretory VSMCs, the fibronectin splice variants V (IIICS) and EIIIA with binding sites for the integrins $\alpha 4\beta 1$, $\alpha 5\beta 1$, and $\alpha 9\beta 1$ are abundant [39]. In capillaries, scattered pericytes, each encapsulated by an own BM, stabilize the endothelium and its subendothelial BM [40–42].

The fibroelastic connective tissue of the tunica adventitia connects the blood vessel with the perivascular connective tissue. It is rich in versican, a glycoprotein, which can interact with fibrillin-1 [43], fibulin-1 [44], and fibulin-2 [45], as well as with other ECM molecules.

2.3. Receptors for ECM Molecules. To interact with their microenvironment and to spatiotemporally regulate their differentiation state, morphology, metabolism, and survival, cells are equipped with a variety of receptors for all the ECM molecules [13]. Integrins are the largest family of these receptors, and they mediate adhesion to collagens, laminins, and fibronectin. In addition, there are other receptors and coreceptors, such as the syndecans [46].

Binding to a wide variety of different ECM molecules and transmitting signals bi-directionally in an outside-in and inside-out manner, integrins constitute functional hubs, which, according to an interesting concept in network theory and systems biology, integrate networks of angiogenic signaling cues that orchestrate the behavior of ECs and VSMCs during angiogenesis [47, 48]. Thus, therapeutically targeting integrins as the operationally important circuit-integrating hubs rather than single pathways of the complex system may result in a more pronounced inhibition of angiogenesis [47].

ECs express the vitronectin receptors $\alpha v\beta 3$ and $\alpha v\beta 5$; moreover, on ECs and pericytes the following integrins are expressed: the collagen receptors $\alpha 1\beta 1$ and $\alpha 2\beta 1$, the laminin receptors $\alpha 3\beta 1$, $\alpha 3\beta 6$, and $\alpha 6\beta 4$, the osteopontin receptor $\alpha 9\beta 1$, and the fibronectin receptors $\alpha 4\beta 1$ and $\alpha 5\beta 1$ [49]. Pericytes additionally express the laminin receptor $\alpha 7\beta 1$, and the osteopontin receptor $\alpha 8\beta 1$, and integrin $\alpha v\beta 3$ is also expressed on glial cells [49].

As EC-derived tumors, angiosarcomas express the integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, and $\alpha 6\beta 1$, and in benign and malignant mesenchymal tumors as well as in the desmoplastic stroma of carcinomas, integrins $\alpha 1\beta 1$ and $\alpha 5\beta 1$ are widely distributed [50]. Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bind to the same ligand in the ECM and are VEGF-dependently upregulated on migrating ECs, and antagonists against both integrins inhibit VEGF-mediated angiogenesis without affecting the existing vasculature [51, 52]. Therefore, and against the background of gene ablation studies, they are believed to differentially regulate angiogenesis [49]. Important coreceptors for integrin $\alpha 2\beta 1$ are the syndecans-1 and -4, which weaken the invasiveness of tumor cells into a collagenous matrix [53].

Cells bind to fibronectin and vitronectin preferentially via the RGD-dependent integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ [54]. Fibronectin can also be bound by the leukocyte-specific integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ [55]. Cell-fibronectin interactions are modulated by proteoglycans, glycoproteins of the ECM, and the coreceptors syndecans [56].

Integrin $\alpha v\beta 3$ was identified as a marker for angiogenic vascular tissue [57]. In contrast to quiescent ECs, integrin $\alpha v\beta 3$ is highly expressed on activated ECs during tumor angiogenesis, as well as on some tumor cells [58, 59]. In the tumor microenvironment, angiogenic ECs can interact due to their increased levels of the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ with provisional matrix proteins, such as vitronectin, fibrinogen, vWF, osteopontin, and fibronectin. Also, partially proteolyzed collagen in the tumor exposes RGD sites and is a further ligand for integrin $\alpha v\beta 3$ [60]. Thus, the ECM of the tumor microenvironment both provides survival signals and facilitates invasion. Integrin-$\alpha v\beta 3$-mediated adhesion to platelets protects malignant cells from clearance through the immune system, and moreover, $\alpha v\beta 3$ integrin also helps tumor cells to adhere to the vessel endothelium and to spread into adjacent tissues [61].

The pharmacological inhibition of integrin-$\alpha v\beta 3$-mediated cell-matrix interaction impedes tumor angiogenesis and growth [62], as does a replacement of the $\beta 3$ subunit with a mutated nonphosphorylatable subunit in a murine model [63], which provides evidence for a proangiogenic role of integrin $\alpha v\beta 3$, in contrast to integrin $\alpha v\beta 5$, which does not seem to play an essential role in angiogenesis [64]. Interestingly, the analysis of αv-knock-out mice revealed that, despite being embryonic or perinatally lethal, the vascular endothelium was not impaired in the absence of the αv subunit, whereas the primary cause of death was brain hemorrhage [65–67]. Also endothelial Tie-2-specific knockout of the αv subunit did not result in any vascular or angiogenesis defect [67]. Moreover, in an integrin subunit $\beta 3$- and also $\beta 5$-deficient mouse model, pathologic angiogenesis and tumor growth are increased [68]. A possible cause for these seemingly contradictory phenomena could be a relief of a transdominant inhibition by $\alpha v\beta 3$ on other integrins or other molecules, which would enhance their proangiogenic function [69, 70]. Likewise, there could be a compensatory role of other integrins with overlapping function [49]. Moreover, inhibition could also stabilize the

integrin $\alpha v\beta 3$ in its unligated conformation and thus induce apoptosis by triggering an integrin-mediated death program [71].

Integrin $\alpha v\beta 8$ is important for vascular development in the embryonic brain and in the yolk sac [72]. It is expressed on astrocytes but not on ECs or pericytes, nevertheless plays an important role in angiogenesis, as it binds in addition to several ECM proteins also to the latency-associated peptide (LAP) of TGFβ1, which in cooperation with the membrane-type metalloproteinase MT1-MMP/MMP14 results in activation of TGFβ and triggering of its downstream signal cascades [73–75].

Collagen IV, an essential component of BMs, is bound by integrin $\alpha 1\beta 1$, which is expressed on mesenchymal cells and can also bind to other collagens [76, 77]. Further collagen-binding integrins are $\alpha 2\beta 1$, the main receptor for fibrillar collagens, which is expressed on epithelial and some mesenchymal cells as well as on thrombocytes [78], $\alpha 10\beta 1$ in cartilage [79], and $\alpha 11\beta 1$, a key receptor for fibrillar collagen on fibroblasts [80]. The integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are involved in the regulation of collagen and MMP synthesis and thus of special importance for ECM turnover [81–83]. Discoidin domain receptors DDR1 on epithelial cells and DDR2 on mesenchymal cells are further collagen receptors with tyrosine kinase function and are relevant for cancer [84]. Other collagen receptors are glycoprotein GPIV on platelets [85], the leukocyte-associated immunoglobulin-like receptor LAIR-1/CD305 [86], and the urokinase-type plasminogen activator receptor-associated protein uPARAP/Endo180, which is involved in matrix turnover during malignancy [87].

Laminin, as a further integral component of BMs, is bound by the integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 7\beta 1$ [88–91] and also by α-dystroglycan [92, 93] and by the 67 kDa laminin receptor 67LR [94]. 67LR is increased in various tumors and correlates with their metastatic potential [95, 96]. The different laminin receptors may also act cooperatively in laminin binding, for example, laminin-binding $\beta 1$ integrins and 67LR [97] or integrin $\alpha 6\beta 4$ and syndecan 1 [98].

Integrin $\alpha 3\beta 1$, which in the vascular wall binds to laminins-411 (laminin 8) and-511 (laminin 10), thrombospondin (TSP), TIMP2, tetraspanin CD151, and to the C-terminal domain of the collagen IV $\alpha 3$ chain, is controversially ascribed either a positive or a negative role in angiogenesis (cf. [99]).

There is controversy whether the hemidesmosomal integrin $\alpha 6\beta 4$, which is expressed on a subset of ECs [100] and on tumor ECs [101], aggravates pathological angiogenesis [101] or whether it is a negative regulator of angiogenesis that is downregulated at its onset [102].

Thus, many molecules of the ECM scaffold, for example, laminins, collagens, fibronectin, and vitronectin, are ligands for integrins that link the cell's cytoskeleton to the ECM. Loss of this matrix-integrin contact triggers apoptotic cell death [103]. Picking up signals from the cell's microenvironment, integrins functionally sense, interpret, and distribute information, which allows the cell to modulate its proliferation, differentiation, migration, and shape [104]. The modulatory and regulating function of integrins is emphasized by direct interaction with a multitude of proteins, such as MMPs, uPA/uPAR, tissue inhibitor of matrixmetalloproteinase-2 (TIMP-2), vWF, TSP-1, osteopontin, syndecan-1, insulin-receptor substrate-1 (IRS-1), cytohesin-1, integrin cytoplasmic domain-associated protein-1 (ICAP-1), integrin-linked kinase (ILK), calcium- and integrin-binding protein (CIB), $\beta 3$-endotoxin, talin, actinin, tensin, nischarin, and the Ras-related protein Rab 25 [9].

The subendothelial BM of the tunica intima serves as a mechanical support to which ECs are anchored by various adhesion molecules, especially integrins [46, 105–108]. Additionally, the subendothelial BM provides microenvironmental information that regulate the metabolic activity of attached ECs, such as their production of leukocyte adhesion molecules [107] or antithrombotic prostacyclins [109], as well as other properties, for example, the tightness of intercellular contacts [108]. Therefore, angiogenesis is regulated not least by integrins which are adhesion receptors for matricellular proteins, ECM proteins, and immunoglobulin superfamily molecules, on nearly all cells including ECs [8, 58].

In addition to their mechanical function [110], integrins also assist growth factor receptors and play important roles in signaling processes, in particular as soluble growth factors, and other signaling molecules are bound by integrins as well [111]. For example, the proangiogenic VEGF-A_{165} is bound by integrins $\alpha v\beta 3$ and a$3\beta 1$ [112] and also by the tenascin-C- and osteopontin-receptor integrin $\alpha 9\beta 1$ [113]. The latter integrin, furthermore, binds the lymphangiogenic growth factors VEGF-C and VEGF-D [114]. Angiopoietins-1 and -2 are bound by integrin $\alpha 5\beta 1$ [115]. Integrin $\alpha 6\beta 1$ is a receptor for the proangiogenic CCN-family member CYR61, and is involved in *in vivo* in tube formation [116, 117]. The fibronectin receptor integrin $\alpha v\beta 3$, which is the best-studied integrin in relation to angiogenesis and is upregulated during wound healing and retinal vascularization and especially on tumor blood vessels, also binds to fibroblast growth factor FGF-1 [118]. Semaphorin 7A binding is also reported for the collagen receptor integrin $\alpha 1\beta 1$ [119].

Stimulated by PDGF, vascular smooth muscle cells express the laminin receptor integrin $\alpha 7\beta 1$, which plays an important role in recruitment and differentiation of VSMCs [120, 121].

Integrin $\alpha 9\beta 1$ is not only involved in lymphangiogenesis [114] but also plays a role in EC adhesion [122]. While binding of TSP-1 to integrin $\alpha 9\beta 1$ promotes angiogenesis [123], VEGF-A is another ligand of integrin $\alpha 9\beta 1$ [113].

2.4. Vascular-Relevant Integrin-Deficient Mouse Models. The crucial involvement of integrins in EC biology has been elucidated substantially by the examination of genetic knock-out studies [124]. By ablation of the respective genes, the EC integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, $\alpha 9\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ and also the VSMC integrin $\alpha 7\beta 1$ and the glial cell integrin $\alpha v\beta 8$ have been implicated in regulation of cell growth, survival, and migration during angiogenesis (for recent reviews of the findings from knock-out mice cf. [8, 10]). However, due to redundancy and compensatory

mechanisms, the interpretation of knock-out results is often difficult.

Itgb1−/− mice die at E5.5 before they start to develop their vasculature [125, 126]. Mice with a conditional knock-out in Tie-2-positive ECs survive until E9.5–E10.5, and they are capable of vasculogenesis, but their angiogenesis is disturbed showing defects in sprouting and branching [127–129]. Another endothelial-specific knockout of the integrin $\beta 1$ subunit is mediated via VE-cadherin-Cre recombinase and becomes manifest later in embryogenesis resulting in lethality between E13.5 and E17.5 [130]. In this mouse model, loss of $\beta 1$ integrin leads to a decreased expression of the cell polarity gene PAR3 and thus to disruption of EC polarity and lumen formation [130].

Itga1−/− mice, deficient for the collagen-binding integrin $\alpha 1\beta 1$, show a normal vascular development and a reduced tumor angiogenesis in adulthood, which has been attributed to increased MMP activity [131], while $\alpha 2\beta 1$-deficient Itga2−/− mice show an enhanced tumor angiogenesis in adulthood, but an otherwise normal vascular development [131, 132], and integrin $\alpha 2\beta 1$ is involved in the PlGF-dependent regulation of VEGFR-1 [132]. Although integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bind to the same ligand in the ECM, their differential knockout results in opposing effects on angiogenesis, suggesting a regulatory role for this pair of integrins.

Da Silva and coworkers generated EC-specific conditional $\alpha 3$ integrin knock-out mice and showed that these mice, in contrast to a global ablation, are viable and fertile but display enhanced tumor growth, elevated hypoxia-induced retinal angiogenesis and tumor angiogenesis, and increased VEGF-mediated neovascularization [99]. The authors also could show that $\alpha 3\beta 1$ is a positive regulator of EC-derived VEGF, which again represses VEGFR2 expression. Their data demonstrated that endothelial $\alpha 3\beta 1$ negatively regulates pathological angiogenesis and implicated an unexpected role for low levels of EC-derived VEGF as an activator of neovascularization.

Itga4−/− mice, deficient for fibronectin- and VCAM1-binding integrin $\alpha 4\beta 1$, are embryonic lethal with 50% dying at E9.5–10.5 due to failure of chorion-allantois fusion and 50% dying at E11.5 due to cardiovascular defects [55].

Mice, which by ablation of Itga5 are deficient for the fibronectin receptor integrin $\alpha 5\beta 1$, show normal vasculogenesis but no angiogenesis, which results in embryonic lethality at E10-11 due to defects in posterior somites, yolk sac, and embryonic vessels [133, 134]. This demonstrates the requirement of the integrin $\alpha 5$ subunit during embryonic development of early blood vessels and other tissues. Accordingly, integrin $\alpha 5\beta 1$, which is poorly expressed on normal quiescent ECs, is markedly upregulated during tumor angiogenesis [135].

Among the laminin-binding integrins, integrin $\alpha 6$ is not essentially required for vascular development, although $\alpha 6$-deficiency is lethal with skin blistering defects resembling epidermolysis bullosa [136]. In line with the $\alpha 6$ knock-out mice, Itgb4−/− mice, lacking a functional laminin-binding integrin $\alpha 6\beta 4$ by deletion of its signaling domain, show normal vascular development, although with reduced angiogenesis [101], but die of severe skin defects [100]. In neovascularization, the endothelial expression of integrin $\alpha 6\beta 1$ is downregulated [102]. While it is not required for EC proliferation and survival, it promotes tumor angiogenesis [101]. In contrast, genetic ablation of $\alpha 7\beta 1$, which is expressed on VSMCs but not on ECs, leads to incomplete cerebral vascularization and hemorrhage and also to placental vascular defects, which results in partial embryonic lethality and demonstrates that integrin $\alpha 7\beta 1$ is important for recruitment and survival of VSMCs [121, 137].

Deletion of Itga8 resulting in lack of integrin $\alpha 8\beta 1$, a receptor for fibronectin and tenascin, results in partial embryonic lethality, but no defects in vascular development (Müller and Reichardt, cited in [138]).

Itga9−/− mice lacking integrin $\alpha 9\beta 1$, which is the receptor for tenascin-C, osteopontin, VCAM-1, and also for VEGF-A, -C, and -D [113, 114], have defects in large lymphatic vessels and die postnatally at P8-12 from a bilateral chylothorax [139].

Ablation of Itgav, resulting in simultaneous loss of the two integrins $\alpha v\beta 5$, a receptor for vitronectin, osteopontin, and Del-1 (developmental locus 1), and $\alpha v\beta 3$, a receptor for a variety of ECM proteins, such as fibronectin, vitronectin, laminins, fibrinogen, fibrin, TSP, tenascin-C, vWF, denatured collagen, osteopontin, MMP-2, Del-1, bone sialoprotein, FGF-2, thrombin, and CCN1 (cystein-rich protein 61), leads to 80% embryonic lethality at E9.5, and the other 20% die at P0 with brain hemorrhage [65]. On the other hand, Itgb3−/− mice, which are just integrin-$\alpha v\beta 3$ deficient, show 50% embryonic and early postnatal lethality and an enhanced angiogenesis in surviving adult animals, indicating that this integrin is not strictly required for vascular development [140]. Surprisingly, animals with an intact but nonfunctional $\beta 3$ integrin subunit develop normally but show defects in angiogenesis in adulthood [63]. In contrast, Itgb5−/− animals lacking integrin $\alpha v\beta 5$ develop normally and angiogenesis is not significantly affected, indicating that this integrin is not mandatory for vascular development [64]. Integrins $\beta 3$ and $\beta 5$ doubly deficient mice show enhanced tumor growth and angiogenesis. This strongly suggests that these integrins are not required for vascular development or for pathological angiogenesis, pointing out that the mode of action of $\alpha v\beta 3$ antagonists and antiangiogenic therapeutics is still insufficiently understood [68]. Ablation of Itgb8 leads to the loss of integrin $\alpha v\beta 8$ on glial cells and thus to disrupted blood vessel formation in the brain, thereby demonstrating that this integrin is mandatory for brain's blood vessel development [72]. Moreover, the phenotype of $\beta 8$-deficient mice resembles that of αv-deficient mice, which provides evidence that most defects in αv-deficient mice are due to the loss of integrin $\alpha v\beta 8$ [72].

2.5. Integrin Structure. The family of integrins contains 24 structurally related N-glycosylated heterodimeric proteins assembled noncovalently from 18 α-subunits and eight β-subunits. Each subunit comprises a large extracellular domain, a single transmembrane domain, and with the exception of the $\beta 4$ integrin subunit, a short noncatalytic cytoplasmic tail [141]. Integrins are of special importance as

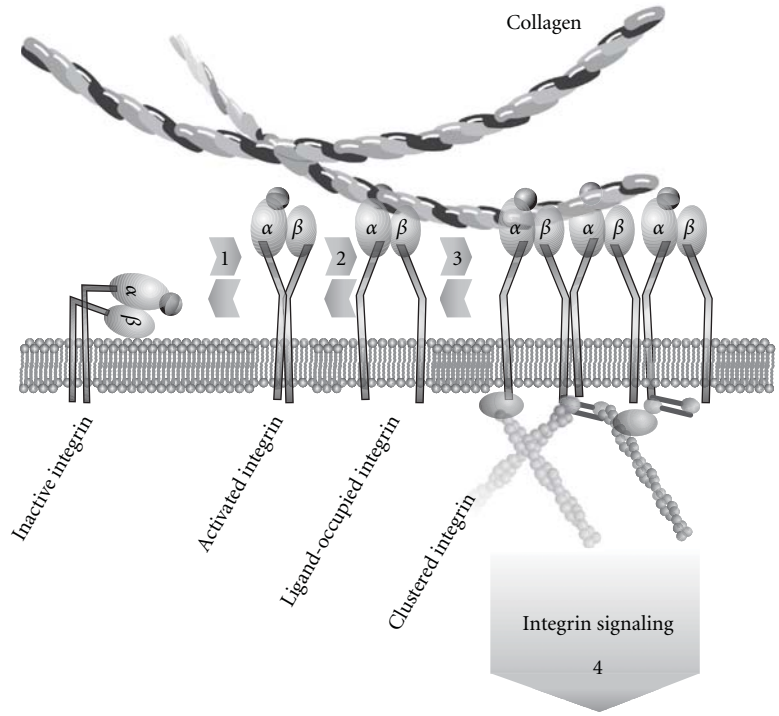

Figure 1: Integrin activation. Integrins are a family of heterodimeric transmembrane adhesion receptors that bidirectionally relay signals with the extracellular matrix (ECM) and also with other cells. When activated, a conformational change increases the affinity, and clustering increases the avidity towards the ligand. (1) By inside-out signaling, integrins can reversibly undergo a conformational change from a bent inactive to an upright activated conformation with intermediate ligand affinity, at which the cytoplasmic domains are still close together. (2) Upon ligand binding, the integrin adopts a high-affinity conformation with a concomitant parting of the legs and a separation of the cytosolic α- and β-tails that unlocks docking sites for cytosolic molecules. (3) Clustering of ligand-occupied and activated integrins establishes a mechanical link between ECM and cytoskeleton and leads to the recruitment of scaffolding molecules and kinases. (4) The assembly of focal adhesions triggers intracellular signaling cascades. Details can be found in the text.

they mediate cell matrix crosstalk via both outside-in and inside-out signaling [54, 142]. Moreover, the 24 different integrins possess promiscuous and redundant ligand specificities, which is of importance when distinct signals are to be transduced or when in a particular context a defined cellular response is elicited, as is discussed by Rüegg and Alghisi [11].

Integrin structure and function have been studied in detail at the molecular level [143, 144]. The extracellular headpiece is formed by a disk-like propeller domain of the α subunit and globular domains of the β subunit [145, 146]. The joint globular head harbors the ligand-binding site [146, 147]. The crystal structure of the integrin-$\alpha v\beta 3$-binding site with an inserted RGD ligand [148] helped to map functional amino acid residues on other integrins [149]. Recently, the binding pocket of integrin $\alpha 5\beta 1$ has been mapped by swapping regions of zebrafish and human $\alpha 5$ subunit in a gain-of-function approach [150].

2.6. Integrin Signaling. Depending on their activity, integrins adopt distinct conformations (Figure 1). In the inactive resting conformation, the headpiece of the heterodimer bends towards the plasma membrane, and the transmembrane domains of the α and β subunits are associated [146]. Upon ligand binding, the previously bent integrin ectodomain adopts an activated upright conformation [106, 151]. This conformational change is conveyed through the transmembrane domains towards the cytoplasmic tails [54, 105, 152], where cytoskeletal proteins and signaling molecules relay the incoming signal intracellularly [153]. In inside-out signaling, the binding of intracellular molecules, such as talin or kindlins [154, 155], to the cytoplasmic integrin tails leads via a separation of the transmembrane domains [156] to a switch blade-like erection of the extracellular domains [147, 157, 158]. Likewise, in outside-in signaling, ECM ligand binding to the integrin headpiece also induces a conformational change in the hybrid domain and thereby a separation of the integrin subunits' legs [144]. This parting of the legs separates the cytosolic tails and allows binding of cytosolic proteins and thus clustering of integrins and formation of focal adhesion sites (Figure 1).

By clustering into focal adhesions, integrins recruit talin, paxillin, α-actinin, tensin, and vinculin and thereby mechanically couple the ECM scaffold to the actin cytoskeleton. Additionally, integrins bind scaffolding molecules, such as p130 CRK/BCAR1, and recruit and activate kinases, such as focal adhesion kinases (FAKs), Src family kinases (SFKs), and integrin-linked kinase (ILK), the latter forming a complex with the adapter molecules parvin and PINCH/LIMS1 [159].

In addition, tetraspanins can recruit integrins to membrane microdomains, thus regulating integrin function [160]. Thereby, the rather unstable nascent adhesions are transformed into focal complexes, focal adhesions, fibrillar adhesions, or podosomes. This clustering of integrins leads to a reorganization of the plasma membrane around the focal adhesion into caveolin-containing lipid rafts, to which also growth factor receptors often localize, and to the assembly of adhesion signaling complexes [161–163]. This allows a regulation of growth factor signals by integrin-mediated caveolae trafficking [164, 165]. In the assembly of such integrin adhesions, up to 156 distinct molecules, amongst other adaptor proteins, kinases, and phosphatases, are involved [48, 163]. Membrane lipid-protein interactions that modulate the homo- or heterotypic association of receptor molecules in the cell surface, or between adjacent cells, have been reviewed recently [166]. From the focal adhesion sites signal pathways diverge that regulate diverse cellular programs, such as adhesion, migration, proliferation, and survival. To provide an overview, integrins generally relay their signals via the FAK, ERK, and NF-κB pathways [153].

In most cases, in mechanosensory signaling FAK, Src, and SH2, domains containing protein tyrosine phosphatase 2 (SHP2) are involved [167]. Upon integrin binding, FAK autophosphorylates and binds to Src, which further phosphorylates FAK and several downstream binding partners, amongst others, JNK and Rho [168–170].

Activated FAK also recruits PI3K, which mediates the activation of AKT and procures integrin-mediated cell survival, and likewise the antiapoptotic AKT can be activated via Ang-1 [171]. Moreover, signals relayed via integrins and Src can be integrated by FAK with growth factor receptor-relayed signals via Ras, MEK, and MAPK [172]. Growth factors can activate Ras signaling independently from integrin-relayed adhesion signals. Nevertheless, MEK1 and Raf1 are important interfaces between integrin-relayed and growth-factor-relayed signaling, because both MEK1 and Raf1 need to be activated via adhesion-mediated activation of Src and FAK in order to activate MAPK [173, 174].

An endothelial-specific ablation of FAK results in impaired blood vessel development and embryonic lethality [175] Downstream of FAK, Src couples integrin-mediated and VEGF-receptor-mediated proangiogenic signaling in ECs [176–178]. However, endostatin can also activate Src via integrin $\alpha5\beta1$ and thereby disassemble actin stress fibers and focal adhesions and thus inhibit cell migration, which is regulated by integrins via the Ras/ERK pathway [179–181]. Important for adhesion and migration of endothelial and VSMCs are also p130Cas and PLC-γ, which can interact with FAK [182–185].

PI3K is of pivotal importance for angiogenesis, because its deletion results in embryonic lethality E9.5 to E10.5, when angiogenesis is important for vascular development. PI3K deletion also causes decreased Tie-2 expression and thus creates a phenotype resembling Tie-2 deficiency [186, 187]. Moreover, EC-specific deletion of the PI3K isoform p110α impairs angiogenesis [188]. In ECs, adhesion via integrins elicits a survival signal via FAK/PI3K/mTOR/4E-BP1 and Cap-dependent translation [189]. Furthermore, the activation of PI3K by Ras is important for lymphangiogenesis [190].

In addition to a direct activation of ERK, integrins can also activate a Raf/MEK/ERK signaling cascade in ECs [189, 191, 192]. Raf-deficient and MEK-deficient mice have severe vascular defects [193, 194]. Growth-factor-mediated ERK signaling is linked with integrin-mediated signaling via FAK [195]. Integrin-mediated ERK signaling is important for cell proliferation and migration of ECs [191, 196]. Integrin $\alpha1\beta1$ is unique among the collagen-binding integrins because it promotes cell proliferation by activating the Ras-Shc-MAPK pathway, and cell cycle progression is regulated via FAK, Rac, and cyclin D by integrin-mediated adhesion and matrix stiffness [197–199].

Integrins can also activate the NF-κB pathway in ECs and protect them from apoptosis [200–202]. Additionally, NF-κB signaling regulates the expression of cyclooxgenase-2 (COX-2), which again is involved in EC spreading and migration and in the induction of VEGF and FGF-2 [177, 203, 204]. However, inhibition of the NF-κB pathway increases angiogenesis pathologically [205].

Integrins alone are not oncogenic, but some oncogenes may depend on integrin signaling for tumor growth and invasion. For example, integrin-triggered FAK signaling is essential for Ras- and PI3K-mediated oncogenesis [206, 207]. Also the expression of the cancer stem cell marker CD44 is integrin-regulated, and it can be speculated that integrin-relayed signals are needed to maintain a cancer stem cell population [12, 208]. On the other hand, there is evidence that the collagen receptor integrin $\alpha2\beta1$ has a tumor-suppressing function [209, 210].

Ligated integrins promote survival, whereas unligated integrins recruit caspase-8 to the plasma membrane and promote apoptosis in a process termed integrin-mediated death [71, 211], which differs from anoikis induced by loss of cell adhesion to the ECM [103, 212]. Loss of caspase-8 confers resistance to integrin-mediated death of tumor cells, and unligated integrin $\alpha v\beta3$ promotes the malignancy of such tumors [213, 214]. Cell survival is promoted by integrin ligation-dependent upregulation of BCL2 and FLIP/CFLAR, activation of the PI3K-AKT pathway, NF-κB signaling, and p53 inactivation [176, 202, 215–217]. Survival is also promoted by crosstalk between integrins and growth factor receptors, for example, $\alpha v\beta3$ and FGFR or $\alpha v\beta5$ and VEGFR2 [195, 218].

In various steps of angiogenesis and tumor progression, crosstalk between integrins and growth factor receptors on tumor cells and also on host cells is important. This crosstalk can consist in either an activation of a latent growth factor, a regulation of common pathways for signaling or internalization and recycling, a collaborative or a direct activation, or also a negative regulation [111]. The outcome of a growth factor signal in a particular context is often determined by a synergistic and reciprocal interaction of integrins with growth factor receptors, such as tyrosine kinase receptors like VEGFRs and Tie-2, Met, and FGFR, and semaphorins regulate integrin function as well [111, 219–221]. A complex of VEGF with the fibronectin heparin II domain increases, upon cell binding via integrin $\alpha5\beta1$ and

the signaling via VEGFR2 synergistically [222]. Expression of integrin $\alpha 11\beta 1$ on tumor-associated fibroblasts has a tumor-promoting effect, because it upregulates the expression of insulin-like growth factor 2 (IGF2), which is another example of integrin-regulated growth factor signaling [223].

Beside binding ECM proteins and thus regulating adhesion and migration, integrins can also directly interact with pro- and antiangiogenic factors [221]. Integrin $\alpha 5\beta 1$ can bind to matrix-bound VEGFR-1 [224]. In addition, integrin $\alpha 9\beta 1$ can directly interact with VEGF-A, -C, and -D and also with hepatocyte growth factor (HGF) [113, 114, 225]. Moreover, integrin $\alpha 3\beta 1$ and $\alpha v\beta 3$ bind VEGF-A_{165} and VEGF-A_{189} [112]. FGF is directly bound by integrin $\alpha v\beta 3$ [226]. Angiopoietins also can directly interact with many integrins [115, 221, 227, 228].

In the context of a hypoxic tumor microenvironment, it is especially interesting that the expression of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ is upregulated by VEGF [51].

3. The Dynamic Process: Connection of a Tumor to the Host Vasculature

Angiogenesis is an important step in the metastatic cascade, which not only provides the tumor with nutrients but also is a route for dissemination. An important trigger for this is hypoxia [229].

3.1. An Angiogenic Switch Triggers the Angiogenic Cascade. In avascular tissue regions, an oxygen diffusion limit of about 150 μm restricts tumor growth to just a few millimeters in diameter. Thus, in this prevascular phase of tumor dormancy, there is a dynamic equilibrium between proliferation and hypoxia-induced apoptosis [230]. The dormant phase ceases when a tumor recruits its own vasculature by the secretion of angiogenic factors into its environment [231], a process denoted as angiogenic switch [2, 6]. After this angiogenic switch is thrown, the tumor hooks up to the body's vascular system and thus resumes its growth.

In tumor development, the establishment of an angiogenic phenotype is a crucial and general step [232–234]. Depending on tumor type and environment, this induction of new vessel sprouting can occur at different stages of the tumor progression pathway, and it leads to exponential macroscopic tumor growth [2, 4, 6]. In addition, recent data indicate that angiogenesis also contributes to the microscopic premalignant phase of neoplastic progression [5].

Infiltration of bone-marrow-derived monocytes that differentiate into macrophages can trigger this angiogenic switch in spontaneous tumors by releasing both numerous proangiogenic cytokines, for example, VEGF, TNFα, IL-8, and bFGF [235, 236] and MMPs (e.g., MMPs-2, -7, and -9) together with elastase and uPA [236]. These matrix-degrading enzymes loosen the avascular ECM for the angiogenic ingrowth of neovessels.

From the multitude of proangiogenic molecules, such as FGF-1 and -2, G-CSF, HGF, IL-8, PD-ECGF, PGE-1 and -2, PlGF-1, and -2, TGF-α and -β, TNF-α, and VEGF-A through E, only the VEGFs and PlGFs are specific for ECs [230]. VEGF-A, which exists in five splice variants, is the most intensively studied one [237]. Mediated by HIF-1, VEGF-A synergizes with FGF-2. VEGF is upregulated under hypoxic and hypoglycemic conditions prevailing within tumor tissue [230].

The role of chemokines in tumor angiogenesis and neovascularization has been reviewed recently [238]. Tumor cells express CCL2/MCP-1 (C-C-motif ligand 2/monocyte chemotactic protein-1), and thus, tumor-associated macrophages (TAMs) are recruited, resulting in an inflammatory response. These TAMs are again a source for angiogenic growth factors, such as, VEGF and FGF-2 [239, 240]. MCP-1 also mediates the recruitment of mural cells in an Ang-1-dependent manner in an *ex vivo* model [241].

Multiple sequential steps are required for angiogenesis to be successful and in all steps of this angiogenic cascade integrins, which mediate interactions of cells with surrounding insoluble ECM proteins, in addition to soluble growth factors, play an important role [15]. In a first step, the BM of an existing vessel is degraded by MMPs that are expressed by ECs, such as MMP-1, MMP-2, MMP-9, and MT1-MMP/MMP14 [242–244], at which MMP-9 is required for tumor vasculogenesis rather than angiogenesis [245]. Subsequently, cell-matrix contact influences the outgrowth of tip cells and the proliferation of stalk cells that thereupon form endothelial tubes [246]. A new BM is assembled by newly synthesized BM proteins. Finally, the newly generated capillaries undergo maturation, pruning, and expansion.

3.2. Tumor Vessels Can Arise by Different Types of Vessel Formation. During embryonic morphogenesis, endothelial precursor cells called angioblasts initiate the body's vasculature by forming tubes in a process called vasculogenesis. This is subsequently accompanied by sprouting (angiogenesis) of new vessels from already existing ones. Once morphogenesis is completed, the adult vasculature is largely quiescent, except for transient events, such as wound healing or menorrhea [247]. However, angiogenesis takes place under many pathological conditions, such as atherosclerosis, endometriosis, osteomyelitis, diabetic retinopathy, rheumatoid arthritis, psoriasis, and tumor growth [230]. During tumor progression, the quiescent vasculature becomes permanently activated to sprout new vessels that enable blood supply and thus help sustain tumor growth [5, 6]. Due to its increased metabolic rate, tumor tissue requires blood supply for expansive growth, which is circumstantiated by the observation that tumor cells, which are p53 deficient and thus show a reduced apoptosis rate, die beyond an oxygen diffusion limit in the range of 150 μm [248]. Tumor cells proliferate around the continuously formed neovessels which markedly differ from normal vessels in morphology and molecular composition [219, 249]. Tumor vasculature generally appears highly tortuous, chaotic, and disorganized. The vessels themselves are leaky due to a discontinuous endothelium, a poorly formed BM, and a lack of mural cells. In addition, tumor cells sometimes mimic ECs. This poor quality of tumor-associated blood vessels compromises blood flow, impairs drug delivery, and facilitates tumor cell intravasation leading to hematogenous or lymphatic metastasis. In addition to histological vessel malformations,

tumor vessels show an anomalous composition of their ECM, for example, tenascin-C and –W, and the oncofetal fibronectin ED-B splice variants are associated with tumor vessels [250, 251]. ED-B fibronectin is synthesized by neoplastic cells [252]. Melanoma and glioblastoma cells secrete tenascin-C as do cancer-associated fibroblasts (CAF) of most carcinomas [253]. Tenascin-C stimulates angiogenesis in ECs, mediates survival of tumor stem cells, enhances proliferation, invasiveness, and metastasis in tumor cells, and blocks immunosurveillance [250, 253]. Tenascin-W is more strictly associated with tumorigenesis and can be used as a tumor biomarker for breast and colon cancer, because it is undetectable in healthy stroma but overexpressed in the tumor stroma [254, 255].

Vascularization mechanisms in cancer have been reviewed recently [256, 257]. New tumor blood vessels can either arise by vessel co-option or be formed by tumor angiogenesis, but there is also evidence for vasculogenesis or recruitment of circulating bone-marrow-derived endothelial progenitor cells that differentiate into ECs [230, 258–260] (Figure 2(A)). Depending on the tumor type, tumor blood vessels build different and characteristic vascular beds, and, according to the function of the vascular bed and the osmotic pressure of the surrounding tissue, endothelia represent highly heterogeneous "vascular addresses" [230]. Tumor vessels constantly change their shape due to persistent growth, and about 30% of the vasculature comprise arteriovenous shunts bypassing capillaries. The concomitant poor perfusion leads to hypoxia of ECs, which consequently synthesize more proangiogenic molecules and thus crank tumor angiogenesis [230].

3.2.1. Endothelial Sprouting. Endothelial sprouting can be triggered by hypoxia, hypoglycemia, and inflammatory or mechanical stimuli, such as blood pressure, and is regulated by many angiogenic growth factors, such as VEGF, and matrix proteases. When neovessels sprout from capillaries, pericytes are selectively lost, and upon receiving an angiogenic stimulus, select ECs differentiate into tip cells that invade the avascular ECM (Figure 2(B)). These tip cells migrate into the ECM following the stimulatory gradient. Behind the tip cells, other ECs begin to proliferate and, as stalk cells, form cord-like structures. These develop into endothelial tubes [130, 261, 262] that subsequently anastomose and thus allow blood flow. Finally, pericytes and smooth muscle cells are recruited, a new BM is synthesized, and the ECs become quiescent again.

The molecular background of capillary sprouting and the key role of VEGF have been reviewed by Carmeliet [231]. Upon a hypoxic stimulus, VEGF is produced, and as a consequence the endothelium's permeability is increased and the BM loosened by the activity of MMPs [243, 263] and the urokinase plasminogen activator system [264]. The MMP inducer EMMPRIN/CD147 also upregulates soluble VEGF isoforms 121 and 165 and VEGFR-2 on ECs and thus promotes sprouting angiogenesis [265]. Integrin $\alpha v\beta 3$ mediates migration into the fibrin-rich cancer stroma and furthermore can associate with MMP-2, thus enabling ECs to maintain the BM in the sol state and to promote tumor

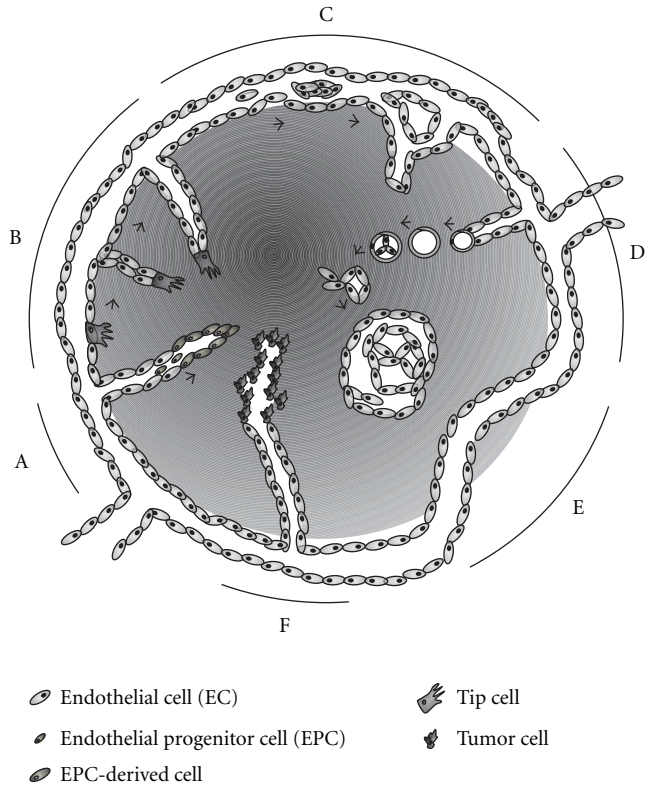

Figure 2: Diverse types of vessel formation. Tumor neovascularization can take place by distinct types of vessel formation, which can proceed simultaneously and also merge seamlessly. (A) Neovessel formation by recruitment of bone-marrow-derived endothelial progenitor cells. (B) Sprouting angiogenesis is initiated by the differentiation of an EC into a migratory but nonproliferating tip cell. (C) Intussusceptive angiogenesis starts with the insertion of a connective tissue pillar into a preexisting vessel, and the vessel is displaced as the pillar extends in size. (D) In glomeruloid angiogenesis, complex vascular aggregates of several closely associated vessels are formed. (E) Vessel co-option is the acquisition of host capillaries by the tumor. (F) In vascular mimicry, tumor cells can partly assume EC function and form vessel-like hollow structures. Arrows denote consecutive stages of vessel formation. Tumor tissue is depicted dark gray. See text for details.

cell invasion [266]. In addition to VEGF, FGF, PDGF, and PlDGF are involved, and Ang-2/Tie-2 signaling regulates the detachment of pericytes. Later, PDGF-BB recruits pericytes and smooth muscle cells to the newly formed EC tube, and TGF-β1 and Ang-1/Tie-2 stabilize the EC-mural cell interaction [231].

3.2.2. Intussusceptive Angiogenesis. Another way of tumor neovascularization is intussusceptive angiogenesis, which represents a nonproliferative and noninvasive mechanism for the enlargement of a capillary plexus by intussusceptive growth, arborization, and remodeling [267] (Figure 2(C)). As this mode of vascularization is mostly independent from EC proliferation and migration, as well as BM degradation, this process is more economical and, occurring within hours or even minutes, is noticeably faster than sprouting

angiogenesis [268]. It begins with the formation of transluminal pillars from the EC walls. Their subsequent expansion splits the preexisting vessel into two, thereby enhancing the vascular surface. In a subsequent process of arborization, the disorganized capillary network is remodeled into a functional tree-like structure by serial pillar formation. In a final remodeling step, the branching angles are modified, and the capillary network is pruned. The formation of new capillaries is initiated by sprouting angiogenesis that is later accompanied or followed by intussusceptive angiogenesis, which increases the EC surface [269]. Intussusceptive angiogenesis is synergistically regulated by VEGF and Ang-1, and it seems to be induced by laminar shear stress on the vessel walls, whereas oscillating shear stress favors sprouting angiogenesis [269].

3.2.3. Glomeruloid Angiogenesis. In many aggressive tumors, glomeruloid angiogenesis gives rise to complex vascular structures termed glomeruloid bodies, in which several microvessels together are ensheathed by a BM of varying thickness containing sparse pericytes [270] (Figure 2(D)). The frequency of occurrence of such glomeruloid bodies is an indication for the tumor's aggressiveness and the patient's survival [271]. The formation of such glomeruloid bodies is rather a remodeling than true angiogenesis, because proliferating and migrating tumor cells can actively pull capillaries of the surrounding host vasculature and adjacent capillary branching points into the tumor node. Thereby, formed coiled vascular structures develop subsequently into glomeruloid bodies that are connected to the surrounding vasculature via numerous narrowed capillaries [256].

3.2.4. Vessel Co-Option. Malignant cells can initially grow in the vicinity and along pre-existing microvessels and thus use the host vasculature for their own benefit (Figure 2(E)). This co-option of the host vasculature was originally believed to be limited to the initial phase of tumorigenesis [272]. Meanwhile, however, there is evidence that vessel co-option might persist during all stages of primary and metastatic growth of various tumors [256], for example, cutaneous melanoma, which appears to grow by co-opting the vascular plexus in its surrounding connective tissue, while there is no sign of directed vessel ingrowth [273].

Vessel co-option is regulated dependent on the tumor type and the host environment, but the key regulators are again VEGF and angiopoietins [272, 274]. Ang-1 binds to Tie-2 and thus triggers signaling cascades, assuring survival and quiescence of ECs, and thus causing tumor vessel maintenance, whereas the nonsignaling Tie-2 ligand Ang-2 acts as a negative regulator and destabilizes the capillary walls by detachment of pericytes [272, 274]. Subsequently, VEGF via its receptor VEGFR-2 promotes both survival of ECs and growth of new vessels [237, 275].

3.2.5. Vascular Mimicry. Aggressive melanomas can form fluid-filled vessel-like channels without any EC lining in a nonangiogenic process termed vascular mimicry [276] (Figure 2(F)). These channels allow perfusion independent of angiogenesis, and they can arise by two types of vasculogenic mimicry, designated the tubular and the patterned matrix type [277]. These tubular vessel-like networks resemble the pattern of embryonic vascular networks, and, in their gene expression pattern, aggressive tumors that form such channels resemble endothelial, pericytes, and other precursor stem cells, suggesting that tumor cells might disguise as embryonic stem-cell-like or other cell types [256]. Vasculogenic mimicry of the patterned matrix type looks completely different and is characterized by a fluid-conducting meshwork of extravascular patterned depositions of matrix proteins such as laminins, collagens IV and VI, and heparin sulfate proteoglycans that anastomose with blood vessels [277–279]. Although it is not yet elucidated how such channels are connected to the vasculature, the latter type of vascular mimicry has been reported for many cancers, such as breast, ovarian, and prostate carcinoma, melanoma, soft tissue sarcomas, osteosarcoma, and phaeochromocytoma [277, 280]. In aggressive melanoma, the expression of tissue factor pathway-associated genes, such as tissue factor (TF), TF pathway inhibitor-1 (TFPI-1), and TFPI-2, is upregulated, suggesting an anticoagulation mechanism in the channel-forming tumor cells [281]. Fluid propelled through these channels by a pressure gradient might facilitate the supply with nutrients and oxygen, and, additionally, this fluid-conducting network could substitute for a lymphatic vascular system and drain extravasated interstitial fluid in tumors that lack lymphatic vessels, for example, uveal melanoma [279, 280].

4. Manipulation of Cell Matrix Interaction in Tumor Angiogenesis

Cell-matrix interactions regulate signaling pathways that are intricately interconnected with cytokine-regulated pathways, which complicates the analysis of their contribution to a particular step in angiogenesis [153]. ECM receptors can be manipulated with a wide variety of different compounds ranging from endogenous compounds, such as matrikines, over their synthetic analogues and peptides mimicking only integrin-binding sites to function-blocking antibodies and small molecules with integrin inhibitory function. Other starting points for an antiangiogenic therapy are the inhibition of signaling cascades downstream of the ECM receptors or cytokine receptors and as a new avenue the blocking of microRNAs with antisense RNAs in ECs [282, 283]. An efficient antivascular cancer therapy can target either the angiogenic signaling pathways or the vascularization mechanism [256]. A combination of conventional chemotherapy with angiosuppressive or vascular disrupting therapy is often problematic and needs careful design [256].

4.1. Pharmacological Intervention of Integrin-ECM Interaction. In addition to soluble growth factors, such as VEGF, there are several endogenous angiogenesis inhibitors, for example, endostatin, endorepellin, and tumstatin, which share the common feature that they all are proteolytic fragments of ECM molecules [284, 285]. In tumor angiogenesis

within a primary tumor, such ECM fragments are generated by the release of MMPs, in order to degrade the BM. This results not only in labile and leaky tumor vessels but at the same time keeps metastases from growing, as these endogenous angiogenesis inhibitors are distributed via the blood stream [230]. Therefore, they are of pharmacological interest with regard to their use as angiogenesis inhibitors. Intensive efforts have been directed towards the development of integrin antagonists for the treatment of cancer and many other diseases, ranging from autoimmune diseases over inflammatory to thrombotic diseases, and their applications seem promising [11, 286]. Integrin-mediated interactions of cells with their surrounding ECM can be manipulated by antibodies, peptides, small nonpeptidic compounds, and endogenous inhibitors (Figure 3). Integrin antagonists with antiangiogenic activities have been reviewed recently with special emphasis on drugs that are in clinical trials [11].

Spurred by the success in pharmacologically targeting RGD-dependent integrins, there are also attempts to pharmacologically manipulate RGD-independent integrins, such as the collagen- and laminin-binding integrins, as reviewed recently [287]. The collagen-binding subgroup of integrins with their common A domain comprises interesting targets in the development of drugs against thrombosis, inflammatory diseases, and cancer. TSPs-1 and -2 are naturally occurring potent angiogenesis inhibitors, and their antiangiogenic effects can be imitated by short-peptide mimetics that among other targets bind to $\beta 1$ integrins [288, 289].

An endogenous inhibitor, which blocks the interaction of integrin $\alpha 1\beta 1$ with collagen I and also binds to heparan sulfate proteoglycans, is arresten, the C-terminal fragment of the collagen IV $\alpha 1$ chain [290, 291]. Endorepellin, a C-terminal fragment of perlecan specifically blocks the function of integrin $\alpha 2\beta 1$ [292] and interestingly also binds to endostatin, thus counteracting its antiangiogenic effect [293]. Additionally, integrin $\alpha 1\beta 1$ can be specifically inhibited with obtustatin from the snake venom of *Vipera lebetina obtusa* [294, 295]. The interaction of integrin $\alpha 2\beta 1$ with collagen can be specifically inhibited with the C-type lectin rhodocetin from the snake venom of *Calloselasma rhodostoma* [296, 297]. In addition, it can also be selectively antagonized by the protein angiocidin, which was first detected in lung carcinoma cells [298, 299]. The aromatic tetracyclic polyketides maggiemycin and anhydromaggiemycin from *Streptomyces*, which have been described as potential antitumor agents [300], inhibit collagen binding by blocking the A domain of the integrin subunits $\alpha 1$, $\alpha 2$, $\alpha 11$, and to a lesser extent $\alpha 10$ while cell adhesion to fibronectin, mediated by integrins $\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, is unaffected [301]. Recently, the sulfonamide derivative BTT-3016 has been described as a potent antithrombotic small-molecule inhibitor of integrin $\alpha 2\beta 1$ with only slight effect on other collagen-binding integrins and no effect on fibronectin- or vitronectin-binding integrins [302]. Another sulfonamide derivative, E7820, which does not interfere with integrin-ligand interaction, reduces integrin $\alpha 2$ expression on the mRNA level [303]. Angiogenesis can be inhibited with antibodies against the α subunits of the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, whereas quiescent vessels are not affected [230].

In a phase I clinical trial, endostatin, the C-terminal fragment of collagen XVIII, blocks the function of integrin $\alpha 5\beta 1$ [179, 304, 305] and also binds to heparin and with lower affinities to other heparan sulfate proteoglycans that are involved in growth factor signaling [306, 307]. Endostatin's antiangiogenic activity can also be mimicked with derived short non-RGD but arginine-rich peptides [308]. Integrin $\alpha 5\beta 1$ can also be blocked by the synthetic non-RGD peptides PHSCN, named ATN-161, [309] and cyclic CRRETAWAC [310], as well as by the peptide mimetics SJ749 [311] and JSM6427 [312], and it can be inhibited by the affinity-matured humanized chimeric monoclonal antibody M200/volociximab [313].

Angiostatin is a proteolytic fragment of plasminogen that effectively inhibits integrin $\alpha v\beta 3$ [314], and its antiangiogenic effect can also be achieved by its isolated kringle-5 domain [315]. Kringle-1 to 3 show the same antiproliferative effect as the whole angiostatin, but hardly inhibit migration, whereas kringle-4 inhibits EC migration but shows only a marginal antiproliferative effect [316]. Other endogenous integrin $\alpha v\beta 3$ inhibitors are the collagen XVIII fragment endostatin [304], and the C-terminal fragment of the collagen IV $\alpha 3$-chain termed tumstatin [317], which also binds to integrin $\alpha 6\beta 1$ [318]. Tumstatin has two binding sites for integrin $\alpha v\beta 3$. The N-terminal site mediates an antiangiogenic signal, whereas the C-terminal binding site is associated with the antitumor cellactivity [318, 319]. Canstatin, the NC1 domain of the collagen IV $\alpha 2$ chain, inhibits both integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ [320] and seems to interact with integrin $\alpha 3\beta 1$ too [321]. A hemopexin-like domain comprising C-terminal fragment of MMP-2, termed PEX, also antagonizes integrin $\alpha v\beta 3$ by preventing its binding to MMP-2 and thus inhibiting proteolytic activity on the cell surface, especially during vessel maturation [322, 323]. Fastatin and other FAS1 domains, which are present in the four human proteins periostin, FEEL1, FEEL2, and βhig-h3, also function via integrin $\alpha v\beta 3$ as endogenous regulators of pathogenic angiogenesis [324]. Next to these natural antagonists there is a variety of synthetic RGD—containing peptide inhibitors that mimic a motif that occurs on many ECM molecules, such as fibronectin, vitronectin, fibrinogen, osteopontin, TSP, vWF, and partially degraded collagen. Most integrins of the αv subfamily and the integrins $\alpha 5\beta 1$ and $\alpha IIb\beta 3$ bind to this motif. Therefore, adhesion and spreading of ECs to the ECM can be competitively inhibited by RGD peptides, whereby anchorage-dependent ECs undergo apoptosis [230]. To this group belong compounds, such as cilengitide/EMD121974 [325], S137 and S247 [326, 327], the TSP-derived peptide TP508/chrysalin [328], and several integrin $\alpha v\beta 3$- and $\alpha v\beta 5$-specific peptidomimetics, such as BCH-14661, which preferentially inhibits $\alpha v\beta 3$ and BCH-15046, which blocks $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ [329], SCH221153 [330], and ST1646 [331]. Another inhibitor is the non-peptide antibiotic thiolutin, which intracellularly blocks paxillin and thus, indirectly, integrin $\alpha v\beta 3$-mediated adhesion to vitronectin [332]. Antibodies against the $\beta 3$ subunit inhibit contact of ECs to vitronectin and concomitantly VEGF-induced tyrosine phosphorylation of VEGFR-2 in cell culture studies [333]. Moreover, integrin

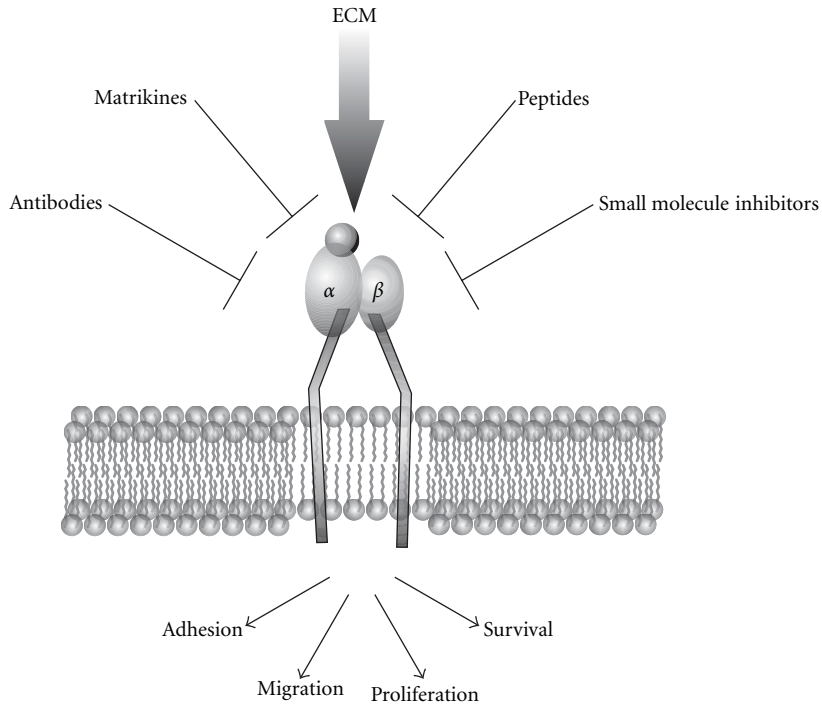

Figure 3: Options to manipulate integrin function. Essential cellular functions, such as adhesion, migration, proliferation, and survival, which all are regulated by integrins, can pharmacologically be manipulated with a panoply of matrikines, antibodies, peptides, and small molecule inhibitors, many of which are used as therapeutic tools in combination with conventional chemo- or radiotherapy to attack tumor cells and vasculature. Details are described in the text.

$\alpha v\beta 3$ can be effectively antagonized with the monoclonal antibody LM609/MEDI-552 and its humanized derivative abegrin/etaracizumab/vitaxin [57, 334–337]. In contrast, the humanized anti-αv antibody CNTO95 targets both integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ [338]. The humanized Fab fragment 17E6/abciximab/ReoPro of the monoclonal antibody c7E3 inhibits the integrins $\alpha v\beta 3$ and also $\alpha M\beta 2$/Mac-1 [339, 340], whereas the human-specific monoclonal antibody 17E6 targets all αv integrins [341]. Currently, humanized or chimeric integrin antibody antagonists of $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$, and peptide inhibitors of these integrins are in clinical trials as antiangiogenic agents [180].

5. Applications and Outlook

Integrins and their binding partners are of special interest as potential therapeutic targets, and several are already in clinical trials. However, the results fall short of the initial expectations, pointing out that monotherapy with a single angiogenesis inhibitor is not sufficient to counteract the numerous angiogenic factors involved in tumor progression [231]. Moreover, there are some caveats in aiming at integrins as therapeutic targets. Obviously, integrins are expressed on virtually all cells under physiological as well as pathological conditions, and it is a major challenge to target exclusively integrins on tumor or tumor-associated cells. Another problem is that low concentrations of antagonists alter the signaling of integrins and other receptors. When administered in nanomolar concentrations, the RGD-containing inhibitors cilengitide and S 36578 alter the trafficking of integrins and VEGFR2 in tumor ECs, thus stimulating angiogenesis and tumor growth [342].

Current tumor therapy aims at vessel eradication in order to disrupt the connection of the tumor to the vascular system and thus cut off the supply of nutrients and oxygen. This can be done with compounds that preferentially affect tumor endothelia rather than normal cells, that is, (i) specific angiogenesis inhibitors, (ii) tumor vessel toxins that attack inherent weaknesses in static tumor vessel endothelia and associated vascular structures, and (iii) dual-action compounds [343]. However, within the last years, a paradigm shift has taken place [344, 345]. Vessel normalization by pruning immature vessels and increasing pericytes and BM coverage of the remaining vessels comes to the fore, rather than vessel eradication, because mere antiangiogenic treatment can worsen malignancy [346]. A malformed tumor vasculature creates and aggravates a hypoxic and acidic milieu which hampers drug delivery and perfusion [347–349], and, due to its leaky endothelium, it promotes tumor cell dissemination [346]. Therefore, chemotherapeutic efficacy can be ameliorated by a concomitant vessel normalization therapy which improves delivery and efficacy of cytotoxic drugs and also sensitizes the tumor cells to radiation [345, 350].

In vessel normalization, the interaction of cells with their surrounding ECM via integrins is of special importance. However, many antiangiogenic compounds, for example, ATN-161, endostatin, and integrin inhibitors, show

hormetic, that is, bell- or U-shaped, dose-response curves and thus present a challenge for clinical translation [351]. Nanomolar concentrations of RGD-mimetic $\alpha v\beta 3$ and $\alpha v\beta 5$ inhibitors (S 36578 and cilengitide) can paradoxically stimulate tumor growth and angiogenesis by altering the trafficking of $\alpha v\beta 3$ integrin and VEGFR2. Thus, they promote the migration of ECs towards VEGF, which has important implications for the use of RGD mimetics in tumor therapy [342]. Thus, depending on tumor type, dose, and manner of application, the currently available-integrin targeting compounds can act either anti- or proangiogenic. A promising approach may be a combination therapy that blocks simultaneously angiogenic integrin $\alpha v\beta 3$ and VEGFR activities [352–355].

To circumvent these problems, instead of targeting the integrins, which are in principle present on both normal and malignant cells, another strategy aims at tumor-promoting integrin ligands, such as ED-B fibronectin, tenascin-C, and tenascin W [252, 253, 255]. Invasive tumor cells partially degrade and denature their surrounding ECM, and the thereby released cryptic collagen IV epitope HU177 may also be a potential target for antiangiogenic and tumor-selective drug delivery [356].

In comparison to a systemic administration of a chemotherapeutic agent, its therapeutic index can be increased by selectively targeting integrins that are overexpressed on tumor cells [357]. Chemotherapeutic small molecules, peptides, and proteins as well as nanoparticle-carried chemotherapeutics, which are conjugated to ligands of integrins that are overexpressed on angiogenic ECs or tumor cells, can be selectively internalized after integrin binding [357]. Especially nanoparticles, such as micelles, liposomes, polymeric nanospheres, and polymersomes loaded with chemotherapeutic or radiotherapeutic drugs and equipped with multivalent integrin ligands show decreased systemic toxicity, prolonged half-life and passive retention in the tumor, improved binding affinity, and facilitated internalization, thus resulting in increased drug delivery [12, 357, 358]. A therapeutic strategy that targets several integrins and receptors by such chemo-, radio-, and possibly gene therapeutic approaches may be more effective than a monotherapy [231, 357].

Coadministration of the αv integrin-targeting cyclic peptide iRGD (CRGDKGPDC), or structurally closely related peptides, with anticancer drugs considerably enhances their efficacy and selectivity [359]. Upon binding to αv integrin-expressing tumor ECs, iRGD is proteolytically processed to CRDGK with a much weaker integrin affinity, whereas this truncated peptide shows an increased affinity to neuropilin-1 (NRP-1), thus increasing vascular and tissue permeability in a tumor-specific and NRP-1-dependent manner [359]. Interestingly, this coadministration does not require chemical conjugation of the drug with the iRGD peptide; that is, approved drugs could be used unmodified [359]. Coadministration of such a tumor-penetrating peptide with either small molecules, such as doxorubicin, antibodies, such as trastuzumab, or nanoparticles, such as Nab-paclitaxel (abraxane) or doxorubicin-loaded liposomes, resulted in equivalent or increased delivery and efficacy, and it improved their therapeutic index by lowering the effective dose [359].

Additionally, integrins can be used as biomarkers to noninvasively assess the efficacy of chemotherapeutic and radiotherapeutic drugs [12]. Integrin-targeted probes can be used to visualize tumor angiogenesis and the response to chemo- and radiotherapy by various imaging methods, such as magnetic resonance imaging (MRI), positron emission tomography (PET), and ultrasonography [360–362]. Moreover, fluorescence labeling of integrin ligands allows intraoperative fluorescence imaging, thus providing a tool to intraoperatively detect and remove metastases of submillimeter size [363].

In summary, the above data illustrate the importance of integrins and integrin-binding and signaling proteins in both physiological and pathological blood vessel formation. Thus, they may be potential targets for antiangiogenic tumor therapy. Although our knowledge concerning this matter has increased remarkably within the last years, the understanding is far from complete.

Abbreviations

Ang:	Angiopoietin
BM:	Basement membrane
CRDGK, CRGDKGPDC, CRRETAWAC, PHSCN, RGD:	Amino acid sequences in single letter code
ECM:	Extracellular matrix
EC:	Endothelial cell
FAK:	Focal adhesion kinase
FGF:	Fibroblast growth factor
G-CSF:	Granulocyte colony-stimulating factor
HGF:	Hepatocyte growth factor
HIF:	Hypoxia-inducible factor
IL:	Interleukin
MAPK:	Mitogen-activated protein kinase
MEK:	MAPK/ERK kinase
MMP:	Matrix metalloproteinase
NC:	Noncollagenous
NF-κB:	Nuclear factor κ-light-chain enhancer of activated B cells
NRP-1:	Neuropilin-1
PDGF:	Platelet-derived growth factor
PD-ECGF:	Platelet-derived endothelial cell growth factor
PGE:	Prostaglandin E
PI3K:	Phosphatidylinositol-3 kinase
PLC:	Phospholipase C
PlGF:	Placenta-derived growth factor
Ras:	Rat sarcoma protein
Src:	Sarcoma oncogene
TGF:	Transforming growth factor
Tie:	Tyrosine kinase with immunoglobulin-like and EGF-like domain
TIMP:	Tissue inhibitor of metalloproteinases

TNF: Tumor necrosis factor
uPA(R): Urokinase-type plasminogen activator (receptor)
VCAM: Vascular cell adhesion molecule
VEGF(R): Vascular endothelial growth factor (receptor)
VSMC: Vascular smooth muscle cell
vWF: Von Willebrand factor
TSP: Thrombospondin.

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Research Article

The Characteristics of Vascular Growth in VX2 Tumor Measured by MRI and Micro-CT

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Blood supply is crucial for rapid growth of a malignant tumor; medical imaging can play an important role in evaluating the vascular characterstics of tumors. Magnetic resonance imaging (MRI) and micro-computed tomography (CT) are able to detect tumors and measure blood volumes of microcirculation in tissue. In this study, we used MR imaging and micro-CT to assess the microcirculation in a VX2 tumor model in rabbits. MRI characterization was performed using the intravascular contrast agent Clariscan (NC100150-Injection); micro-CT with Microfil was used to directly depict blood vessels with diameters as low as 17 um in tissue. Relative blood volume fraction (rBVF) in the tumor rim and blood vessel density (rBVD) over the whole tumor was calculated using the two imaging methods. Our study indicates that rBVF is negatively related to the volume of the tumor measured by ultrasound ($R = 0.90$). rBVF in the tissue of a VX2 tumor measured by MRI *in vivo* was qualitatively consistent with the rBVD demonstrated by micro-CT *in vitro* ($R = 0.97$). The good correlation between the two methods indicates that MRI studies are potentially valuable for assessing characteristics or tumor vascularity and for assessing response to therapy noninvasively.

1. Introduction

Medical imaging is an important and useful tool for assessing the shape and structure of a tumor as it grows and for monitoring the effects of clinical treatments [1–3]. It has been documented that increased vascularity in the neoplastic tissue provides a valuable indication of tumor aggressiveness; this blood supply is crucial for the malignant tumor to grow rapidly. Traditionally, the response to cancer treatment is judged by the reduction in tumor volume. Recently, switching the target of cancer treatment from the exceptionally heterogeneous tumor cell population to the considerably more homogeneous tumor vasculature has emerged as a revolutionary therapeutic approach. Development of a large number of antivascular and antiangiogenic therapies has created the need for techniques that noninvasively quantify vascular volume and flow changes in response to the therapy. Assessment of structural and functional abnormalities of a tumor's blood vessels for prognostic reasons, therapy monitoring, or prediction of therapy success is increasingly gaining attention. The relative blood volume fraction in selected regions (rBVF: a ratio of average signal contribution in a region of the tumor due to intravascular agents relative to that in whole blood, measured *in vivo*), can be determined by 2D MRI using intravascular contrast agents [4, 5]. The relative blood vessel density in the tumor (rBVD: percentage of pixels within identifiable blood vessels relative to total number of voxels in the tumor or tumor region) is best measured by high-resolution micro-CT *in vitro* but may be approximated by high-resolution 3D MRI *in vivo*.

A rabbit model with VX2 tumor has been studied widely to assess tumor development and treatment response [6–8]. In general, the tumor mass can be visualized as two distinct regions: the highly vascular advancing rim where tortuous blood vessels are abundant in the peripheral area and the hypovascular central region where the number of blood vessels gradually decreases, resulting in hypoxia and necrosis. Tumors usually develop a disorganized microvascular network during growth; it has been shown that the density of the blood vessels in the tumor changes at different stages of the tumor growth [9]. The rBVF and rBVD and the changes of blood volume and vascular density with tumor growth can be monitored using medical imaging. However, the relationships between the functional rBVF measured by 2D

MRI *in vivo*, morphological rBVD measured by micro-CT *in vitro*, and the size (volume) of the tumor have not yet been reported. Therefore, the purpose of this study was to evaluate the rBVF characteristics in the highly vascular advancing rim of a rabbit VX2 tumor model by 2D MRI *in vivo* and compared to rBVD measured by micro-CT *in vitro* to validate a practical *in vivo* MRI method which could be used to characterize the earlier functional response of a tumor to clinical intervention and treatments. The blood vessel density (rBVD) in the tumor demonstrated by micro-CT and rBVD by 3-dimensional (3-D) MRI were also compared. The relationships among rBVF and rBVD and the volume of the VX2 tumor as it grows were evaluated as well. Micro-CT can demonstrate the tumor's 3D microangioarchitecture and therefore can serve as a gold standard for perfused blood volume; the minimal diameter of the blood vessels detectable on micro-CT images was about 9 to 17 μm [10, 11]. Histopathology was also performed to demonstrate differences in the structure of blood vessels in small and large tumors.

2. Materials and Methods

2.1. Animal Model. Nine New Zealand white rabbits (4 to 4.5 kg) were used in this study. All animals were prepared in accordance with the "*Guide to the Care and use of Experimental Animals* (Canadian Council on Animal Care)" under approved protocols. The rabbits were each injected intramuscularly with about 1×10^6 VX2 tumor cells in a hind leg. The tumor was monitored daily by B-mode ultrasound starting at 7 days after injection of the tumor cells. When the tumor reached at least 2 cm^3, an MRI study was scheduled. Tumor volume was calculated at the time of the MRI study from the maximum length, width, and height measured on ultrasound images acquired the same day as length (cm) $*$ width (cm) $*$ height (cm) $* \pi/6$, assuming a prolate ellipsoid shape. Tumors were included with volumes ranging from 2.4 cm^3 to 15.37 cm^3 based on studies at time points ranging from 10 to 20 days after the tumor cells were injected. MRI and micro-CT studies, for each tumor were conducted within 24 hours. Rabbits were anaesthetized with Ketamine (50 mg/kg) and Xylazine (5 mg/kg).

2.2. MRI. Studies were performed on a GE Signa 1.5 T CV/i equipped with 40 mT/m gradients with a 150 mT/m/s slew rate using a 3-inch receive-only surface coil for signal reception. The maximal center cross-section of the tumor were imaged by 2D MRI with a T_1-measurement acquisition.

2.2.1. 2D MRI. 2D Quantitative T_1 measurements for rBVF were made using a Look-Looker sequence with spiral readouts (225 ms acquisition spacing, TR = 3000 ms, RBW = 125 kHz, 20° flip angle, 7 mm slice thickness, FOV = 20 cm, 4096×8 spiral readouts), 1.1 mm resolution. The intravascular contrast agent, Clariscan, (NC100150-Injection, GE Healthcare) was used in this study. T_1 measurements were made both prior to and approximately 4 minutes following an intravenous bolus injection of Clariscan (0.05 mL/kg = 0.15 mg Fe/kg), and then at 15-minute intervals of different time points thereafter. Dynamic T_1-weighted imaging for perfusion was performed immediately following Clariscan injection and used for identifying the region of interest (ROI) in the tumor rim (fast spoiled gradient echo 2DFT sequence, TR = 12 ms, TE = 5 ms, 30° flip angle, bandwidth = 15.63 kHz, Matrix = 256×128, FOV = 20 cm).

A total of 10 mL (5×2.0 mL tubes) of arterial blood was drawn both before and after the Clariscan injection at different time points and T_1 decay times for each sample was measured (TR = 3000 ms, TE = 50 ms, RBW = 125 kHz, 10° flip angle, 10 mm slice thickness, FOV = 20 cm, 6 NEX, 4096×4 spiral readouts) to calculate the T_1 due to the contrast agent in whole blood. The changes in longitudinal relaxation rates R_1 $(1/T_1)$ in blood after administration of contrast medium is proportional to contrast agent concentration. Under the assumption that Clariscan remains intravascular in the tumor, the blood volume fraction can be calculated using the equation:

$$\mathrm{BVF} = \frac{(1/T_1 t)\mathrm{postCA} - (1/T_1 t)\mathrm{preCA}}{(1/T_1 b)\mathrm{postCA} - (1/T_1 b)\mathrm{preCA}}, \quad (1)$$

where preCA and postCA refers to measures before and after injection of Clariscan. $T_1 t$ and $T_1 b$ refer to the measurements from the tissue *in vivo* and the blood sample *in vitro*, respectively. This equation assumes that we can model the tumor as two pools, consisting of an intravascular and extravascular compartments, and the distribution of protons between the pools is equal (partition coefficient = 1). In addition, the exchange of protons between the pools is fast enough that the system can be regarded as well mixed within a time on the order of T_1 [12]. Previous studies showed that the condition of fast water exchange appears to be met when T_1 in blood is greater than 150 ms [13, 14]. The postCA measurement of Clariscan for rBVF measurement was taken at the last time point (at 60 to 90 min after injection) when T_1 of blood was >150 ms to satisfy this condition.

2.2.2. 3D MRI. The 3D spoiled gradient echo acquisition (3D SPGR, TR = 6 ms, TE = 2 ms, FOV = 20 cm, 30° Flip angle, Bandwidth = 125 kHz, slice thickness = 0.8 mm, 320×320 pixels in-plane yielding approximately 0.6-mm resolution) was also conducted before and 10 minutes after Clariscan injection. Maximum Intensity Projection (MIP) was obtained by subtraction of pre- from post-contrast agent.

2.3. Micro-CT. When MRI *in vivo* studies was finished, tumors were perfused via the femoral artery with saline followed by microfil (Flow Tech inc., Carver, MA) for the micro-CT study. The tumor was excised 90 minutes after microfil perfusion to allow time for the microfil to harden; the tumors were then stored in 10% formalin for 24 hours. The specimen was then mounted in 10% gelatin and 3D cone beam CT data sets were acquired over 2.5 hours with 905 views at 35 μm-resolution using a micro-CT scanner (MS-8, GE Medical Systems, London, Ontario). An X-ray source of voltage 80 kvp and a beam current 90 μA were used.

A 3D data volume was reconstructed using the Feldkamp algorithm for cone beam CT geometry [11]. 3D surface rendering of the vasculature of tumor was accomplished using Display software (Montreal Neurological Institute, McGill, Montreal, Canada).

2.4. Tissue Preparation and Histology. Since microfil used for micro-CT makes it difficult to interpret histology, we examined samples from four additional VX2 tumors of similar sizes histologically to confirm vascular characteristics. These were fixed in 4% PFA for 4 hours, washed in PBS 3 times for 15 minutes, and cryoprotected in 15% sucrose in PBS for 1 hour followed by 30% sucrose in PBS overnight at 4°C. Samples were then incubated in Tissue-Tek OCT (Sakura) at 4°C for 4 hours prior to embedding in OCT over dry ice. The tissue blocks were cryosectioned at 7 μm, placed onto L-polylysine-coated slides (Fisher Scientific), and dried for 1–4 hours at room temperature before storage at −20°C. Histology study was conducted by Immunohistochemistry staning, the slides were incubated with alkaline phosphatase conjugated monoclonal anti-α-Smooth muscle actin (1 : 100; Sigma) for 1 hour at room temperature followed by washing 4 times in PBS for 5 min. This stain was used to identify microvessels based on their smooth muscle layer and was found to be adequate for this purpose in the rabbit model. Then the sections were incubated 10 min in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$) and then stained with BM Purple AP substrate (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 337 mg/mL NBT (nitroblue tetrazolium salt; Boehringer Ingelheim), and 175 mg/mL BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt; Boehringer Mannheim). The staining reaction was allowed to proceed for 10 min at room temperature. The slides were then washed extensively in PBS and covered with cytoseal mounting medium (Richard-Allan Scientific).

3. Data Analysis

3.1. 2D MRI. ROIs were carefully placed around the abundant microvascular rim of the tumor in both large and small tumors for 2D measurements. The ROI in the rim of tumor tissue avoids necrosis and bigger vessels in the center of the tumor for correctly assessing the rBVF in microcirculation. The ROIs were determined from serial images taken immediately after injection of the contrast agent Clariscan (published data) [15] in MRI as areas that were filled with contrast agent at early phases and showed clearer outlines for the tumor.

3.2. 3D MRI and Micro-CT. Measurements of vessel density, rBVD, from 3D MRI and 3D micro-CT were determined by rendering 3D isosurfaces in Amira (AmiraDev 4.1.1; TGS, Berlin, Germany). Micro-CT images *in vitro* showed the isolated tumor's vessel clearly, however, Images of 3D MRI *in vivo* demonstrated not only the tumors' vessels but also the blood vessels of whole leg, Therefore the MRI images and micro-CT images were aligned using the big blood vessels in the tumor, then the tumor was segmented by manually drawing a volume using "Label/Voxel" to encompass the whole tumor defined by the vascular rim in the micro-CT data. The blood vessel density over the tumor (rBVD) was determined by counting voxels inside the surface-rendered vessels and comparing that to the total number of voxels in the tumor using the "TissueStatistics" package in Amira.

4. Statistical Analysis

The correlations between rBVF in the rim measured by 2D MRI and the volume of the tumor by ultrasound were assessed with Pearson's correlation coefficient. The relative blood volume fraction in 2D and the blood vessel density in 3D MRI were compared. The blood vessel density determined by 3D MRI and micro-CT were also compared. Statistical significance was set as $P < 0.05$.

5. Results

5.1. rBVF Relative to Volume of the Tumor. Our results indicated for the first time that rBVF in the rim of the tumor seems to decrease with increasing volume of VX2 tumors (as measured by ultrasound) once the tumors were detectable and measurable for rBVF (i.e., those larger than 2.4 cm^3 in this study). There was a significant inverse relationship between the rBVF and the volume of the tumor, $R = 0.90$, $P < 0.001$ (Figure 1). In our study, the rBVF in the tumor's rim varied from 3.05 to 16.55% with an average of 10.38 ± 5.36%, as measured by MRI; the volume of the tumors ranged from 2.40 to 15.37 cm^3 with an average volume of 8.98 ± 4.82 cm^3, obtained by ultrasound.

5.2. rBVF in 2D Compared to rBVD in 3D MRI. The rBVF in the rim measured by 2D MRI was significantly correlated with the rBVD across the tumor calculated from 3D MRI (Figure 2, $R = 0.95$, $P < 0.001$). The rBVF in the rim measured in this study was derived in a slice at the maximum cross-section of the tumor, and rBVD in 3D was calculated from the whole volume of the tumor. The average rBVD in 3D was higher than the rBVF measured in 2D, likely due to the greater coverage of tumor and inclusion of larger vessels in the 3D acquisition.

5.3. Blood Vessel Density in Micro-CT Compared to 3D MRI. The vessel density obtained by micro-CT yielded results similar to corresponding measurements by 3D MRI (Figure 3, $R = 0.97$, $P < 0.001$). A representative large tumor (>9.6 cm^3 in volume) had a lower density of blood vessels (8.8% in micro-CT compared to the 4.0% derived from MRI). The blood vessel density increased to 20% by micro-CT and to 17% by 3D MRI in a small tumor around 2.8 cm^3 in volume. The smaller tumor demonstrated a thicker rim with more blood vessels (Figure 4) while the larger tumor showed a thin rim with fewer blood vessels (Figure 5) by 3D MRI and micro-CT.

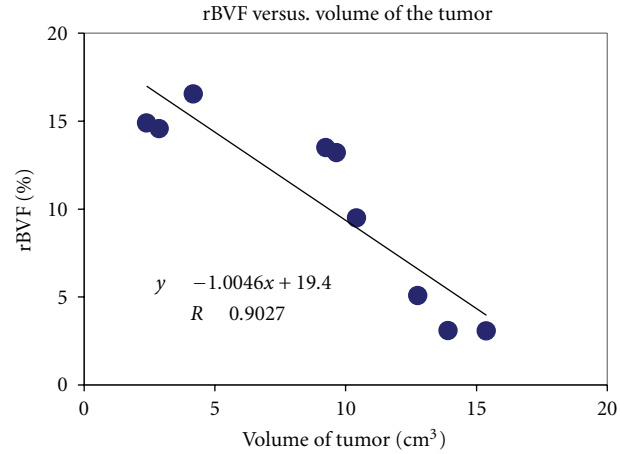

Figure 1: rBVF measured by MRI in a VX2 tumor is inversely related with volume of tumor (cm^3). $R = 0.90$, $P < 0.001$.

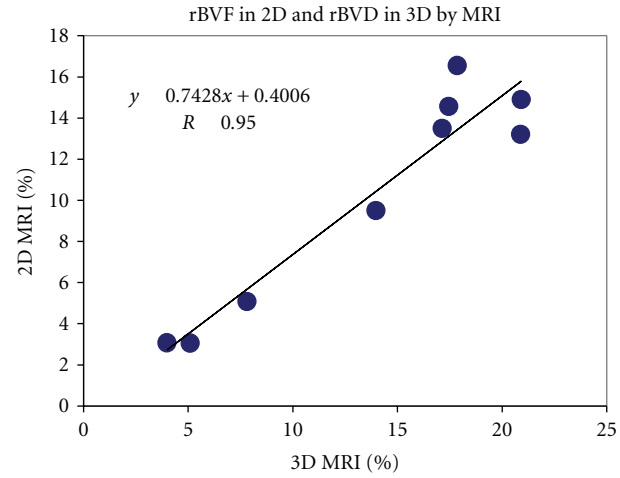

Figure 2: Relationship between rBVF measured by 2D MRI and rBVD by 3D MRI. $R = 0.95$, $P < 0.001$.

The imaging results were consistent with histopathological observations (Figures 6 and 7). In the histology specimens, the small tumor showed more small vessels in the section, while the large tumor showed fewer blood vessels with greater diameters as well as more necrosis.

6. Discussion

Our results indicate that the rBVF in the tumor rim measured by noninvasive imaging methods was negatively related to the volume of the tumor and that small tumors at earlier stages of growth have a higher density of blood vessels than in large tumors at the later stages of growth. Therefore, the vasculature of the tumors changed at different growth stages [16, 17]. The higher rBVF and density of blood vessels in small tumors may be related to their rapid growth and the relative absence of necrosis. The large mature tumor has a lower BVF and bigger vessels with more arteriovenous shunts; these characteristics may be related to

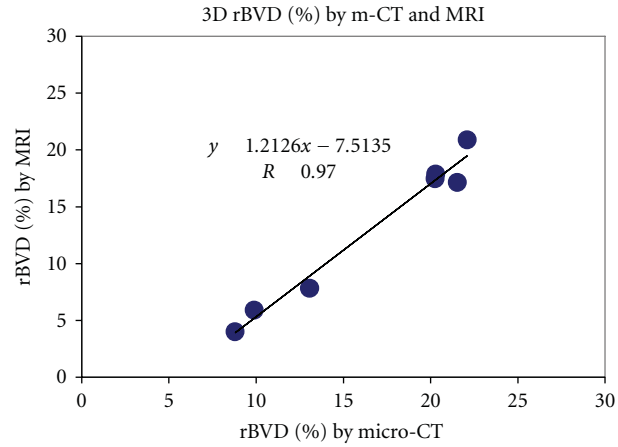

Figure 3: Relationship of blood vessel density measured in 3D by m-CT and MRI. $R = 0.97$, $P < 0.001$.

the greater metastatic potential and the lower efficiency of the treatments at later stages.

Micro-CT, using a microfocus X-ray source and high-resolution detectors, could clearly observe the 3D microangioarchitecture of the tumor *in vitro*. Interestingly, the images were comparable with 3D MRI at much lower resolution using intravascular contrast agents *in vivo* (Figures 4 and 5). The minimal diameter of the microvessel demonstrated by micro-CT was 17 μm. The resolution in 3D MRI *in vivo* was 0.6-0.7 mm in this study. This suggests that most of the vessel area across the whole tumor is dominated by the larger vessels.

Studies over the last decade have demonstrated that although the microvessel density in tumors is heterogeneous, the vasculature of the tumor may differ substantially at the different stages of the growth [9]. Our results provided imaging and histopathological evidence supporting these conclusions. At the earlier stages of the tumor's growth, more blood vessels were shown in the peripheral rim of the tumor. This could explain the rapid tumor growth and the greater efficiency of treatments in earlier stages.

Clinical investigations have suggested that the incidence of metastases in many cancer types is positively correlated with the microvessel density in vascular hot spots of the primary tumor [18–20]. Metastatic potential also could be related to the increased prevalence of arteriovenous shunts. These may facilitate extravasation and transportation of larger cancer cell clusters. The structural and functional abnormalities of the vessels in the bigger tumors also result in regions that are poorly perfused and hence hypoxic. Some studies have indicated that tumor hypoxia may also promote tissue metastasis. Rofstad has stated in detail the theoretic reasons of several mechanisms involved [21]. Hypoxia is also a cause of resistance to certain medical and cytotoxic agents. The poorly perfused, hypoxic tumor microenvironment may also be resistant to radiation therapy, photodynamic therapy, chemotherapy, and some forms of gene therapy.

Measurement of microvessel characteristics can aid in assessing the stage of disease, the likelihood of metastasis,

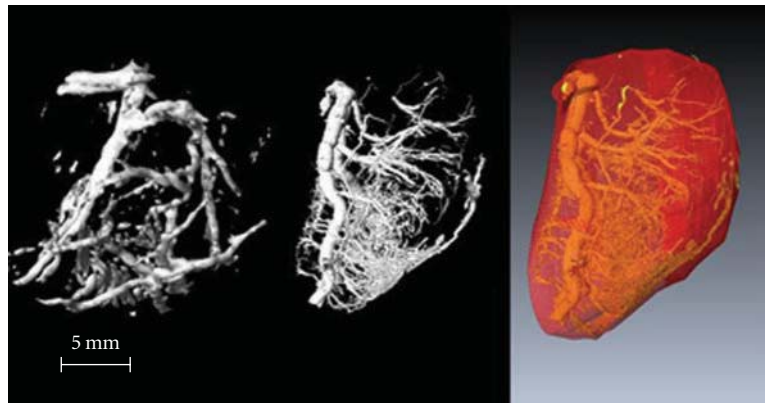

Figure 4: Small tumor (2.8 cm^3)—left: 3D MRI, middle: micro-CT, right: whole tumor volume. A higher density of blood vessels is seen in the peripheral rim. The large artery on the left side of the images is the feeding blood vessel for the tumor.

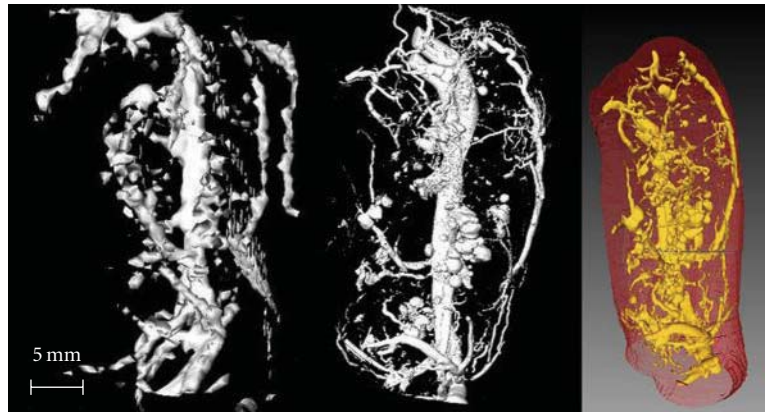

Figure 5: Large tumor (9.6 cm^3)—left: 3D MRI, middle: micro-CT, right: whole tumor volume. A lower density of blood vessels is seen in the peripheral rim.

and prognosis under various treatment strategies for a wide range of cancers [22–25]. Many studies have demonstrated reductions in blood volume and blood vessel density in tumors after antivascular and anti-angiogenic treatments [26]. However, there are some reports indicating that microvessel density in the tumor is not always an indicator of anti-angiogenic therapeutic efficiency. These data demonstrate that the absence of a drop in microvessel density does not indicate that the agent is ineffective [27–29]. Because anti-angiogenic agents act to inhibit neovessel formation, measurements of preexisting microvessel density are not sufficient to reveal the functional or angiogenic status of the tumor neovasculature. Therefore, blood flow in the tumor, including the blood volume and the blood flow velocity in the microcirculation of tumor tissue, may be more sensitive than the blood vessel density for evaluating the efficiency of antiangiogenesis agents in the earlier stage of the drug administration. As a histological index, the blood vessel density could not be expected to change as quickly as the blood volume and velocity in the micro-circulation of the tumor in response to the treatments. Therefore MRI and ultrasound, as noninvasive modalities for determining the morphological and functional blood volume changes in the tissue are useful and valuable tools [15] for evaluation of the microvasculature during tumor growth, and also for monitoring as well as evaluating the functional characteristics of vasculature in the tumor as an indicator of the efficiency of anti-vascular and anti-angiogenic agents. One study recently demonstrated that tumor blood flow measured by destruction-replenishment ultrasound was highly correlated with the contrast enhancement estimated by CT images [30]. Our data indicated that relative blood volume and blood vessel density in the microcirculation of tissue measured by MRI is consistent with the vessel density determined by micro-CT. The results indicated also that the MRI measurements of rBVF and rBVD in the tumor reflect the histological structure in the tissue.

7. Study Limitations

One limitation of the rBVF and rBVD evaluation is that the magnetic resonance macromolecular agent, Clariscan, may

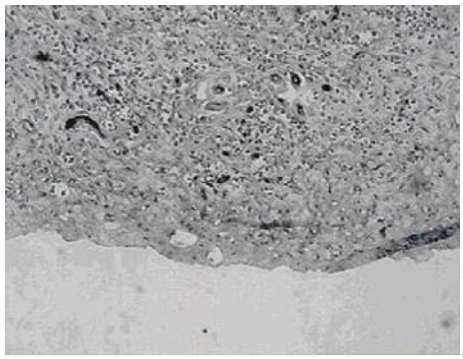

Figure 6: Histology section from a small tumor shows a higher density of smaller vessels.

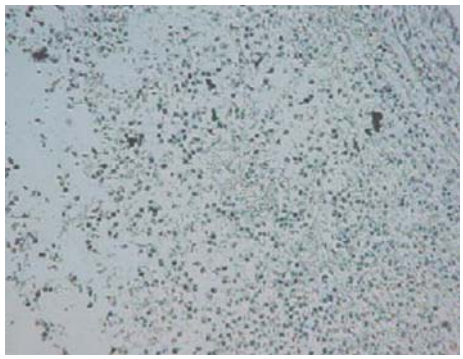

Figure 7: Histology section from a large tumor shows fewer and relatively bigger vessels and necrosis.

slowly leak out of the blood vessels. All data was collected within 1.5 hours of contrast injection in this study; this should minimize the effects of leaking. Another problem is that we need blood samples for contrast agent concentration calculation in the blood pool, which is time consuming. One could measure the signal from a relatively large blood vessel *in vivo* instead in future studies.

The relationship of the vascularity and volume of the tumor may be different when the volume of tumor is less than 2.0 cm^3; however that was not included in this study. Micro-CT and high-frequency ultrasound may be helpful for evaluation of smaller tumors.

8. Conclusion

Relative blood volume in the microcirculation of tissue as measured by MRI is valuable for determination of the vascular, functional, and anatomic characteristics of a tumor, as well as the changes with growth. The observations using MRI and micro-CT have shown that rBVF and rBVD are significantly related to the characteristics and status of tumor growth. This has immediate utility for preclinical research studying the impact of novel therapeutics on tumor blood supply. Specifically, the blood volume determination in the tumor using the noninvasive MRI study may be useful for monitoring and evaluating the efficiency of anticancer drugs, especially anti-vascular and anti-angiogenic agents. Such studies may also help elucidate optimal timing in the administration of anti-vascular cancer therapies. This noninvasive MRI technique could be extended to clinical studies if and when an intravascular contrast agent similar to Clariscan is approved for patient use.

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Review Article

Angiogenesis in Acute Myeloid Leukemia and Opportunities for Novel Therapies

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Acute myeloid leukemia (AML) arises from neoplastic transformation of hematopoietic stem and progenitor cells, and relapsed disease remains one of the greater challenges in treating this hematologic malignancy. This paper focuses on angiogenic aspects of AML including the significance and prognostic value of bone marrow microvessel density and circulating cytokine levels. We show three general mechanisms whereby AML exploits angiogenic pathways, including direct induction of angiogenesis, paracrine regulation, and autocrine stimulation. We also present early evidence that leukemia cells contribute directly to vascular endothelia. Novel treatment strategies are proposed, and a review of relevant antiangiogenic clinical trials is presented. By understanding how blood vessels can serve as a reservoir for refractory and relapsed AML, new diagnostics and promising treatment strategies can be developed.

1. Introduction

Acute myeloid leukemia (AML) is a cancer of the bone marrow characterized by a mutation in a hematopoietic stem or progenitor cell (HSPC), which develops into a highly proliferative accumulation of dysfunctional and immature myeloid cells. These abnormal cells eventually dominate hematopoietic niches like the bone marrow and result in abnormal peripheral blood counts (anemia, thrombocytopenia, leukocytosis due to high number of myeloblasts number and/or neutropenia) [1]. Initial disease remission can be achieved in 30–70% of AML patients after standard induction chemotherapy regimens such as 7 + 3 (seven days of cytarabine and 3 days of an anthracycline). However, refractory and relapsed disease remains a major challenge in all patients, especially in older individuals. In patients 60 years and older, the 5-year survival prognosis for AML is only 20% with the majority of patients succumbing to disease relapse [2, 3]. Mechanisms for AML relapse are related to leukemia cell insensitivity and potential sanctuary sites. When considering that functional blood vessel networks are essential to these mechanisms of relapse, the investigation of angiogenesis in leukemia is highly significant.

Until recently, leukemia studies have focused primarily on the leukemia cell. However, with mounting evidence showing the importance of the bone marrow microenvironment in regulating hematopoiesis, it is necessary to broaden the scope of investigation beyond the leukemia cell. A better understanding of the pathobiology surrounding and supporting leukemia cell survival has great potential to lead to promising new therapies.

2. Endothelial Cells in Support of Leukemia

The importance of the cancer microenvironment is widely recognized in solid tumors. Cancer cells interact with the stromal microenvironment in complex ways to promote their own survival and proliferation. However, in the case of hematologic malignancies like AML, the leukemia microenvironment is highly dynamic. The typical leukemia niche is within the bone marrow microenvironment. But AML cells can also migrate systemically to other organs that support hematopoiesis, such as the liver and spleen [4]. Monocytic AML subtypes (M4 and M5 FAB subtypes) can also migrate across blood-organ barriers and into privileged areas such as the central nervous system.

Although the complete model of the bone marrow microenvironment is not yet fully understood, it has been simplistically divided into three compartments: an endosteal niche that maintains quiescent hematopoietic stem cells, a vascular niche which regulates entry and exit from the bone marrow, and the central marrow space filled with various hematopoietic progenitors in the process of differentiation [5] (Figure 1).

One of the earlier investigations in the relationship between AML cells and the vascular niche was performed by Fiedler et al. [6]. These investigators found that a large proportion of AML patients had disease that expressed vascular endothelial growth factor (VEGF), as well as VEGFR1 and VEGFR2. They also found that VEGF induced human umbilical vein endothelial cells (HUVECs) secrete GM-CSF, which is a known mitogen for AML cells. Together, these results were one of the first to suggest that AML cells (i) exploit angiogenic signaling for autocrine stimulation and (ii) provoke endothelial cells to secrete proleukemic factors for survival and proliferation.

Recent evidence indicates that leukemia cells, like tumor cells, depend on angiogenesis in the bone marrow. Clinically, increased angiogenesis has been reported in the bone marrow of patients with AML. Hussong et al. stained bone marrow biopsies for blood vessels in 20 patients with untreated AML, compared with 20 control patients and quantified the number of vessels/mm in each case [7]. They found significantly increased microvessel density (MVD) in the bone marrow of AML patients ($P < 0.001$), suggesting a role of angiogenesis in AML. This is particularly significant when considering the strong positive correlation between increased bone marrow vasculature and overall survival of leukemia [8, 9]. A higher microvessel density predicted for poor prognosis and suggests that blood vessel-AML interactions may contribute to refractory disease [10].

Endothelial cells support adhesion and transmigration of subsets of normal CD34+ HSPCs. *In vitro* studies have shown that upon transwell or direct coculture with HUVECs, AML blasts proliferate to a higher degree and are less susceptible to traditional chemotherapeutic agents such as cytarabine [11]. We have found similar results when coculturing human promyelocytic leukemia cells (HL60) with HUVECs and then exposing the cells to cytarabine. HUVECs protect AML cells from chemotherapy (Figure 2). Together, these results provide compelling evidence that endothelial cells are protective of leukemia and may be a site of leukemia reinitiation after chemotherapy. Although the protective effect of endothelial cells on leukemic myeloblasts is evident, a thorough understanding of the detailed interactions and mechanisms is necessary in order to rationally design new therapeutic strategies.

While endothelial cells enhance leukemia proliferation, emerging evidence indicates that leukemia cells may have a reciprocal effect of enhancing endothelial cell proliferation. Hatfield et al. investigated the hypothesis of interdependence using transwell and direct contact experiments between primary AML cells and dermal microvascular endothelial cells (DMVECs). ^{3}H-Thymidine incorporation assays were used to quantify proliferation of endothelial cells. Their results showed enhanced endothelial cell proliferation in transwell coculture with AML blasts [12]. At the very least, this codependence is mediated by secreted cytokines between leukemia cells and endothelial cells. Cytokines that are involved in this bidirectional crosstalk include, for example, VEGF, angiopoietins, GM-CSF, CXCL8, and IL-6. A study by Kruizinga et al. [13], evaluated the leukemia cell expression levels of several VEGF isoforms in a pediatric AML population. The study used PCR arrays to analyze AML myeloblast cells for the presence of the cytokines. Various isoforms of VEGF including VEGF-121, VEGF-165, and VEGF-189 were expressed in the AML cells. Particularly, the VEGF-165 and VEGF-189 isoforms stimulated endothelial proliferation and angiogenesis. However, correlations between VEGF mRNA isoform expression levels and known prognostic factors were not found, nor was there a relationship between VEGF expression and overall survival or relapse-free survival.

With ECs promoting AML cells, and AML cells promoting ECs, a cyclical positive feedback loop is established and strongly favors the potential for refractory and relapsed disease (Figure 3).

Clinically, increased levels of circulating angiogenic factors correlate with increased angiogenesis in the bone marrow [14] and are high-risk indicators of disease relapse and early mortality [15]. Some of these circulating proangiogenic factors include VEGF and the angiopoietins. The clinical relevance of other angiogenic mediators, such as basic fibroblast growth factor (bFGF) and IL-6, has yet to be defined. The presence of angiogenic mediators in leukemia is complicated by the variabilities of gene expression and factor secretion. Cytokine levels vary on a patient-by-patient (disease-by-disease) basis, but evaluating the presence of these cytokines can be used as a prognostic indicator. Hou et al. [16] assessed the expression of a few of these cytokines in mononuclear cells of the bone marrow in 126 newly diagnosed AML patients prior to treatment to correlate prognostic outcome. By PCR screening, they found that a high level of pretreatment angiopoietin-2 (Ang-2) was a prognostic indicator of poor outcome. There is contrary evidence from others [17] that showed that pretreatment levels of Ang-2 were a prognostic indicator of good clinical outcomes. However, in common between these seemingly conflicting reports were the observations that in the presence of high VEGF-A levels, high Ang-2 correlated with poor outcome. Thus, the significance of Ang-2 in AML is complex and most likely influenced by VEGF activity. The angiopoietin/Tie2 axis may be important in AML and certainly needs further definition.

3. Leukemia Cells with Endothelial Cell-Like Phenotype and Function

The ability of leukemia cells to respond to angiogenic signals from endothelial cells suggests a close relationship between the two cell types. In fact, AML cells from patients have been reported to express VEGF and VEGFRs [6, 13, 18]. Fiedler et al. were the first to report that primary AML cells can express VEGF, VEGFR1, and VEGFR2. A greater proportion of AML

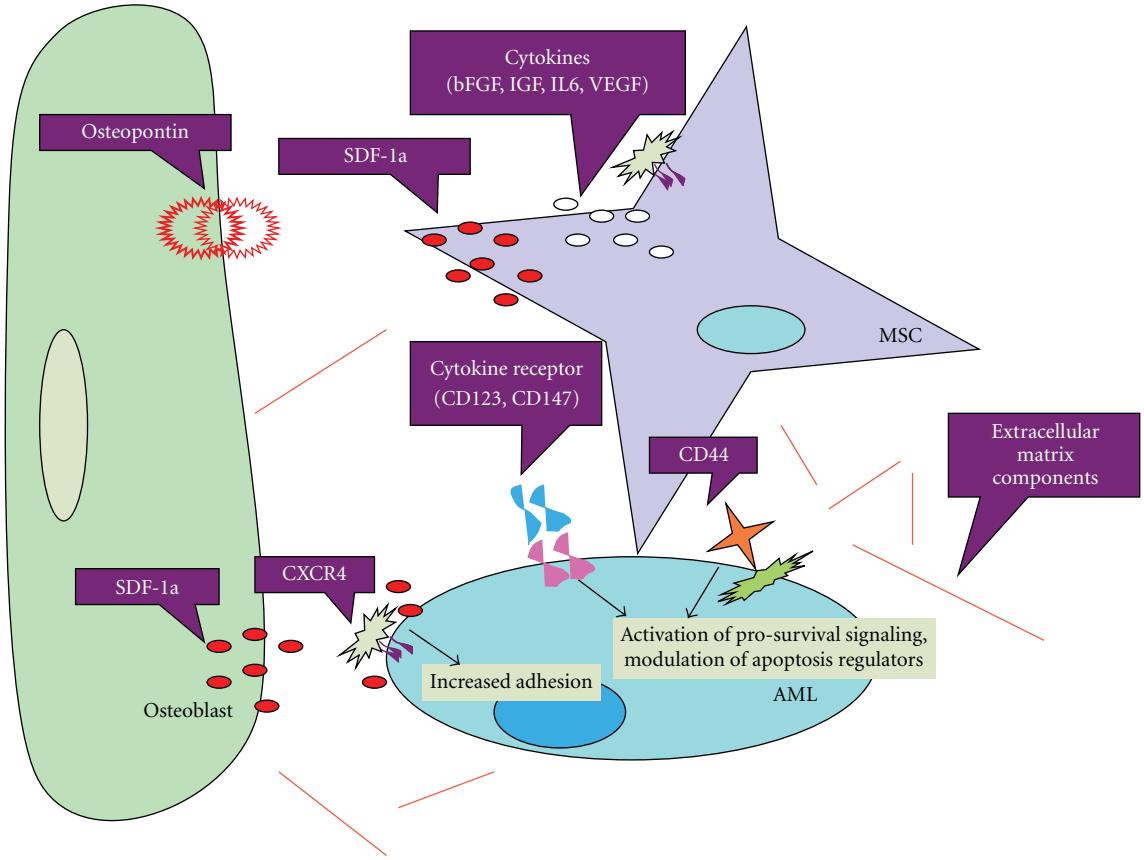

Figure 1: Acute Myeloid Leukemia Cells Within the Bone Marrow Microenvironment. The bone marrow niche can be simplistically divided into the endosteal niche or osteoblastic niche which is located on the inner bone surface. Hematopoietic stem cells (HSCs) have been found to reside here in a quiescent state. The vascular niche is made up a central sinusoid and lined by endothelial cells, macrophages, and perivascular cells. In the central marrow region, between the endosteal niche and vascular niche, acute myeloid leukemia (AML) cells hijiack the entire bone marrow anatomy and induce angiogenesis. The location of AML initiation and relapse within the bone marrow has yet to be defined.

patients had disease that expressed VEGFR1 compared to VEGFR2 [6]. Padro et al. also screened AML patients, but found that VEGFR2 was more commonly expressed on AML myeloblasts [19]. Together these data support the notion that AML blasts can exhibit a hybrid EC phenotype and that the coexpression of EC surface proteins is variable. The variability of VEGFR expression on AML cells introduces the possibility of a personalized medicine approach to treating AML. For example, in patients who have AML that expresses VEGFRs, treatment with anti-VEGF agents may bring about improved outcomes.

Given that AML cells can secrete VEGF and express VEGFRs, it stands to reason that AML cells may benefit from an autocrine loop. Indeed, investigators have reported a possible autocrine loop including VEGF produced by leukemia cells and their own VEGF receptors. In particular, VEGFR2+ AML cells were treated with VEGFR2 neutralizing antibodies in serum-free growth conditions. Blocking VEGFR2 resulted in decreased leukemic cell growth, supporting the notion of a VEGF/VEGFR2 autocrine loop [20].

The angiopoietin/Tie2 axis represents another potential autocrine loop in AML. We and others have found that certain myeloid leukemia cell lines, namely, K-562, express angiopoietin-1 (Ang-1), Ang-2, and their receptor, Tie2. Primary human AML specimens have also been reported to express these endothelial cell associated factors and receptor [21]. An *in vitro* study by Reikvam et al. found that blocking angiopoietin interactions with the Tie2 receptor using antibodies led to marked decreases in AML cell proliferation [22]. Interestingly, they found that some primary AML specimens were dependent on autocrine stimulation in order to proliferate, whereas others that did express the angiopoietins and Tie2 proliferated independently of autocrine stimulation. Yet to be defined is the effect of angiopoietin peptibodies such as AMG386 (Genetech) in hematologic malignancies. Clinical studies have begun using these peptibodies in prostate and ovarian cancers [23].

Another explanation for AML myeloblasts that coexpress EC phenotype is the possibility of fusion. Studies by Skinner et al. [24] further explored this idea using an *in vivo* model of primary human AML cells into immunocompromised mice. Hepatic sinusoids were lined by hybrid human AML-murine ECs (AML-EC). Moreover, these fused AML-ECs contained separated human DNA and murine DNA typically found in syncytia. Yet to be determined is whether the expression of EC proteins endows AML myeloblasts with resistance

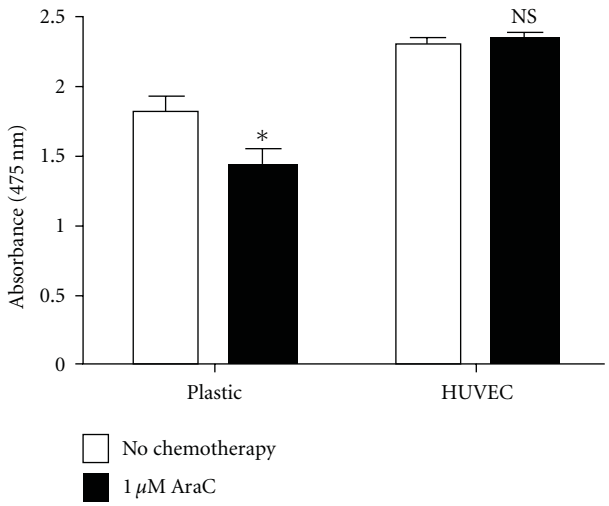

Figure 2: Endothelial Cells Protect Acute Myeloid Leukemia Cells from Chemotherapy. Human acute promyelocytic leukemia cells (HL60) were cultured in two conditions: over plastic and in the presence of human umbilical vein endothelial cells (HUVECs). The leukemia cells were then exposed to cytarabine chemotherapy, which is commonly administered to patients with AML. Cell proliferation was subsequently measured by XTT assay. HL60 cells in coculture with HUVECs showed no decrease in cell proliferation after chemotherapy exposure (NS) as compared to HL60 cells cultured over plastic ($P < 0.05$).

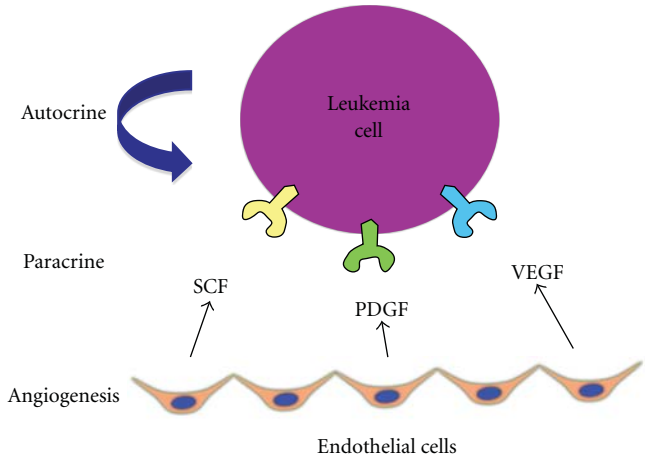

Figure 3: Multiple Mechanisms of Angiogenic Pathways Regulate Acute Myeloid Leukemia Survival and Proliferation. Acute myeloid leukemia cells exploit angiogenic mechanisms by (1) inducing angiogenesis directly, (2) expressing receptors for specific angiogenic growth factors (paracrine regulation), and (3) secreting their own angiogenic factors for their own angiogenic growth factor receptors (autocrine stimulation). Thus, angiogenesis has both cell-extrinsic and cell-intrinsic significance in leukemia. Stem cell factor (SCF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) are a few of many yet to be defined angiogenic factors that regulate leukemia cell survival and proliferation.

to chemotherapy. If AML-EC hybrids are more resistant to chemotherapy and can regenerate and proliferate AML cell population, then these cells may represent sources of refractory and relapsed disease.

4. Leukemia Hemangioblast Activity: Leukemia Cell Differentiation into Endothelial Cells

Blood and blood vessels are closely linked in developmental biology. In the embryo, the hematopoietic and endothelial lineages are generated from a common mesodermal progenitor, the hemangioblast [25, 26]. Our group and others have demonstrated that adult hematopoietic stem and progenitor cells also exhibit this hemangioblast activity [27–30]. Given that AML cells arise from malignant hematopoietic stem and progenitor cells, it is therefore possible that there may also be a leukemia hemangioblast—generating both malignant leukocytes and malignant ECs.

In one report, a subpopulation of vascular progenitor cells (VEGFR2+ $CD31^-$ $CD34^-$) harboring the *BCR/ABL* gene fusion was identified in the BM of patients with CML [31]. These cells possessed the potential to form malignant hematopoietic and endothelial cells *in vitro* at the single-cell level. Moreover, when transplanted into NOD/scid mice, these $VEGFR2^+$ $CD31^-$ $CD34^-$ cells were capable of reproducibly transferring CML to transplanted mice and generating ECs within blood vessels that expressed *BCR/ABL*. In other studies, it was shown that transformed genotypes including *BCR/ABL* and the Janus kinase 2 (JAK2) V617F mutation are not readily found in colonies generated in endothelial colony forming cell (ECFC) culture conditions, whereas angiogenic monocytes that form CFU-Hill colonies can harbor such mutations [32, 33]. Together, these results demonstrate the existence of an adult hemangioblast population even in settings of hematological malignancies; however, these data also suggest that ECs harboring cytogenetic mutations may not be derived from putative endothelial progenitor cells (EPCs), which are defined by their ability to form ECFC colonies, but instead from a population of hematopoietic-derived cells.

Certainly, the existence of a bipotential malignant hematopoietic stem cell could explain a source of relapsed disease and would represent a new target for therapy.

5. Potential Novel Therapeutic Approaches to Targeting Leukemia and Endothelial Cell Interactions

The importance of angiogenesis in AML has led to clinical studies of vascular targeting agents in patients with AML (Table 1). Both angio-inhibitory and vascular disrupting strategies are being studied.

Given the presence of VEGF in AML and signs of increased angiogenesis in the bone marrow, investigators have tested anti-VEGF strategies for the treatment of AML. Bevacizumab (anti-VEGF-A antibodies, Avastin) brings about modest clinical efficacy in the treatment of colon cancer when combined with cytotoxic chemotherapy [45]. In the case of AML, bevacizumab was proposed to inhibit

Table 1: Vascular targeting strategies for patients with acute myeloid leukemia.

Agent	References	Target	Phase of clinical study	# of Patients in AML trial(s)	Clinical activity
Bevacizumab	[34, 35]	VEGF-A	Phase 2	48, 9	None as monotherapy; minimal when combined with chemotherapy
Aflibercept	[36]	VEGF-A, VEGF-B, PlGF	Preclinical		
Sunitinib	[37]	VEGFR-1,-2,-3, PDGFRs, c-KIT, FLT3, CSF-1, RET	Phase 1	15	Minimal as monotherapy
Semaxanib	[38]	VEGFR-1, -2, c-KIT, FLT3	Phase 2	6	Minimal as monotherapy
Sorafenib	[39–41]	FLT3, VEGFR-2, -3, PDGFR, Raf, c-KIT	Phase 3	127	None
Axitinib	[42]	VEGFR-1, -2, -3, PDGFR-β, c-KIT	Phase 2	12	Minimal as monotherapy
Cediranib	[37]	VEGFR-1, -2, -3, PDGFR-β, c-KIT	Phase 1	35	Modest as monotherapy
Vatalinib	[43]	VEGFR-1, -2, -3, PDGFR-β, c-KIT, FMS	Phase 1	17	None with monotherapy; minimal when combined with chemotherapy
Combretastatin A-4-Phosphate (Zybrestat)	[44]	Microtubule depolymerization in endothelial cells, direct cytotoxicity to AML cells	Preclinical		
Combretastatin A-1-Phosphate (OXi4503)	[15]	Microtubule depolymerization in endothelial cells, direct cytotoxicity to AML cells	Phase 1	Ongoing	

leukemia cells by two proposed mechanisms: first, when considering that AML myeloblasts can express VEGF isoforms and VEGFR2, anti-VEGF therapies may have a direct inhibitory effect on malignant myeloblasts. Second, VEGF is an important factor in the angiogenesis of the leukemia niche. Therefore, it was reasoned that bevacizumab may result in AML regression in patients. Two clinical studies of bevacizumab in relapsed and refractory AML have been reported [34, 35]. Dr. Karp and colleagues at Johns Hopkins conducted a phase II clinical study of bevacizumab using a timed sequential therapy approach in 48 patients with either relapsed or refractory AML. Cytarabine was administered on days1–4, followed by 40–60 minutes of mitoxantrone, and finally bevacizumab on day 8. Serum VEGF levels were elevated prior to bevacizumab infusion on day 8 and decreased markedly after infusion. The clinical outcomes of this time sequential therapy showed complete response in 33% of patients, partial response in 15%, and no response in 35%. In the 33% of patients with complete response, the median disease-free survival was about 7 months. These clinical outcomes in a heavily pretreated group are higher than expected with chemotherapy alone and suggest an additive effect of bevacizumab in remitting disease. In the second study, Mesters and colleagues assessed the activity and efficacy of single agent bevacizumab in a small trial of 9 patients with relapsed and refractory AML. In their study, VEGF expression in the bone marrow was decreased after bevacizumab; however, there was no decrease in VEGFR2 and VEGFR2y, suggesting little inhibition of their phosphorylation activity. Furthermore, despite reduced VEGF expression in the bone marrow, there was no significant decrease in blast count after bevacizumab monotherapy. Indeed, we have found similar findings of minimal benefit in preclinical models of AML [15]. Overall, there is low enthusiasm for single agent bevacizumab in AML. However, anti-VEGF-A antibodies may be useful in combination with other chemotherapeutic or vascular disrupting agents.

Another agent that binds and targets the VEGF ligand, and with higher affinity than bevacizumab, is aflibercept VEGF trap. This fusion protein binds to VEGF-A, VEGF-B, and placental growth factor (PlGF). Aflibercept has only been evaluated in solid tumors for tolerability and shows modest antitumor activity [36]. In AML, the study of aflibercept has been restricted to human AML through *in vitro* and mouse xenograft models. The preliminary *in vivo* studies of Lal et al. showed that aflibercept slowed disease progression in two systemic human AML mouse xenograft models. Combining aflibercept with doxorubicin enhanced antileukemia effects, decreased microvessels, and induced perivascular apoptosis [46].

Another strategy in targeting VEGF activity is to target VEGFRs. In specific, small molecule tyrosine kinase inhibitors (TKIs) have been designed to impair VEGFR phosphorylation activity.

Vatalanib, active against VEGFRs and PDGFRs, is well tolerated and has shown clinical activity in multiple solid tumors. Vatalanib has been tested in AML and MDS. A two-armed phase I clinical trial of dose-escalated vatalanib showed that the agent is well tolerated in AML and MDS patients with minimal side effects [43]. Hypertension was reported and mitigated by medical management. A vatalanib dose of 750 mg by mouth daily was established as

the maximum tolerated dose (MTD). While the safety profile for this TKI is favorable, the clinical efficacy of Arm 1 monotherapy showed no significant response to treatment, with the greatest efficacy in 2 patients that had prolonged disease stabilization. Combination therapy resulted in 5 complete remission events. This study supports the recurring theme of minimal efficacy in terms of monotherapy small-molecule TKIs in the treatment of AML.

Cediranib, which was designed with even greater affinity for VEGFR-1 and VEGFR-2, has also shown clinical activity in certain solid tumors such as glioblastoma multiforme and nonsmall cell lung cancer [47, 48]. This TKI also inhibits receptor signaling at nanomolar ranges against c-kit, PDGFR-β, and VEGFR-3. In AML, a phase I clinical study of cediranib in 35 leukemia patients showed a correlation between cediranib exposure and plasma VEGF levels and dose- and time-dependent reductions of soluble VEGFR-2 [37]. Also, although there was no connection between clinical activity and microvessel density from treatment, the majority of patients who received the maximum tolerated dose (30 mg/day) did show significant decreases in their bone marrow MVD. In terms of clinical response, only modest benefit was reported. Only four patients out of 31 evaluable subjects showed an objective response. Taking this into consideration, future clinical studies will consider cediranib at 20 mg and 30 mg in combination with standard induction chemotherapies to improve clinical efficacy.

In AML, activating mutations in FLT3, especially internal tandem duplications, predict for a higher chance for refractory and relapsed disease. Thus, the current standard of care is to refer patients with FLT3 mutant AML for allogeneic hematopoietic cell transplant (allo-HCT), as this is the only potential for cure. However, few older AML patients are candidates for allo-HCT because of comorbidities, difficulties in finding a donor and financial/insurance reasons. Therefore, attempts have been made to target FLT3 activity with inhibitors.

Sorafenib, a TKI designed to target Ras-Raf/MEK/ERK signaling, but also targets FLT3, has shown clinical activity in renal cell carcinoma and hepatocellular carcinoma [49, 50]. Given that approximately 30% of AML patients have an activating FLT3 mutation, sorafenib was recently tested in AML patients to establish feasibility [39]. Metzelder et al. administered sorafenib in 8 AML patients (FLT3 mutant) between 2007 and 2010. All patients showed rapid hematological responses and complete molecular remissions were observed. The study is ongoing and longer followup is needed and planned [40]. On a much larger scale, Serve et al. conducted a multicenter, randomized, placebo-controlled, double-blind trial of this sorafenib in AML. Investigators administered oral sorafenib versus placebo in combination with standard induction chemotherapy (seven days of cytarabine and three days of idarubicin chemotherapy followed by two cycles of intermediate dose AraC consolidation therapy in 197 AML patients over the age of 60 [41]). One hundred two patients received sorafenib (400 mg daily) and 95 patients received placebo. Hand-foot-skin reactions which were commonly seen in early phase solid tumor trials were also observed in a few AML patients ($n = 5$) receiving sorafenib. Prior to the consolidation AraC cycles, there was a trend in slower regeneration of leukocytes and thrombocytes in the sorafenib arm. However, in terms of clinical response, there were no improvements in event-free survival and overall survival compared to the placebo group. This trial suggests that while targeting FLT3 with sorafenib is tolerable in AML, it shows little clinical activity.

Sunitinib (SU11248) is another angio-inhibitory TKI that has been tested as an antineoplastic agent [37]. In addition to targeting c-Kit, VEGFRs, and PDGFRs, sunitinib also inhibits FLT3. This inhibition profile, therefore, made sunitinib an attractive agent for AML—especially for patients with high risk FLT3 activating mutation. In a phase I study of 15 patients with refractory AML, patients received sunitinib 50 mg daily. No dose limiting toxicities occurred. Adverse events were limited to grade 2 edema, fatigue, and oral ulcerations. There were two fatal hemorrhages, which were potentially related to underlying disease. Escalating the dose to 75 mg resulted in grade 4 toxicities of fatigue, hypertension, and cardiac failure and led to the abandonment of the dose level. In terms of efficacy, there were only partial responses of short duration. Levels of both plasma VEGF and plasma FLT3 ligand (FL) significantly increased from baseline in most of the 16 evaluated patients with no correlation to clinical responses. The significance of VEGF plasma level increases has been reported in other clinical studies and may owe itself to the hypoxic induction of VEGF, whereas the significance of FL has yet to be determined. Given the adverse events at low doses of sunitinib and minimal clinical response, there is low enthusiasm to continue testing this TKI as a monotherapy agent. However, this multitarget agent may enhance response to other agents such as chemotherapy and/or vascular disrupting agents.

The small-molecule TKI semaxanib (SU5416) targets the common VEGF receptors 1 and 2, cKit, and FLT3. In a multicenter phase 2 trial of semaxanib, 42 patients with advanced, c-kit positive AML, either refractory or elderly patients not fit for intensive induction chemotherapy, received at least one dose of treatment [38]. At a dose of 145 mg/m^2 twice a week, the drug was well tolerated, with mostly mild to moderately severe adverse events. The most striking adverse event, not seen in solid tumor studies of semaxanib, was severe bone pain, which may be attributed to the activity of the drug in bone marrow. Of 25 patients who were evaluable for clinical response, 1 patient achieved a morphological response followed by a relapse after 8 weeks, and 7 of the 25 patients achieved partial response with decreases in bone marrow and peripheral blood leukemic blasts of at least 50%. In terms of biological response, VEGF levels and bone marrow MVD correlated with each other and decreased after semaxanib treatment. Again, the modest clinical activity of this small-molecule TKI dampens enthusiasm for monotherapy in AML. However, the agent did show biological activity and this merits further study, including combination therapy.

Due to their multitargeted potential, TKIs could open the door to more personalized cancer treatments. However, many are metabolized through hepatic cytochrome P450 enzymes and have the potential to interact with many

medications commonly prescribed to patients with AML (e.g., antifungal agents such as azoles and chemotherapy). It is conceivable that in the future, AML cell surface expression or genetic mutation may dictate what type of TKI to prescribe. This is particularly attractive in older patients with AML who may be ineligible for high intensity chemotherapy. These older patients may fare better with an oral TKI and low intensity chemotherapy.

Our own studies have led further efforts to develop multitargeted therapies. Our lab has identified a promising multi-target antivascular treatment strategy, using a novel endothelial cell targeting agent, combretastatin A-1 (OXi4503) [15], alone and in combination with bevacizumab. Combretastatins were discovered in the 1970s from the South African Bush Willow. These agents are structurally similar to colchicine and, like colchicine, bind to β-tubulin, lead to microtubule depolymerization and selectively target rapidly proliferating ECs. Unlike colchicine, combretastatins exhibit vascular disruption below maximum tolerated dose (MTD). Therefore, these agents are highly attractive for clinical translation. We and others have shown that naturally occurring combretastatins, CA4 and CA1, potently regress AML in xenograft models. We also showed that the combretastatin CA1 results in a VEGF-driven reactive angiogenesis which supports disease relapse. Therefore we devised a strategy to combine combretastatin (CA1) and anti-VEGF antibodies (bevacizumab). This novel strategy resulted in potent regression of AML. We therefore translated this work into a phase I clinical study of CA1 (OXi4503) in patients with relapsed and refractory AML and MDS (ClinicalTrials.gov Identifier NCT01085656). After establishing MTD, we plan to combine combretastatins with other angio-inhibitor agents like bevacizumab in future clinical trials.

6. Summary and Future Directions

Whereas cancer angiogenesis is classically thought of in context to solid tumors, there is mounting evidence that angiogenesis is also significant in leukemia. With specific regard to leukemia and endothelial cells, there are several aspects to consider. First, endothelial cells can support leukemia cells via secreted factors. Whereas a few key axes have been identified (e.g., VEGF/VEGFR and Ang-1/2/Tie2), the full panoply of secreted factors has yet to be defined. Second, leukemia cells can promote endothelial cells. The observation of a codependent relationship creating a vicious cycle of support substantiates the strategy of using endothelial cell targeting agents such as combretastatins. Third, studies identifying critical adhesion molecules between leukemia and endothelial cells are lacking, and this represents an open area of research. These studies will enlighten us of how leukemia cells enter and exit the bone marrow. Understanding how sinusoidal endothelial cells—gatekeeper cells of the marrow—regulate emigration and immigration will lead to novel strategies for mobilizing leukemia out of protective niches and towards heightened sensitivity to treatment. Fourth, leukemia cells can coexpress endothelial cell features. How these features impact sensitivity and protection from conventional treatment need to be better elucidated. Part of these studies will involve inhibiting endothelial cell-associated expression, and it will be important to determine whether this inhibition results in increased sensitivity to treatment. If so, then this would represent a novel strategy for sensitizing leukemia-EC hybrids to treatment. Fifth, the vascular niche in the bone marrow contains many other cell types. Two in particular, perivascular pericytes and intercalated megakaryocytes, participate in the way that the sinusoidal vessels regulate hematopoietic stem and progenitor cell function. Therefore, it is reasonable to consider these other cells types as important players in the governance of leukemia behavior. Finally, beyond cell types, there are many noncellular elements of the bone marrow microenvironment that need to be assessed. These bone marrow conditions include chronic physiological hypoxia, low pH, low glucose levels, and extracellular matrices. All of these factors affect angiogenesis and vice versa; therefore, these factors need to be defined in the context of leukemia pathology to have a more complete picture of how the disease develops, responds to treatment, and relapses.

One important clinical translation of a better understanding of the relationship between angiogenesis and leukemia is in the realm of improved diagnostics. Currently bone marrow biopsies and repeated blood draws are the mainstay for diagnosis, prognosis, and response assessment. With advent of new biologics that have antiangiogenic and antivascular activity, there is a calling for novel biomarkers and methods to measure response and predict for responders. Through the development of a pre-treatment biomarker screening method, we may be able to utilize existing and future antiangiogenic agents to their full potential on a more personalized basis.

Another clinical translational consideration is how to assess treatment response after administering vascular targeting agents in AML. Currently, there are no established methods. Consider that the primary target is bone marrow blood vessels, it would reason to follow that changes in bone marrow vascularity indicate treatment response. However, there are several methods to quantify bone marrow vascular activity. Serial bone marrow biopsies for measurement of microvessel density, EC function, and angiogenic cytokinesare involve procedures that are painful and possibly affected by sampling area. Furthermore, histologic and biochemical testing takes time and do not provide results in real time. Another method to monitor bone marrow blood vessel activity is via serial computed tomography (CT) scans. However, this method exposes the patient to harmful ionizing irradiation and intravenous contrast carries the risk for nephrotoxic reactions. Magnetic resonance imaging (MRI) is another method to measure changes in bone marrow blood vessels. This method is rapid and does not deliver harmful ionizing irradiation. Moreover, the risks of a contrast (gadolinium) toxicity is lower than with CT scans. Recently, two groups have shown early data that dynamic contrast enhanced- (DCE-) MRI can be used to assess bone marrow vascular perfusion and predict for response to chemotherapy [51, 52].

The last clinical translational consideration is that many of the vascular targeting clinical studies in AML have lacked investigation of mechanisms of action. It is assumed that the administered antivascular agents operated through blood vessel targeting mechanisms, but without an accurate measurement of response this theory has yet to be proven. Thus, it is imperative that rationally designed biomarkers be used to evaluate response to vascular targeting agents. In addition, AML cells themselves can aberrantly express an endothelial-like phenotype, and therefore may be direct targets of vascular targeting agents.

Finally, defining how leukemia exploits the bone marrow vascular niche may lead to promising new therapeutics. We have already translated the vascular disrupting agent, CA1P (OXi4503), into the clinic, and there are many more on the way. As with many antiangiogenic and antivascular agents, cytopenias have been observed in early clinical studies. This is to be expected considering the crosstalk between angiogenesis and hematopoiesis. Therefore, the application of these novel agents into the leukemia unit and clinic will require careful administration and an "induction" mindset. Blood product transfusions and prophylactic antibiotics will likely be required in these AML patients. However, with adequate support, these agents may show promising results over time.

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Review Article

The Role of PTEN in Tumor Angiogenesis

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During the past 20 years, the phosphatase and tensin homolog PTEN has been shown to be involved in major physiological processes, and its mutation or loss is often associated with tumor formation. In addition PTEN regulates angiogenesis not only through its antagonizing effect on the PI3 kinase pathway mainly, but also through some phosphatase-independent functions. In this paper we delineate the role of this powerful tumor suppressor in tumor angiogenesis and dissect the underlying molecular mechanisms. Furthermore, it appears that, in a number of cancers, the PTEN status determines the response to chemotherapy, highlighting the need to monitor PTEN expression and to develop PTEN-targeted therapies.

1. Physiological Angiogenesis

Vasculogenesis and *angiogenesis* are two distinct processes, whereby the first represents vessel formation from differentiated precursors while the second originates from the preexisting vasculature. Capillaries formed during these processes are mainly constituted by endothelial cells which face the lumen of the vessel and sometimes, depending on their size, are surrounded by mural cells comprising pericytes and smooth muscle cells. Angiogenesis is essential during development while in adulthood the vasculature is usually quiescent, except during wound healing and the female reproductive cycle [1]. It is governed by several factors secreted by the targeted tissues and consists of four steps. The first step originates from the existing vessel from which the sprout arises. The combination of nitric oxide and Vascular Endothelial Growth Factor (VEGF) enhances vessel permeability by increasing capillary dilatation and leakiness, respectively. This allows extravasation of various plasma proteins which facilitate endothelial cell migration. Second, endothelial cells, to invade the hypoxic tissue, have to detach from the basement membrane they are laying on. This is mediated by the secretion of proteases such as proteins from the matrix metalloproteinase family (MMP-2, -3, -9) or through the inhibition of protease inhibitors such as TIMP (tissue inhibitor of MMP) proteins family. Third, after endothelial cells have detached, they proliferate and migrate to invade the hypoxic area, the source of proangiogenic factors, until they find contact to another capillary. During this process, as during axonal guidance, some cells lead the elongation of the sprouting vessel towards the angiogenic chemoattractant source. The endothelial cells forming the sprouting blood vessels exhibit distinct phenotypes. We can distinguish the tip cells at the leading edge of the sprout, stalk cells which follow the tip and phalanx cells which are quiescent cells from the mature vessel. Fate determination of the tip cells is dynamically regulated by VEGF-A/VEGFR1 and 2 and Notch/Dll-4 signaling pathways. Briefly, endothelial cells having the greater VEGFR2 to 1 ratio and higher Dll4 expression are more likely to adopt and keep the tip cell phenotype. VEGF-A-stimulated candidate cells will, therefore, signal through VEGFR2 which has a lower affinity for this ligand but a better protein kinase activity. VEGFR2 signaling leads to Dll-4 expression, activating Notch on the neighbour cell. Notch signaling in these endothelial cells leads to VEGFR1 upregulation while VEGFR2 is downregulated reducing the chance to become a tip cell. This system results in fate determination of the tip cells while keeping their neighbouring cells under a stalk cell phenotype; however, these phenotypes are not fixed over time [2]. First, the neighbourhood of tip cells is constantly changing due to endothelial cell migration, and this impacts on cells' VEGFR

expression. Second, VEGFR2 to 1 ratio determines the time length of tip cell turnover, and, altogether, this result in an oscillatory Dll4 expression. Third, the main source of VEGF-A may differs during vessel elongation. After formation of tip cells, the sprout elongates through proliferation of stalk cells and reaches its target under the drive of tip cells. During the last step, cells start to differentiate and form a tube which will be stabilized through the recruitment of mural cells and secretion of extracellular matrix (ECM) [3]. An intact, functional vasculature requires a right balance between pro- and antiangiogenic factors; therefore, physiological angiogenesis is the result of a tightly controlled excess of proangiogenic factors. By contrast, tumor angiogenesis (Figure 1) originates from a disturbed balance between pro- and anti-angiogenic factors rendering endothelial cells unable to become quiescent and consequently support a constant growth of new blood tumor vessels [4]. In this work, we will focus on the following factors involved in tumor angiogenesis: VEGF/VEGFR, angiopoietins, and their receptors: Tie-1 and -2, Eph receptors, and their ephrins ligands, HIF1α (hypoxia-inducible factor 1 alpha), MMPs, and delineate their relationship with the important tumor-suppressor PTEN (phosphatase and tensin homologue).

2. Key Players in Angiogenesis

VEGF is the most important regulator of endothelial cell fate and acts as ligand of the VEGF receptor which belongs to the receptor tyrosine kinase (RTK) superfamily. VEGF is phylogenetically ancient: in *Drosophila* it is thought to be involved in blood cell positioning and comprises only one member; however, in mammals, 5 members of the VEGF family: VEGF-A, -B, -C, -D, and placenta growth factor: PLGF have been described. VEGF-A plays a prominent role in angiogenesis as shown by genetic studies of VEGF-A knock-out mice which die embryonically [5]. VEGF-A interacts preferentially with two of the three human members of VEGF receptors: VEGFR1 (Flt-1) and VEGFR2 (Flk1), while VEGF-B and PLGF bind to VEGFR1. Here we will not discuss in details VEGF-C and -D, which bind to VEGFR3 (Flt-4) and are involved in lymphangiogenesis. VEGF signaling through VEGFR1 and -2 results in an upregulation of proteases expression, recruitment of mural cells due to PDGFβ secretion, increased vessel permeability, endothelial cell proliferation, migration, survival, and specialization [6].

Angiopoietin (Ang) ligands bind to *Tie* receptors, which are single transmembrane RTK. These proteins possess three immunoglobulin-like (Ig-like) domains, three epithelial growth factor domains (EGF) and three fibronectin type III domains giving the name Tie: Tyr-kinase with Ig and EGF homology domains. The two isoforms of Tie: Tie-1 and -2 are involved in vascular development as demonstrated by loss-of-function studies [7, 8]; however, Tie-2 is much more studied than its isoform. Tie-2 is required early in embryonic development for plexus remodeling and maturation. Tie-2 is predominantly expressed on endothelial cells and principally binds to Ang-1 and -2 which are agonist and antagonist ligands, respectively. Ang-1 stimulation of Tie-2 leads to endothelial cell survival and migration, limits vessel permeability, and stimulates smooth muscle cells recruitment. Tie-2 signaling triggers a negative feedback loop involving the forkhead transcription factor FOXO1 and Ang-2 expression [9].

Another system of receptor/ligand involved in angiogenesis regulation is the Eph/ephrin couple. *Eph receptors* (first cloned from an erythropoietin producing hepatocellular carcinoma cell line) belong to the RTK superfamily and are involved in embryogenesis, axonal guidance, and angiogenesis [10–12]. Eph receptors are divided into two subfamilies: A and B, which generally bind to ephrin A and B ligands, respectively. In contrast to the other RTK whose ligands are secreted molecules, ephrin ligands are membrane bound. This renders this couple quite unique as (a) it requires two neighbouring cells to signal, and (b) numerous studies have demonstrated that this signal is transmitted by both receptor and ligand. This bidirectional signaling is involved in the regulation of the cytoskeleton leading to modulation of cell adhesion, repulsion, and motility and contributing to cell positioning [13]. Wang et al. first showed that EphB4 and ephrinB2 are key players in the development of the vascular system by the use of transgenic mice. They demonstrated that EphB4 is restricted to veins while ephrinB2 is expressed only by arteries, and a lack of one of these actors is lethal due to perturbed arteriovenous differentiation [14]. Indeed ephrinB2 reverse signaling was demonstrated to participate to tip cell guidance through control of VEGFR2 signaling guarantying a successful vasculature formation [15]. Moreover, ephrinB2 was shown to contribute to vessel maturation as it is expressed on mesenchymal cells such as smooth muscle cells, and its binding to EphB leads to mural cells recruitment [16].

As angiogenesis responds to an increased need in oxygen, hypoxia is a crucial part of the process. The main effector of the adaptive response to hypoxia is the transcription factor HIF1. HIF1 is a member of the basic helix-loop-helix/PAS protein transcription factor family and comprises HIF1α and HIF2α, which to be active require heterodimerization with HIF1β [17]. The α subunit acts as an oxygen sensor as it is degraded under normoxia while the β subunit is constitutively expressed. In normoxic condition, the prolyl hydroxylase enzymes (PHD1-3) are in an active state and hydroxylate HIF1α on two proline residues (402 and 564) within its oxygen-dependent degradation domain (ODD) [18]. Hydoxy-HIF1α is recognized by the complex formed by the ubiquitin ligase VHL (von Hippel-Lindau) and the elongin proteins, and this binding triggers the rapid HIF1α degradation through the proteasomal pathway. In contrast, under hypoxic conditions, HIF1α forms a heterodimer with HIF1β, and their translocation to the nucleus acts on gene expression through their binding to the hypoxia response element (HRE). Numerous genes are regulated by HIF1, some related to red blood cell production (erythropoietin), some to the vascular architecture and tone (VEGF, VEGFR1, or Nitric Oxide Synthase 2), some to the metabolism (Glut-1 and Glut-3), and some to cellular proliferation and differentiation (TGFβ, Cyclin G2, or p21) [19]. In 2005 our group demonstrated an upregulation of EphB4, ephrinB2,

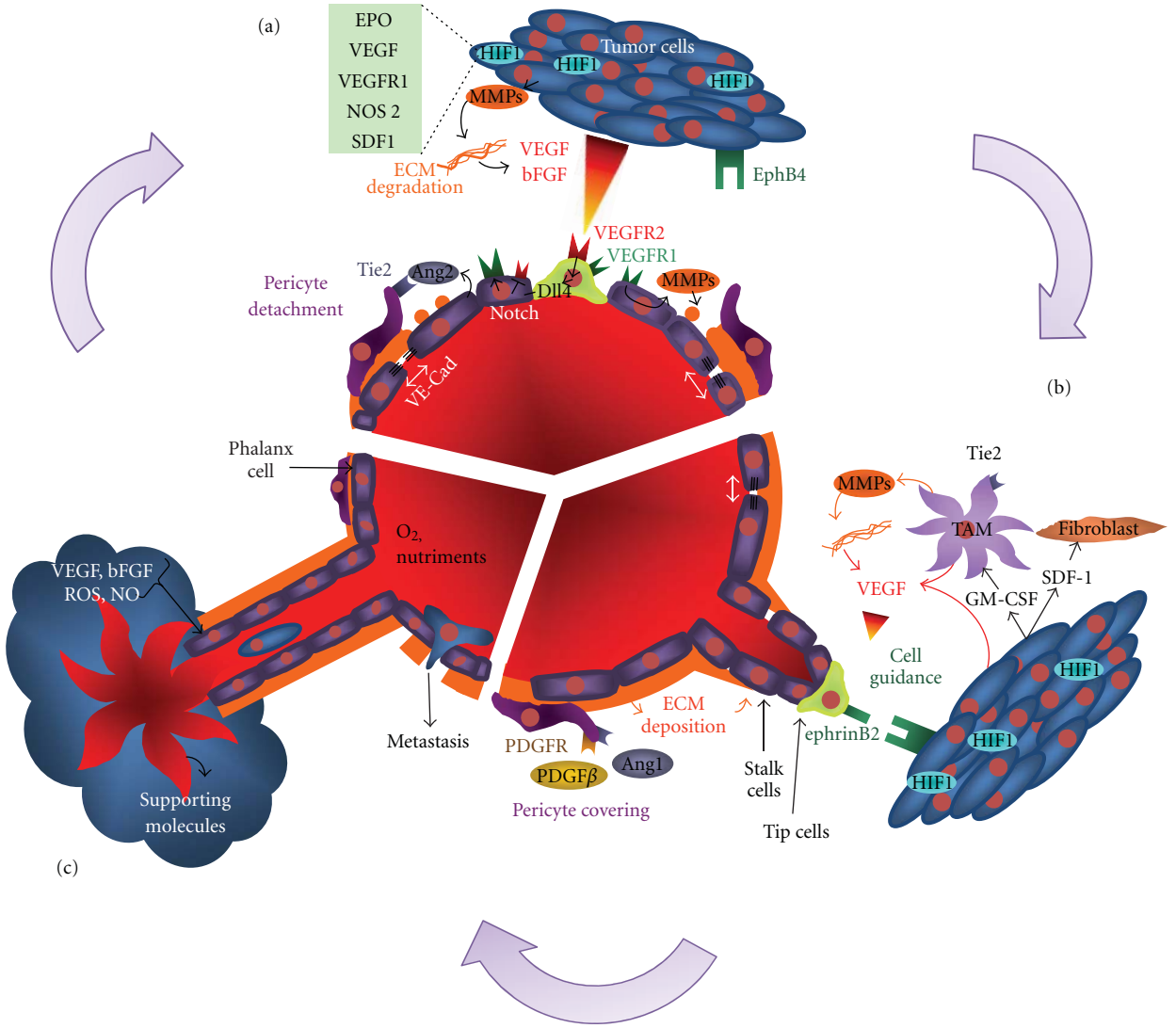

Figure 1: Molecular basics of tumor angiogenesis. Key players of tumor angiogenesis and their main functions are depicted here. (a) Once a tumor has reached a volume of 1-2 mm^3, tumor cells start to be hypoxic, and HIF1α is stabilized. Activated HIF pathway leads to the expression of several genes (within green square) which greatly contribute to VEGF production. VEGF amplify this system through VEGF-dependent MMPs expression involved in ECM degradation and growth factors release. VEGF acts as a chemoattractant on endothelial cells from the nearest vessels and triggers vessel sprouting. Stimulated VEGFR2 leads to the expression of Dll4, a Notch ligand which inhibits the tip cell transformation through VEGFR1 upregulation. VEGFR activation mediates proteases expression, VE-Cad complex disruption leading to cell/cell and cell/matrix detachment. In parallel, Tie2 stimulation by Ang2 induces pericytes detachment. This step is required for endothelial cell migration and proliferation. (b) While tip cells drive vessel elongation towards the source of VEGF and through EphB4/ephrinB2 signaling, tumor cells attract stromal cells. These stromal cells, comprising tumor-associated macrophages (TAM) and fibroblasts contribute to tumor angiogenesis through secretion of proangiogenic factors. During vessel elongation, new ECM is synthesized, and few pericytes will cover the neovessel. This—in conjunction with NO production and disruption of adherens junctions—results in a leaky vessel. (c) The resulting vasculature is tortuous with many dead ends and is prone to cell extravasation. Moreover, endothelial cells contribute to tumor growth by secreting supporting molecules in addition to carrying nutrients and oxygen.

EphA2, ephrinA1 mRNA and protein expression in hypoxic tissues [20].

Finally, one of the major sources of angiogenic factors is the ECM, first because it can store growth factors such as VEGF, second because degradation of some of its component leads to the formation of pro- or anti-angiogenic factors. Among the proteases involved in the release of such factors, MMP proteins which are members of a zinc-dependent family of endopeptidases and more precisely MMP2 and 9 are key players. MMP9 was shown to be activated in numerous cancers and to contribute to tumor angiogenesis by increasing the bioavailability of VEGF, basic fibroblast growth factor (bFGF) by degrading collagen types IV, XVIII and perlecan [21, 22]. Moreover, MMPs are involved in cell migration by interfering with the cell-ECM and cell-cell interactions.

3. Tumor Angiogenesis

All the factors mentioned above are not only involved in physiological but also in tumor angiogenesis. Tumor development is first limited to 1-2 mm^3 due to the lack of oxygen, nutrients, and growth factors. This restriction may be the cause of tumor latency until the so-called *angiogenic switch* occurs. Angiogenic switch is defined as the acquisition by tumor cells of properties required for their unopposed growth, where the balance is skewed towards a proangiogenic phenotype [28]. As tumor cells are in a hypoxic state prior to the angiogenic switch, the HIF1 pathway is constitutively active and leads to the expression of angiogenesis-related genes (VEGF/VEGFR, MMPs, Eph/ephrins). Noteworthy, even after the tumor vasculature is fully developed and functional, most of the tumor cells still maintain an activated HIF1 pathway [29]. As mentioned earlier, HIF1 target genes will lead to endothelial cell recruitment to the tumor site mainly through VEGF/VEGFR activity. This effect is amplified by tumor cells, which also have the capability to attract stromal cells as cancer-associated fibroblasts (CAF) [30] and tumor-associated macrophages (TAM) [31]. TAMs seem to play a pivotal role as they secrete VEGF, MMP9, and even immunosuppressive molecules. The resulting vessels are often dilated due to VEGF and leaky because of nitric oxide overproduction and deficient mural cell recruitment [32]. Tumor vessels are characterized by their irregular and tortuous shape consecutive to aberrant endothelial cell proliferation and often possess dead ends leading to a higher risk of hemorrhage. Therefore, a high tumor-associated microvascular density does not reflect high oxygen level in the tumor as blood flows only irregularly through tumor vessels, leading to hypoxic areas within the tumor. Beside the angiogenic factors directly depending on HIF1 pathway, Ang-2/Tie and Eph/ephrins receptor/ligand pairs have also been shown to play a role during the angiogenic switch. Cancer-dependent Ang-2 upregulation occurs principally in tumor-associated endothelial cells and can be used as a biomarker of tumor progression [33]. High levels of Ang-2 trigger endothelial cells apoptosis mediating vessel pruning. This leads to the formation of hypoxic area which in turn upregulates VEGF expression and results in higher vascular density [34]. The roles of EphB4 and ephrinB2 expressed on blood vessels are more controversial. Although several reports showed that EphB4 stimulation impairs tumor growth due to a defect in tumor angiogenesis [35, 36], Kumar et al. demonstrated the opposite [37–39]. Concerning ephrin reverse signaling, Martiny-Baron et al. demonstrated that blocking ephrinB2 stimulation by the use of monomeric soluble EphB4 impaired tumor growth in nude mice and correlates with a decreased microvessel density [40].

4. PTEN and Tumor Angiogenesis

A remarkable property of tumor angiogenesis is that all involved factors described earlier signal through the PI3 Kinase (PI3K) pathway [41–44]. The PI3K pathway is a signaling route involved in many cellular processes such as cell survival, proliferation, or migration. The PI3K protein class IA is activated in response to RTK stimulation, while the class IB is activated by G-protein-coupled receptors [45]. PI3K activation leads to the transformation of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). This in turn will activate Akt, a serine-threonine protein kinase which has numerous targets comprising the mammalian target of rapamycin (mTOR), a protein complex stimulated in hypoxic and nutrient-poor environment. The major regulator of this signaling pathway is PTEN, also known as MMAC1 (Mutated in Multiple Advanced Cancer1) or TEP1 (TGFβ-regulated and epithelial cell-enriched phosphatase). PTEN gene in human locates on chromosome 10q23.3 and encodes protein of 40–50 kilodaltons as in many organisms, with the exception of PTEN from *Caenorhabditis elegans*. This protein comprise 4 domains: at the N terminal a PIP2 binding site, then a phosphatase domain, a C2 domain containing phosphorylation sites, followed by a PDZ binding motif at the C-terminal end [46]. PTEN acts as a phosphatase on both lipids and proteins; it antagonizes PI3K pathway by transforming PIP3 into PIP2 (Figure 2) [47] and dephosphorylates proteins such as SHC or FAK [48, 49]. Several studies also report a role for PTEN in cell migration, independent of its phosphatase activity as the expression of a truncated form of PTEN possessing only the PTEN C2 domain inhibits cell migration [50, 51]. PTEN is regulated posttranscriptionally by miRNA, such as miR-21 [52] and posttranslationally through phosphorylation, acetylation, ubiquitylation, or by regulation of its localization [53]. As the PI3K pathway is activated during angiogenesis, PTEN can be considered as a major intracellular regulator of this process.

Before discussing the role of PTEN in tumor endothelial cells, we will first summarize its functions in cancer cells. PTEN is frequently found deleted, mutated, or downregulated in human malignancies. PTEN mutation primarily affects the PTEN phosphatase domain and leads to several diseases including Cowden's and the Bannayan-Riley-Ruvalcaba syndrome, the Lhermitte-Duclos disease, as well as an increased risk of breast, thyroid, and endometrial cancers (Table 1).

Regarding HIF1, PTEN was found to inhibit its stabilization and its transcription factor activity in glioblastoma cell lines [54]. Moreover, several studies report a concomitant loss of VHL and PTEN, two important regulators of the HIF1 pathway, in clear cell renal cell carcinoma [55, 56]. Since HIF1 signals through PI3K pathway, leading to an increased VEGF expression, PTEN loss in cancer cells leads to an increased VEGF expression due to upregulation of HIF1. This was shown by several investigators using different models: Fang with PC-3 cells [57], Tian with HepG2 cells [58]. Takei et al. [59] demonstrated that PTEN reintroduction led to a decreased HIF1-α, VEGF, and PCNA expression in ovarian cancer cells. As to MMPs, a study using a multiple myeloma cell line and cells originating from patients showed that PTEN transfection results in a decreased mRNA and protein expression of MMP2, MMP9 and FAK (Focal Adhesion Kinase), leading to decreased cell migration [60]. Furthermore, microarray analysis of gastric

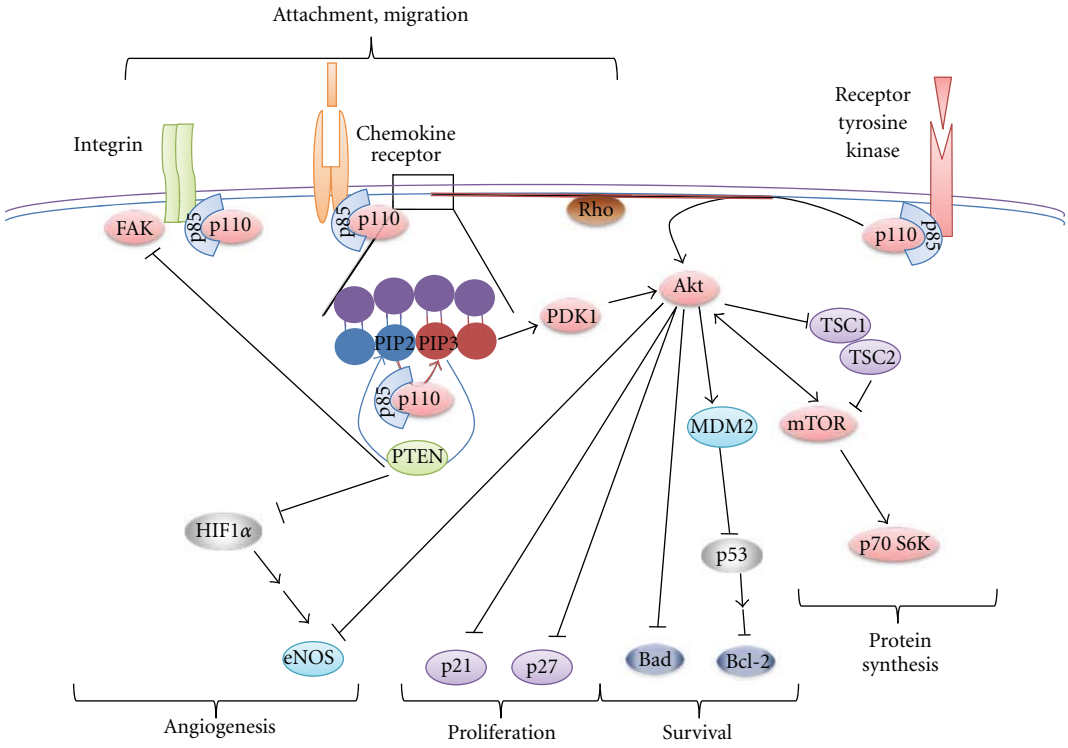

Figure 2: The PI3 kinase pathway and PTEN.

Table 1: PTEN-related diseases and associated cancer susceptibility. Percentages are indicated when available.

Disease	PTEN defect	Clinical symptoms	Cancer susceptibility
Cowden's syndrome [23, 24]	Splice variants Deletion: coding sequence promoter (10%) Nonsense mutation Missense mutation (85%): C124: no phosphatase activities, G129: no lipid phosphatase activity, K289: no nuclear translocation	Trichilemmonas, macrocephaly, papillomatous papules	Breast (65%), thyroid (75%), endometrium (5–10%)
The Bannayan-Riley-Ruvalcaba syndrome [23, 24]	Deletion (11%) Nonsense mutation Missense mutation (60%)	Macrocephaly, intestinal polyposis, developmental delay, lipomas, speckled penis in male	Breast, thyroid, endometrium, rare colorectal carcinoma
The Lhermitte-Duclos disease [25]	Splice variants Deletion Nonsense mutation Missense mutation (80%)	Ataxia, increased intracranial pressure, seizures	Not demonstrated
Proteus/Proteus-like syndrome [26]	Missense mutation (20 and 50%, resp.)	Epidermal nevus, disproportionate overgrowth of the skull, limbs, vertebrate, Lipomas, vascular malformation	rare events: cystadenoma of the ovary, testicular tumors, central nervous system tumors, parotid monomorphic adenomas
Autism [27]	Missense mutation (around 10%)	Sometimes associated with macrocephaly	Not demonstrated

carcinomas highlighted a negative correlation between PTEN expression and VEGF, MMP2, and MMP9 expression, and the authors concluded that PTEN has an inhibitory effect on microvascular density [61]. PTEN loss at post-transcriptional level is also involved in tumor angiogenesis. Giovannetti et al. [62] reported that in pancreatic ductal adenocarcinoma, miR-21 is responsible for elevated expression of MMP2, MMP9, and VEGF and this could be abrogated by treatment of the cells with a PI3K inhibitor. As miR-21 is overexpressed in many tumors, this strategy may represent a valuable tool to control tumor angiogenesis [63]. Concerning the Ang/Tie system, it was shown that Ang-1 stimulation of Tie-2 triggers PI3K pathway activation [64]. Moreover, Findley et al. described a Tie2 receptor shedding which is able to bind Ang-1 and -2 and consequently inhibits Tie2 phosphorylation and activation [65]. This process is regulated by VEGF-dependent activation of PI3K pathway, and PTEN overexpression increases Tie-2 shedding.

PTEN localization within the cell is also part of PTEN regulation, since defective PTEN translocation to the cell membrane impairs its control of PI3K signaling [87]. This was shown by Molina et al., who found that the adaptor protein NHERF1 is involved in PTEN translocation to the cell membrane [88]. They proved that impaired PTEN positioning leads to sustained Akt activation. This can be of primarily importance as NHERF1 has been shown to interact with several RTK such as PDGFRβ and EGFR [89, 90], which all play a role in tumor angiogenesis. Moreover, PTEN translocation to the cell membrane is not solely involved in regulation of RTK's downstream signaling, but is also emerging as a modulator of their expression [91]. Recent work from our lab has shown that PTEN also interacts with EphB1 signaling (manuscript submitted). As mentioned above, Eph receptors and ephrins are frequently overexpressed by cancer and tumor endothelial cells, and this may lead to an enhanced tumor angiogenesis. Among the various pathways activated by Eph receptors, PI3K has been reported in a number of studies [13, 92]. Moreover, Brisbin showed a direct linkage between VAB-1 and DAF-18, the *C. elegans* form of Eph and PTEN, respectively [93], an interesting observation in line with our findings. As during tumor angiogenesis both cancer and endothelial cells are involved, PTEN status in endothelial cells is also crucial. Many studies have shown that PTEN expression can be disturbed in both stromal cells and endothelial cells [94, 95]. This can be primordial as reduced PTEN signaling in endothelial cells as well as PI3K activation leads to an enhanced cell proliferation, survival, and migration—all important features for angiogenesis [96–98]. Moreover, tumor vessels are characterized by their inability to become quiescent, and it was recently shown that PTEN is involved in endothelial cell aging [99].

Interestingly, PTEN is susceptible to oxidation. As highly proliferative cells overproduce reactive oxygen species (ROS), this mechanism may account for the reduction of PTEN levels in cancer and cancer-related cells [100]. A vicious circle, therefore, begins as cancer cells produce more ROS, affecting PTEN level and enhancing VEGF secretion [101, 102]. VEGF in turn acts on endothelial cells and stimulates ROS production mainly by NOX1 (NADPH Oxydase 1) [103], potentially affecting PTEN level as well as cell proliferation. VEGF will also amplify endothelial cell migration and contribute to the angiogenic switch enhancing tumor growth [104]. Another derivative of oxygen is NO, which also plays a role in angiogenesis as it contributes to vessel dilatation. Church et al. demonstrated that PTEN is involved in endothelial Nitric Oxide Synthase regulation in both cancer and endothelial cells [105] and should, therefore, affect tumor angiogenesis.

Cancer cells have a supportive effect on endothelial cells; however, the reverse is also true, with the formation of the so-called vascular niche. Koistinen et al. demonstrated that VEGF-stimulated endothelial NO production maintains Acute Myeloid Leukemia (AML) cell growth, and this involved the PI3K pathway [106]. Several examples of endothelial cells stimulating solid tumor growth have also been described. VEGF promotes endothelial cell survival through PI3K activation and increased expression of the anti-apoptotic protein Bcl-2, and this can be antagonized by an increased PTEN expression [107]. Recently, it was shown that Bcl-2-expressing endothelial cells also produce IL-8, which acts on tumor cells and leads to an increased invasiveness and metastatic ability [108]. Finally Park et al. demonstrated that Nerve Growth factor (NGF) stimulates endothelial cells to produce MMP2, a process inhibited by PTEN transfection [109].

5. Anti-tumor Therapies and PTEN

It now clearly appears that PTEN is involved in all the different steps leading to tumor angiogenesis. As a consequence, although the PI3K pathway is considered as the major signaling node in physiological and tumor angiogenesis, PTEN can be seen as the main intracellular antagonist to this process. Many efforts have been made to control tumor angiogenesis and related tumor growth, but here again PTEN status appears to be critical [110, 111]. Initial strategies to restrict tumor-associated angiogenesis used anti-VEGF compounds such as monoclonal antibodies against VEGF-A (Bevacizumab) or VEGFR tyrosine kinase inhibitors (Sorafenib, Sunitinib). However, after promising results in clinical trials, tumor resistance to these treatments has emerged as a major problem. These resistances are often related to a switch from VEGF- to bFGF- or EGF-dependent angiogenesis and require the HIF-1α and PI3K pathways signaling [112–114]. Many studies have now confirmed that resistance to anti-EGFR therapies is often linked to PTEN status in cancer cells, and PTEN loss negatively correlates with clinical response to these therapies [115, 116] or others (Table 2). This problem can be overcome by the use of combined therapies which show synergistic effects and are now tested in clinical trials [117, 118]. MMPs overexpression is another resistance mechanism. We previously discussed the negative correlation between PTEN and MMPs expression; therefore, cancer cells with reduced or lost PTEN activity are more prone to develop this kind of resistance. To avoid the emergence of such resistance, different strategies aiming

Table 2: Clinical trials having shown an impact of the PTEN status on the response to cancer treatment.

Type of cancer	Metastatic form	Treatments	References
Colorectal		Cetuximab, panitumab	[66–69]
	×	Cetuximab (+irinotecan)	[70–72]
Breast		Trastuzumab, lapatinib	[73–76]
		Trastuzumab	[76–78]
	×	Endocrine therapy	
Glioblastoma		Gefitinib, erlotinib	[79–81]
		Erlotinib + temozolomid	
Gastric		Streptozotocin, doxorubicin, 5-fluorouracil, etoposide/cisplatinum	[82, 83]
	×	Streptozotocin, doxorubicin	[82]
Lung		Gefitinib, erlotinib	[84, 85]
	×	Gefitinib, erlotinib	[85]
Pancreas		Gemcitabine	[62]
Esophageal		5-fluoropyrimidine, taxane, platinum, PI3K pathway inhibitor	[86]

at antagonizing the PI3K pathway or reintroducing the expression and/or activity of PTEN have been tried. Many PI3K inhibitors, as well as Akt and mTOR inhibitors, are now being tested in clinical trials [119, 120]. Although there are numerous inhibitors of the PI3K pathway, to date no PTEN inducer has been found, explaining why only PTEN transfection or gene therapy has been tried. Transfection of wild-type PTEN into human prostate cancer cells sensitizes cells to radiation and leads to a decreased tumor-induced angiogenesis [121]. In this work, both cancer- and tumor-associated endothelial cells were affected by this treatment. As previously mentioned, PTEN reintroduction decreases HIF-1α and VEGF levels [57–59], and Lee et al. showed promising results using this strategy in mice [122]. As PI3K inhibitors monotherapy has often shown mitigated results and gene therapy experiments are just emerging, PTEN-stimulating molecules are urgently needed. The greatest challenge, however, remains the difficulty to set up a robust PTEN activation model, where molecular stimulators of PTEN could be tested.

6. Concluding Remarks

A myriad of studies have now demonstrated the central role of the PI3K pathway in tumorigenesis and angiogenesis. Overactivation of this pathway, a common event in cancer cells, leads to enhanced and uncontrolled tumor angiogenesis and can be antagonized by the phosphatase and tumor-suppressor PTEN. Moreover, PTEN is involved in the control of cell proliferation, migration, and survival of both cancer and tumor-associated endothelial cells. Ideally, combining PTEN-targeted therapy to classical chemotherapy should be a promising strategy, leading to (a) normalization of the tumor vasculature, (b) sensitization of cancer cells to further chemo- and radiotherapy, and (c) avoidance of resistance to such treatments. Further progress and development of PTEN-targeted therapy are, therefore, required and may lead to improvement of the current anticancerogenic strategies.

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Review Article

Targeting Angiogenesis for Controlling Neuroblastoma

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Neuroblastoma, a progressive solid tumor in childhood, continues to be a clinical challenge. It is highly vascular, heterogeneous, and extracranial tumor that originates from neural crest. Angiogenesis, genetic abnormalities, and oncogene amplification are mainly responsible for malignant phenotype of this tumor. Survivability of malignant neuroblastoma patients remains poor despite the use of traditional therapeutic strategies. Angiogenesis is a very common and necessary pre-requisite for tumor progression and metastasis. Angiogenesis is also a major factor in making malignant neuroblastoma. Thus, prevention of angiogenesis can be a highly significant strategy in the treatment of malignant neuroblastoma. Here, we summarize our current understanding of angiogenesis in malignant neuroblstoma and describe the use of experimental anti-angiogenic agents either alone or in combination therapy. This review will clearly indicate the importance of angiogenesis in the pathogenesis of malignant neuroblastoma, its prevention as a promising therapy in preclinical models of malignant neuroblastoma, and prospective clinical trials.

1. Introduction

Neuroblastoma is the most common, extracranial, and heterogeneous solid tumor in children, and it accounts for approximately 15% of pediatric cancer deaths with an estimated incidence of 1 per 7000 births in the USA [1, 2]. This embryonal cancer of postganglionic sympathetic nervous system arises from adrenal gland and less frequently metastasizes in other places such as chest, neck, lymph nodes, pelvis, liver, and bone. The prognosis is variable and depends on several factors. Neuroblastoma is characterized by its intriguing clinical behaviors that include spontaneous differentiation and regression, maturation into benign ganglioneuroma, and deadly metastatic tumor. This childhood neoplasm is staged clinically according to the International Neuroblastoma Staging System (INSS) (Figure 1). The genetic features of neuroblastoma include *N-Myc* oncogene amplification or allelic loss, near triploid karyotype, deletion of short arm of chromosome 1, and high expression of neurotrophin receptors (TrkA and TrkB), all of which are associated with malignant transformation and progression of this disease. Multimodal treatment approaches including myeloablative chemotherapy, radionuclide therapy, immunotherapy, and apoptosis-inducing therapy are evaluated as traditional therapeutic strategies for controlling the malignant growth of the tumors. Despite aggressive conventional treatments and diagnosis techniques in neurosurgery, the survival rate for patients with neuroblastoma remains poor because the majority of children older than 1 year of age with advanced stage neuroblastoma die from progressive disease and only 40% of children over 4 years old with neuroblastoma survive for 5 years, emphasizing the urgent need for the development of innovative therapeutic strategies for treatment of malignant neuroblastoma. Malignant neuroblastoma is a highly vascularized solid tumor that requires access to blood vessels for growth, invasion, and metastasis [3]. Emerging treatments with the delivery of antiangiogenic molecules can thereby hinder neovascularization and arrest the spread of this pediatric tumor. Novel therapeutic approaches with the angiogenic

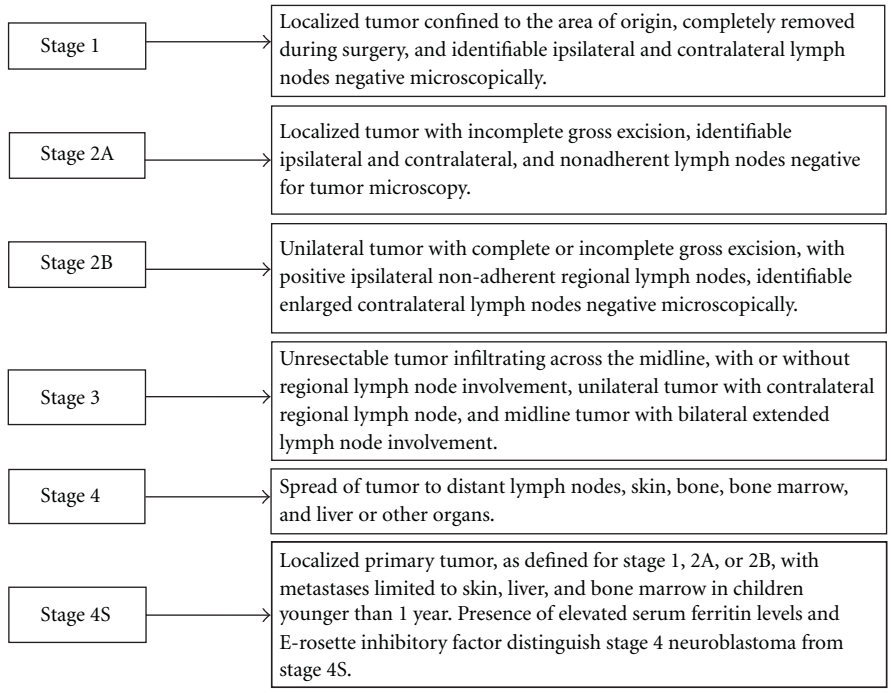

Figure 1: Staging system for neuroblastoma according to the INSS.

inhibitors are expected to improve patient survivability by reducing morbidity, mortality, and drug-related toxicity.

2. Angiogenesis in Human Neuroblastoma

Angiogenesis is a process of development of intrinsic vascular network, and it is a prerequisite for progression and metastatic spread of solid tumors like neuroblastoma where new capillaries sprout from preexisting vessels and the transition from avascular to vascular phase occurs via neovascularization. Tumor angiogenesis is characterized by cascade of events involving mainly dissolution of vascular basal membrane, increased vascular permeability, and degradation of extracellular matrix resulting in endothelial cell migration, invasion, proliferation, and tube formation [4–7]. Finally, the recruitment of perivascular supporting cells such as pericytes, subsequent inhibition of endothelial proliferation, basement membrane reconstitution, and structural reorganization into a functional complex formation stabilize the microvasculature.

Angiogenesis is mediated by multiple regulatory factors such as growth factors, adhesion molecules, and matrix degrading enzymes. Activation of endothelial cell proliferation and migration are mainly regulated by receptor tyrosine kinase ligands such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), and angiopoietins (Ang-1 and Ang-2). The naturally occurring endogenous angiogenesis inhibitors that affect neuroblastoma growth *in vivo* includes angiostatin, endostatin, tumstatin, canstatin, tissue inhibitors of matrix metalloproteinases (MMP), and so forth. An angiogenic switch actually maintains the balance between angiogenic activators and inhibitors and maintains the endothelial cells in an angiogenic or quiescent stage. Malignant growth of human neuroblastoma is highly dependent on angiogenesis. Therefore, anti-angiogenic strategies can be effective in inhibiting tumor cell dissemination and metastasis in highly vascular neuroblastoma [3–7].

3. Angiogenesis Stimulatory Factors in Human Neuroblastoma

3.1. Angiogenic Growth Factors and Their Implications

3.1.1. VEGF and VEGFR Family. VEGF (46 kDa) is an endothelial specific mitogen that plays a crucial role in pathogenesis and neovascularization of neuroblastoma. VEGF signaling plays a regulatory role in neuroblastoma angiogenesis via a paracrine mechanism through two specific tyrosine kinase VEGF receptors: VEGFR-1 (or Flt-1) and VEGFR-2 (or KDR) at the surface of the endothelial cells. The most potent angiogenic factor to promote endothelial cell proliferation is VEGF-A. Encoded by a single gene, VEGF-A has several isoforms such as VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}, which are generated by alternative exon splicing. The other members of the VEGF family include VEGF-B, VEGF-C, VEGF-D, and VEGF-E [8, 9]. VEGFR-2 is the major mediator of VEGF-induced endothelial cell proliferation, migration, and survival, and it acts as a potent

microvascular permeability enhancer of VEGF. VEGFR-3/Flt-4 is a member of the same tyrosine kinase receptors of VEGF-C and VEGF-D. Another coreceptor known as neuropilin (NP) also modulates receptor ligand interactions of the VEGF family. NP-1 enhances the VEGF-A_{165} binding with VEGFR-2 whereas NP-2 acts as a functional receptor for VEGF-A_{145} and VEGF-A_{165}. VEGF is expressed and secreted by majority of neuroblastoma cell lines and primary tumors that contribute to the growth of endothelial cells *in vitro* and angiogenesis *in vivo* leading to poor prognosis in high-risk neuroblastoma [10]. VEGF-A_{165} mRNA expression is upregulated in stage III neuroblastoma [11]. The presence of VEGF, VEGFR-1, and VEGFR-2 mRNA expression is evident in neuroblastoma surgical specimens and cell lines by reverse transcriptase-polymerase chain reaction (RT-PCR), but neuroblastoma SK-N-BE cell line expresses only the mRNA of VEGFRs [12]. Hypoxia upregulates VEGF expression and in conjugation with Flt-1 plays a pivotal role in VEGF-mediated autocrine signaling of tumor growth and angiogenesis in neuroblastoma cell line. A hypoxia-driven VEGF/Flt-1 autocrine loop acts together with hypoxia inducible factor-1 alpha (HIF-1α) through a mitogen-activated protein kinase and extracellular signal-regulated kinase-1/2-mediated pathway in neuroblastoma SK-N-BE2 cell line resulting in tumor cell survivability, multiple drug resistance, and neuroblastoma vascularity [13]. Neuroblastoma cells utilize VEGF both as a stimulator of angiogenesis and an inhibitor of apoptosis through upregulation of Bcl-2 expression [14]. Treatment of neuroblastoma in both *in vitro* and *in vivo* models with anti-VEGF agent results in decrease in tumor vascularity. In both neuroblastoma cell line and *in vivo* models, treatment with bortezomib, a proteasome inhibitor, showed anti-tumor activity with marked decrease in intratumoral vessel counts and reduction in VEGF expression suggesting diversified role of VEGF in progression of advanced stage neuroblastoma [15, 16]. The anti-VEGF antibody bevacizumab markedly reduced the growth rate of three malignant neuroblastoma SK-N-AS, IMR-32, and SH-SY5Y xenografts in immunodeficient mice [17]. ZD6474, a dual tyrosine kinase inhibitor of VEGFR-2 and EGFR, inhibited the phosphorylation of receptor tyrosine kinase in neuroblastoma cells leading to an increase in endothelial cell apoptosis and showed significant anti-tumor activity in seven neuroblastoma cell lines (SK-N-SH, SK-N-SH, SK-NAS, NGP, CHP-134, SH-SY5Y, and SH-EP) [18]. Though VEGF is indispensable for neuroblastoma angiogenesis, treatment of neuroblastoma xenograft model with anti-VEGF antibody shows decrease in vascularity and only partial regression of tumor, suggesting that VEGF blockade is not sufficient to prevent neuroblastoma angiogenesis or apoptosis and multiple angiogenic factors are needed for progression of neuroblastoma [19]. High levels of expression of VEGF-A_{121}, VEGF-A_{165}, VEGF-B, VEGF-C, FGF-2, Ang-2, TGF-α, and PDGF-A have been documented in an analysis of 22 neuroblastoma cell lines and 37 tumor samples suggesting that multiple angiogenic growth factors interplay in the regulation of neovascularisation in advanced stage neuroblastoma [20]. Expression of mRNAs in 24 neuroblastoma cell lines and 40 tumor samples using RT-PCR suggests that expression of c-Kit, PDGFR-β, and Flt-3 mRNA is associated with neuroblastoma in patients under 1 year, while the loss of expression of these kinases is associated with N-Myc amplification in patients over 1 year of age with advanced stage of neuroblastoma [21].

3.1.2. PDGF and PDGFR Family. PDGF and their cognate receptor tyrosine kinases have potent implication in modulating endothelial cell proliferation and angiogenesis in solid tumors. Neuroblastoma cell lines also express dimeric isoforms of PDGF: PDGF-AA and PDGF-BB and their functional receptors PDGFR-α and PDGFR-β, respectively. PDGF has trophic effects on dopaminergic neurons *in vitro*. NB41, a mouse neuroblastoma cell line produces PDGF-AA, PDGF-BB and PDGFR-β, responding to PDGF-BB but not to PDGF-AA. Introduction of an antisense PDGFR-β RNA in NB41 cells completely suppressed neurite extension and cell growth indicating proliferative activity of PDGF in tumors [22]. Treatment of 7 neuroblastoma cell lines with imatinib mesylate displayed concentration-dependent decreases in cell viability and induction of apoptosis due to suppression of c-Kit and PDGFR phosphorylation, leading to inhibition of growth of neuroblastoma [10]. Treatment of 3 neuroblastoma cell lines such as SK-N-AS, IMR-32, and SH-SY5Y with SU11657, which is a multiple inhibitor targeting PDGFR, VEGFR, and c-Kit, reduced their expression and tumor angiogenesis by 63–96% also signifying the crucial roles of these angiogenic ligands and their receptors in tumor cell proliferation and survival [23]. PDGF mainly induces its activity through Ras activation followed by MAPK mediated action. Treatment of neuroblastoma SH-SY5Y cell line with somatostatin causes anti-proliferative effect by inhibition of PDGF-induced PDGFR phosphorylation followed by Ras inactivation providing multiple regulatory role of PDGF in cell proliferation and angiogenesis in high risk neuroblastoma [24].

3.1.3. Stem Cell Factor and c-Kit Receptor Family. Produced by marrow stromal cells, stem cell factor (SCF) is a glycoprotein hemopoietin growth factor that acts by binding to its specific surface protein receptor encoded by the *c-Kit* proto-oncogene. SCF and c-Kit mRNAs are expressed in neuroblastoma cell lines and tumors, and they regulate tumor growth, survivability, and angiogenesis [25]. Neuroblastoma cells treated with an antibody to c-Kit augment apoptosis [26]. Both SCF and c-Kit are preferentially expressed in N-Myc amplified neuroblastoma tumors, and their signaling is active in promoting neuroblastoma cell proliferation that can be selectively inhibited by treatment with STI-571, a tyrosine kinase inhibitor [27]. Retinoic acid treatment has been shown to induce neuronal differentiation as well as enhanced SCF production in neuroblastoma cell lines [28]. Anti-c-Kit administration in 5 neuroblastoma cell lines IMR-5, SK-N-SH, SK-N-BE, AF8, and SJ-N-KP and the neuroepithelioma (NE) line CHP-100 showed significant decrease in cell viability due to induction of apoptosis suggesting that SCF is produced by some neuroblastoma

cell lines via an autocrine loop to protect them from apoptosis and stimulate neuroblastoma growth and metastasis [29].

3.1.4. Flt-3 and Receptor Family. Flt-3 is a transmembrane glycoprotein receptor structurally related to macrophage colony stimulating factor receptor-1 (CSF-1) and c-Kit, and it is expressed on several cell types including neuroblastoma cells. Flt-3 ligand (FL) is a cytokine that promotes the survival, proliferation, and differentiation of hematopoietic progenitors in synergy with other growth factors such as SCF, IL-3, IL-6, IL-12, and GM-CSF. The levels of expression of Flt-3 and FL in 12 tumor cell lines from neuroectodermal tumor (NET), Ewing's sarcoma (ES), and peripheral neuroectodermal tumor (PNET) and in 38 biopsies were analyzed [26]. RT-PCR and flow cytometry confirmed the presence of membrane and cytoplasmic Flt-3 and membrane FL in all these lines. FL shows a significant proliferating and anti-apoptotic activity in neuroblastoma and neuroepithelioma lines suggesting that Flt-3 and its ligand are expressed in neural crest-derived tumors and promote survival and proliferation of their cell lines [26].

3.1.5. MMP Expression in Human Neuroblastoma. In advanced stages of neuroblastoma, tumor cell secrets MMP favoring degradation of extracellular matrix and enhancing tumor dissemination. The family of MMPs includes 72 kDa MMP-2 (or gelatinase A) and 92 kDa MMP-9 (or gelatinase B) that are collagenases. MMP-2 and MMP-9 facilitate invasion and metastasis, and they degrade important constituents of the interstitial stroma and subendothelial basement membrane type IV, V, VII, and X collagens and fibronectin [14]. Immunohistochemical analysis of the expression patterns of MMP-2, MMP-9, and their specific inhibitor, namely, tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) in 31 neuroblastoma patients revealed that increased expression of MMP-2 but not that of MMP-9 and decreased expression of TIMP-2 in stromal tissues of neuroblastoma had significant association with progression of advanced stage disease [30]. Stromal MMP-9 regulates the vascular architecture in neuroblastoma by promoting pericyte recruitment. Prinomastat, a synthetic inhibitor of MMPs, shows inhibition of tumor cell proliferation in human neuroblastoma SK-N-BE2 orthotopically xenotransplanted tumors in immunodeficient mice and prolonged survivability, suggesting that advanced stages of neuroblastoma show increased expression of both MMP-2 and MMP-9 [31]. PEGylated SN38 (EZN-2208), a novel topoisomerase I inhibitor, showed promising anti-neuroblastoma efficacy through increasing apoptosis and reducing expression of VEGF, MMP-2, and MMP-9 in preclinical *in vitro* and *in vivo* models of human neuroblastoma [32]. MMP-2 remains in an inactive form in tumor cell lines and tumor tissues in the absence of expression of a membrane-type 1-MMP (MT1-MMP), which converts pro-MMP-2 to its activate form. MMP-9 is not expressed in neuroblastoma cell lines, but it is present in both inactive and active forms in tumor tissues [33]. Overexpression of MT1-MMP is highly associated with the advanced stage high-risk neuroblastoma. Increased expression of both MMP-2 and MMP-9 is also evident from studies in two neuroblastoma cell lines (LAN-5 and GL-LI-N) and immunohistochemical analysis of tissue biopsies of human neuroblastoma indicating that expression of these MMPs is correlated with angiogenesis in advanced stages [14]. MMPs have a role in retinoic acid-induced differentiation in neuroblastoma cell lines. For example, retinoic acid treatment for 24 h transiently increased invasion and expression of MMP-9 in SH-SY5Y and LAN-5 cells and MMP-2 in SMS-KCNR cells. MMP inhibition prevented retinoic acid-induced neurite formation indicating a regulatory role of MMP in differentiation [34]. Human neuroblastoma tumors xenotransplanted in MMP-9 (+/+) and MMP-9 (−/−) mice showed that bone marrow-derived MMP-9 regulates the recruitment of leukocytes and endothelial cells along with pericytes from bone marrow into tumor stoma leading to neovascularization and tumor progression [35]. N-Myc and Bcl-2 co-expression induces MMP-2 secretion and activation leading to tumorigenic phenotype in human neuroblastoma cells [36].

3.1.6. Association of N-Myc Amplification with Human Neuroblastoma. *N-Myc*, a proto-oncogene normally expressed in the developing nervous system, is frequently overexpressed in high-risk neuroblastoma manifesting increased vasculature and poor prognosis. Amplified N-Myc down regulates both the production and activity of angiogenesis inhibitors and also provokes tumor malignancy [37]. N-Myc contains an N-terminal transactivation domain (Myc box) and a C-terminal basic helix-loop-helix/leucine zipper (bHLH-LZ) motif. The bHLH-LZ region is responsible for both DNA binding and interactions with other bHLH-LZ proteins (Max and Mad).

N-Myc is normally located on the distal short arm of chromosome 2 (2p24), but in cells with N-Myc amplification it also maps to the double minute chromatin bodies (DM) or homogenously staining regions. Genetic material from chromosome 2p24 is amplified to form an extrachromosomal circular element or is transposed to the DM with retention of the normal copies of N-Myc at 2p24. Amplification of the N-Myc occurs in 20% to 25% of neuroblastomas and is a marker of aggressive tumor phenotype [38].

After genomic amplification, N-Myc acts as transcription activator by heterodimerization with Max, a ubiquitously expressed nuclear protein lacking N-terminal transactivating domain. Interaction of the N-Myc/Max complex with the promoter region of target genes through the DNA sequence E-box motif (CACGTG) leads to progression of the cell cycle through the G1 phase ensuing increased transcription of several genes like *ODC* , *MCM7*, and *MRPI*. In the absence of N-Myc, other nuclear proteins Mad and Mxi1 competitively bind with Max and act as transcriptional repressor protein complex to inhibit transcription (Figure 2). Treatment of the N-Myc-amplified neuroblastoma cells with combination of IFN-γ and retinoic acid down regulates N-Myc expression through increased protein turnover, upregulates Mad1 mRNA and protein, and reduces N-Myc/Max heterodimerization, and predominates Mad1/Max network resulting in repression of the N-Myc target genes and potentiating

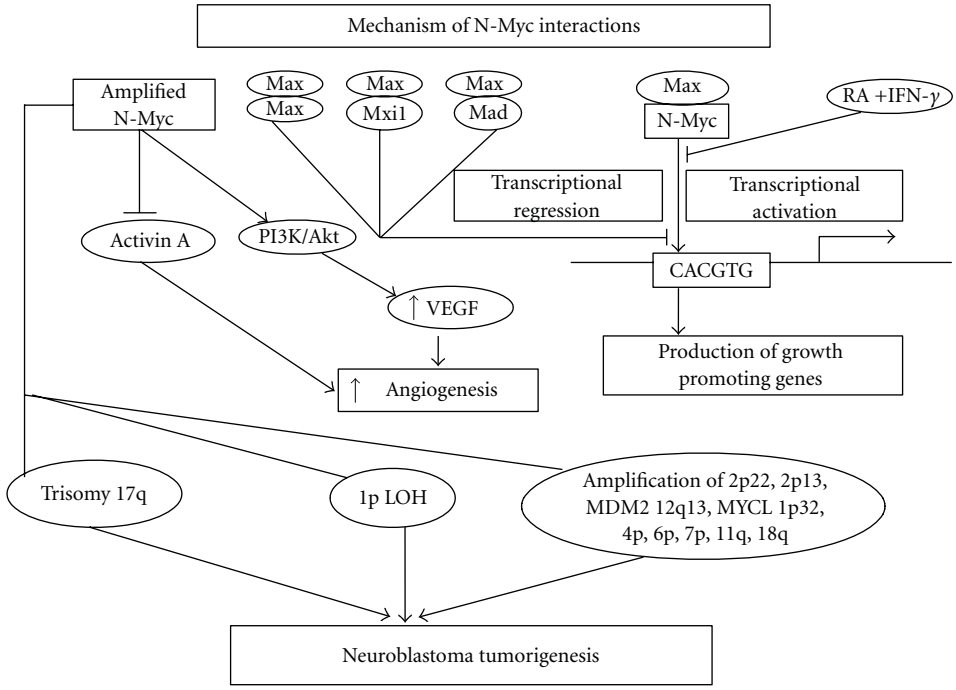

Figure 2: Schematic presentation of molecular changes associated with N-Myc amplification in neuroblastoma. Amplified N-Myc after heterodimerization with Max augments synthesis of several genes and through PI3K/Akt-mediated pathway regulates VEGF signaling resulting in neuroblastoma tumorigenesis.

differentiation and growth inhibition in neuroblastoma cells [39] (Figure 2).

Overexpression of N-Myc down regulates the production of the anti-angiogenic factor activin A (Figure 2). Increased activin A expression inhibits neuroblastoma growth and angiogenesis in a neuroblastoma xenograft model, shows anti-proliferative activity, decreases colony formation of human neuroblastoma cell lines with amplified N-Myc, and induces differentiation. Amplified N-Myc through its interaction with the activin A promoter suppresses activin A synthesis resulting in enhanced vascularization to allow neuroblastoma progression [37].

Other genetic changes associated with N-Myc amplification in high-risk neuroblastoma are loss of heterozygosity on chromosome 1p and amplification of DNA on 2p22, 2p13, the *MDM2* gene on 12q13, and the *MYCL* gene on 1p32. Comparative genomic hybridization reveals that amplifications of 4p, 6p, 7q, 11q, and 18q concurrently with N-Myc amplification have occurrence in advanced stage neuroblastoma. Allelotyping and comparative genomic hybridization studies indicate that trisomy for the long arm of chromosome 17 (17q) along with amplified N-Myc is indicative marker of aggressive neuroblastoma. Deletion of 11q is a predictive pointer in clinically high-risk neuroblastoma patients without N-Myc amplification [38] (Figure 2). N-Myc-amplified neuroblastoma cell lines either do not express CD44 or express a non-functional receptor. Absence of functional CD44 hyaluronan receptor on human N-Myc-amplified neuroblastoma cells predicts risk of disease progression and dissemination [40].

N-Myc plays an important role in the phosphatidylinositol-3-kinase- (PI3K-) mediated VEGF regulation in neuroblastoma cells as evident from the study where inhibition of N-Myc expression by siRNA transfection significantly blocks VEGF secretion [41]. Targeted inhibition of N-Myc by peptide nucleic acid (PNA) in human neuroblastoma N-Myc-amplified (GI-LI-N) and N-Myc-unamplified (GI-CA-N) cells showed cell cycle inhibition with induction of neuronal differentiation and apoptosis in N-Myc amplified cells and caused significant reduction in cell viability with N-Myc translation inhibition, accumulation of cells in G1, and induction of differentiation and apoptosis [42]. N-Myc silencing induces differentiation and apoptosis in human neuroblastoma cells. Also, siRNA directed to N-Myc may provide a novel therapeutic approach for an effective treatment of aggressive neuroblastomas [43].

3.1.7. Neurotrophin Signaling Pathways in Human Neuroblastoma. Malignant transformation of sympathetic neuroblasts to neuroblastoma cells is regulated by neurotrophin receptor pathways. The tropomyosin related kinase (Trk) family consists of three receptor tyrosine kinases (TrkA, TrkB, and TrkC) each of which can be activated by one or more of the 4 neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4). Neurotrophins through intracellular signal transduction pathways mediate diversified biological activities such as cell survival, proliferation, and differentiation in normal and neoplastic neuronal cells.

High expression of TrkA is present in neuroblastomas with favorable prognosis correlating with patient survival and absence of N-Myc amplification. Binding of transmembrane homodimer receptor TrkA to a homodimer of NGF leads to autophosphorylation of the receptor, docking of signaling proteins, signal transduction, and induction of gene transcription. TrkA through direct interaction with proteins SHC, PLCγ1, SH2B, IAPs, and Ras/MAPK signaling pathway regulates survival and differentiation and via activation of PI3K/Akt pathway mediates an alternative survival signaling pathway. TrkB is mainly expressed on unfavorable, aggressive neuroblastomas, responsible for both enhanced angiogenesis and drug resistance and strongly associated with N-Myc amplified tumors. Biological effects of TrkA and TrkB expression on neuroblastoma angiogenesis were examined in human neuroblastoma SH-SY5Y cell line and its TrkA and TrkB transfectants. TrkA expression inhibits tumor growth and down regulates the angiogenic factors VEGF and FGF-2 in SH-SY5Y cells whereas TrkB transfectants and parental SH-SY5Y cells induced endothelial cell proliferation and migration [44]. TrkA expression resulted in severely impaired tumorigenicity and invasiveness in SH-SY5Y xenografted mice and was associated with reduced angiogenic factor expression and vascularization of tumors [44]. NT expression pattern and mutational events leading to tumorigenesis divided neuroblastomas in distinct three types [38]. The first type is characterized by TrkA expressing tumors having mitotic dysfunction leading to near triploid (3N) karyotype. They can differentiate in response to NGF or undergo apoptosis in absence of NGF. Second type is characterized by TrkB expressing tumors having near diploid (2N) or tetraploid karyotype with genomic instability (gain of distal 17q material, loss of 11 q and/or 14 q material with 17q gain, and without 1p deletion and N-Myc amplification) and chromosomal structure alterations. Third type is characterized by tumors with 1p loss with N-Myc amplification [38]. Expression of different members of Trk family thus plays a key role in heterogenous clinical outcome of neuroblastoma.

3.1.8. Expression of Vascular Integrins Associated with Human Neuroblastoma. Angiogenic neovascularization is also influenced by selective expression of adhesion receptor integrins. Cell adhesion to the extracellular matrix is mediated by integrins, which are heterodimeric transmembrane proteins comprising of a diverse family with over 15α and 8β subunits. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are expressed in high-risk neuroblastoma [45]. The binding of the integrin $\alpha_v\beta_3$ to its receptor provides a signal that causes reduction in p53, $p21^{Waf1}$, and Bax expression and increase in Bcl-2 expression implicating survival of endothelial cells [46]. Expression of the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ by microvascular endothelium was demonstrated by immunohistochemical analysis [45]. The integrin $\alpha_v\beta_3$ was expressed by 61% of microvessels in high-risk neuroblastomas (stage IV and N-Myc-amplified stage III) and 18% of microvessels in low-risk tumors (stages I and II and non-N-Myc amplified stage III). The integrin $\alpha_v\beta_5$ was produced by 60% of microvessels in stage IV tumors suggesting their association with neuroblastoma aggressiveness [45]. *In vivo* echographic evidence of tumoral vascularization and microenvironment interactions in metastatic orthotopic human neuroblastoma xenografts showed highly angiogenic integrin $\alpha_v\beta_3$ marker indicating neovascularization to promote the role of cell adhesion molecules [47].

3.1.9. Osteopontin in Neuroblastoma. Osteopontin (OPN) is a multifunctional phosphoprotein secreted by multiple cell types including osteoclasts, lymphocytes, and macrophages, and OPN is expressed in various tumor cells to act as a potent angiogenic factor contributing to tumor growth. OPN through its interaction with α_v integrins regulates both cell attachment and cell signaling. OPN in association with VEGF stimulates endothelial cell migration [48]. OPN plays an important role in increasing tumorigenicity through the enhancement of angiogenesis *in vivo*. Culture medium with murine neuroblastoma C1300 cells transfected with OPN gene significantly stimulates human umbilical vein endothelial cell migration and induces neovascularization in mice resulting in significant increase in tumor growth [49]. Presence of FGF-2 stimulates OPN upregulation in endothelial cells resulting in OPN-mediated recruitment of pro-angiogenic monocytes, induction of expression of the angiogenic cytokines TNF-α and IL-8 to contribute to amplification of FGF-2-induced neovascularization during inflammation, wound healing, and tumor growth [50].

3.1.10. Role of HGF/c-Met Signaling in Neuroblastoma. Both N-Myc amplification and elevated expression of the neurotrophin receptor TrkB are correlated with the malignant phenotype of neuroblastoma. The TrkB-mediated invasiveness is associated with upregulation of hepatocyte growth factor (HGF) and its receptor c-Met [51]. HGF and c-Met are heterodimers composed of a α-chain subunit and a β-chain subunit linked by a disulfide bond. Mature HGF is a heterodimer composed of a α-chain (69 kDa), which contains an N-terminal hairpin domain and four kringle domains, and a β-chain (34 kDa) having serine-protease-like domain. Mature c-Met is comprised of a glycosylated α-subunit (50 kDa) and a transmembrane β-subunit (145 kDa). The extracellular region of mature c-Met contains a Sema domain, which is a cysteine-rich Met-related sequence (MRS) domain, and four immunoglobulin-like structure domain. The intracellular region is composed of a juxtamembrane domain, which is a tyrosine kinase domain (Tyr 1234 and Tyr 1235) that regulates kinase activity of c-Met, and a C-terminal regulatory tail (Tyr 1349 and Tyr1356) that is responsible for c-Met-regulated signal transduction pathway [52].

After binding of HGF to c-Met or other receptor tyrosine kinases, the HGF/c-Met signaling is initiated. Dimerization of c-Met causes transphosphorylation of tyrosines (Tyr 1234 and Tyr 1235) in the kinase domain followed by additional phosphorylation of other tyrosines (Tyr 1349 and Tyr 1356) in the C-terminal regulatory tail. c-Met transmits HGF signaling in cells via various downstream effectors such as Src/FAK, which regulates cell migration and adhesion. The other HGF/c-Met-associated effectors such as p120/STAT3

pathway augments neovasculature of cells whereas the PI3K/Akt and Ras/MEK pathways are responsible for cell proliferation and cell survival [52]. Thus, HGF mediates these multiple signaling cascades in endothelial cells directly or via involvement of VEGF and its receptor to promote angiogenesis. The important downstream effector of HGF/c-Met signaling that promotes neuroblastoma progression is STAT3 [53], one of the well-recognized signal transduction pathways involved in angiogenesis and other steps in cancers.

4. Anti-Angiogenic Therapy for Controlling Neuroblastoma

4.1. Single Anti-Angiogenic Agent Therapy for Controlling Neuroblastoma

4.1.1. Inhibition of VEGF and VEGFR. VEGF is a critical mitogen regulating growth, neovascularization, and migration of endothelial cells and is associated with poor prognosis in neuroblastoma. VEGF and its receptors are expressed in human neuroblastoma tumors and cell lines. VEGF acts primarily by binding to one of its cognate receptors (VEGFR-1, VEGFR-2, and VEGFR-3) on endothelial cells, leading to autophosphorylation of tyrosine residues followed by subsequent activation of multiple intracellular signal transduction pathways such as the MAPK and PI3K/Akt pathways. Several experimental therapeutic strategies including VEGF inhibitors, antibodies directed against VEGF or against its receptors, soluble truncated receptors, introduction of antisense VEGF RNA, development of dominant-negative VEGF mutants and agents interfering directly with VEGF signal transduction have been emerged to target the interaction of VEGF with its receptors and thereby to suppress growth of neuroblastoma [54].

AZD2171, a selective inhibitor of the VEGF receptor family, displayed inhibition of tumor growth in neuroblastoma xenografts showing a promising anti-angiogenic treatment strategy for solid tumors [55]. Treatments with SB202190 (the p38 MAPK inhibitor) enhanced VEGF-mediated protection of the serum-deprived neuroblastoma SK-N-SH cells by reducing caspase-3 and caspase-7 activities and increasing the phosphorylation of the extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt signaling pathway through activation of VEGFR-2. A blockade of VEGFR-2 signaling with the selective inhibitor SU1498 or gene silencing with VEGFR-2 siRNA in SB202190 treated cells hindered this prosurvival response and highly induced activation of caspase-3 and caspase-7, indicating that p38 MAPK exerts a negative effect on VEGF-mediated signaling through VEGFR-2 in serum-starved neuroblastoma cells [56]. Combinatorial treatment with vinblastine, a monoclonal antibody (DC101) targeting VEGFR-2 and rapamycin (mTOR inhibitor) in both neuroblastoma cells and orthotopic xenografted mice showed significant inhibition of tumor growth, angiogenesis, and reduction in microvessel formation suggesting that this combination may be relevant to design new curative strategies against neuroblastoma [57]. Argatroban (a derivative of arginine and a potent anti-coagulant and anti-thrombin agent) serves as a useful therapeutic tool for inhibition of thrombin-induced VEGF production in human neuroblastoma (NB-1) cells, and it may be effective in controlling disorders linked to thrombin-induced VEGF production in neuronal cells [58].

Inhibitors of VEGF with different specificities have been evaluated in human neuroblastoma NGP-GFP xenografts in nude mice [19]. These anti-VEGF agents are NX1838 (an antihuman $VEGF_{165}$ RNA-based fluoropyrimidine aptamer), monoclonal anti-human VEGF antibody, and VEGF-Trap (a soluble composite decoy receptor consists of Ig-like domains of VEGFR-1 and VEGFR-2). High-dose VEGF-Trap showed the greatest inhibition of tumor growth leading to regression of tumor vasculature in xenograft model of neuroblastoma [19]. Combination of topotecan (a topoisomerase I inhibitor) with anti-VEGF antibody significantly suppressed tumor growth in neuroblastoma xenograft [59]. Continuous low-dose therapy with vinblastine and VEGFR-2 antibody induces sustained tumor regression in neuroblastoma xenograft, diminishes tumor vascularity, and directs inhibition of angiogenesis [60], suggesting that metronomic therapy can inhibit endothelial cell proliferation, angiogenesis, and tumor growth.

4.1.2. Inhibition of Multiple Angiogenic-Related Factors. VEGF, FGF-2, PDGF, SCF, and their cognate receptor tyrosine kinases are strongly implicated in angiogenesis in solid tumors like neuroblastoma. SU11657 (SUGEN) is a selective multitargeted (class III/V) tyrosine kinase inhibitor with anti-tumor and anti-angiogenic activity exerted by targeting PDGFR, VEGFR, SCF receptor (c-Kit), and FMS-related tyrosine kinase 3. Oral administration of SU11657 caused significant inhibition of tumor growth as well as of angiogenesis in SK-N-AS, N-Myc amplified IMR-32, and SH-SY5Y human neuroblastoma xenografted athymic nude mice. Immunohistochemical analysis revealed down regulation of the expression of VEGFR-2, PDGFR-β, and c-Kit suggesting that targeting of class III/V receptor tyrosine kinases and their ligands can suppress neuroblastoma tumorigenicity and angiogenesis [23]. Imatinib mesylate, a selective inhibitor of the tyrosine kinase c-Kit and PDGFR, displayed concentration-dependent decreases in cell viability, induction of apoptosis with ligand-stimulated phosphorylation of c-Kit and PDGFR, and inhibition of VEGF expression in 7 neuroblastoma cell lines. Oral imatinib therapy showed anti-tumor efficacy in xenografted SCID mice [10]. SU6668, another receptor tyrosine kinase inhibitor of VEGFR2, PDGFR, and FGFR1, in combination with cyclophosphamide significantly inhibited VEGFR-2 and tumor growth in human neuroblastoma xenografts [37]. Therefore, therapy targeted to multiple angiogenic factors appears to be a novel treatment modality in neuroblastoma.

4.1.3. Retinoids for Inhibition of Angiogenesis in Neuroblastoma. Retinoids, a class of natural or synthetic compounds structurally related to vitamin A, hold great promises for the prevention and treatment of cancer. The cell differentiating

properties and anti-cancer activity of the retinoids all-*trans* retinoic acid (ATRA) and 13-*cis* retinoic acid (13-CRA) are well established in several *in vitro* and *in vivo* models [61, 62]. Retinoids are signaling molecules that are involved in proliferation, differentiation, and apoptosis both via non-receptor and nuclear-receptor-mediated pathways thereby altering gene expression. ATRA induces the expression of both mRNA and protein of the differentiation marker manganese superoxide dismutase (MnSOD) in human neuroblastoma (SK-N-SH) cells with involvement of NF-κB and SOD2 genes, contributing to the concept of using retinoids in cancer therapy [63]. Retinoic acid reduces human neuroblastoma cell migration and invasiveness as evident from down regulation of expression of doublecortin (a microtubule-associated protein involved in neuronal migration) and lissencephaly-1 (another protein involved in neuronal migration) and upregulation of expression of neurofilament protein-68 in human neuroblastoma SK-N-SH cell line [64]. Retinoic acid causes PI3K and PKC-dependent upregulation of 2 putative α-secretases and the disintegrin metalloproteinases ADAM10 and TACE, stimulates α-secretase processing of amyloid precursor protein (APP), and down regulates β-secretase cleavage thereby leading to suppression of amyloid-β formation in human neuroblastoma SH-SY5Y cells (Figure 3). The PI3K inhibitor LY294002 and the PKC inhibitor bisindolylmaleimide XI reduced the retinoid-mediated effect on ADAM10 protein levels and completely abolished the effect on TACE indicating involvement of PI3K- and PKC-mediated signaling pathway in retinoic acid-induced upregulation of secretase [65]. Combined IFN-γ and retinoic acid treatment targets the N-Myc/Max/Mad1 signaling pathway and represses expression of the N-Myc/Mad1 target genes ornithine decarboxylase and hTERT, indicating that this combination strategy may have therapeutic benefits in targeting N-Myc function in high-risk, N-Myc-amplified neuroblastoma patients [39]. Non-genomic actions of retinoic acid on neuroblastoma SH-SY5Y cells is mediated by the classical nuclear receptor, retinoic acid receptor (RAR), resulting in activation of PI3K and MAPK signaling pathways contributing to retinoic acid-induced differentiation [66]. Combination of retinoic acid with histone deacetylase inhibitors (HDACi) could result in improved anti-tumorigenic activity. The HDACi trichostatin A (TSA), sodium butyrate, and suberoylanilide hydroxamic acid (SAHA) alone and in combination with retinoic acid can increase cyclin kinase inhibitor (CKI) mRNA levels in human neuroblastoma SH-SY5Y cells, and activate CKI promoters to inhibit tumor cell growth in neuroblastoma [67]. ATRA through involvement and activation of RAR and ERK 1/2 induces COX-2 and prostaglandin E2 synthesis in human neuroblastoma SH-SY5Y cells and this effect is completely abolished by the RAR pan-antagonist LE540 or the MEK-1 inhibitor PD98059 suggesting involvement of RAR and kinase-dependent mechanisms for ATRA-induced COX-2 activity [68] (Figure 3). Genomic action of ATRA includes caspase-8 transcription via CREB-phosphorylation leading to apoptosis in neuroblastoma cells [69]. Retinoic acid induces cell cycle arrest and differentiation through degradation of the F-box protein Skp2 and stabilization of the cyclin-dependent kinase inhibitor p27 (Figure 3). Skp2 is degraded by anaphase-promoting complex (APC) (Cdh1). Retinoic acid downregulates Rae1 (a nuclear export factor), facilitates APC- (Cdh1-) mediated Skp2 degradation leading to the arrest of cell cycle progression and differentiation in neuroblastoma SH-SY5Y cells [70] (Figure 3).

N-(4-hydroxyphenyl) retinamide (4-HPR), also known as fenretinide, is a synthetic retinoid that induces anti-proliferative activity and apoptosis, inhibits angiogenesis and cell motility, and decreases invasiveness in a wide variety of human cancer cell lines and mammary, prostate, and ovarian tumors in transgenic mice. 4-HPR can activate retinoid receptor-dependent and independent pathways for induction of apoptosis. 4-HPR induces the gene expression of BBC3, pro-apoptotic member of the Bcl-2 family, to trigger apoptosis in neuroblastoma cell lines [71]. 4-HPR also potentiates NF-κB activity, IκBα phosphorylation, production of reactive oxygen species (ROS), 12-lipoxygenase activity, and GADD153 transcription factor activity to elicit apoptotic response in neuroblastoma SH-SY5Y cells [72]. 4-HPR, IFN-γ, and demethylating agent 5-aza-cytidine activate the promoter region of caspase-8 in neuroblastoma cells and regulate both constitutive and inducible caspase-8 expression in pathophysiological condition [73]. 4-HPR is also found to upregulate ceramide level and metabolism of ceramide to gangliosides via glucosylceramide synthase and GD3 synthase leading to activation of ROS signaling pathway via 12-LOX resulting in oxidative stress and neuroblastoma cell death by induction of the transcription factor GADD153 and the Bcl-2-related Bak protein [74]. 4-HPR caused sustained activation of JNK/p38 MAPK pathway and augmented apoptosis in a ROS-dependent manner in neuroblastoma cells [75]. 4-HPR through inhibition of both VEGF and FGF-2 induced endothelial cell proliferation to downregulate angiogenesis in neuroblastoma [76]. Retinoid in combination with an anti-angiogenic factor may provide an effective treatment strategy for controlling neuroblastoma.

4.1.4. Anti-Angiogenic Inhibitors of Methionine Aminopeptidase. The anti-angiogenic inhibitors of methionine aminopeptidase (MetAP2) may hold a key role in therapeutic management of neuroblastoma. MetAP2 is a cytoplasmic enzyme responsible for promoting endothelial cell proliferation, migration, and induction of angiogenesis in neuroblastoma [54]. The concentration of MetAP2 has been found to be higher in neuroblastomas [37].

TNP-470, an irreversible inhibitor of MetAP2, showed anti-angiogenic activity in preclinical models of neuroblastoma. TNP-470 as monotherapy or in combination with a cytotoxic agent such as cisplatin, paclitaxel, or cyclophosphamide significantly inhibited tumor angiogenesis [54]. TNP-470 significantly inhibited growth rate and tumorigenecity in several neuroblastoma xenografts [54, 77]. TNP-470 treatment in small neuroblastoma tumors was found to exhibit chromaffin differentiation and induction of apoptosis [54]. Microspheres containing TNP-470 strongly inhibit *in vivo* hepatic metastasis of neuroblastoma [78]. However, TNP-470 has a few limitations such as neurologic toxicities.

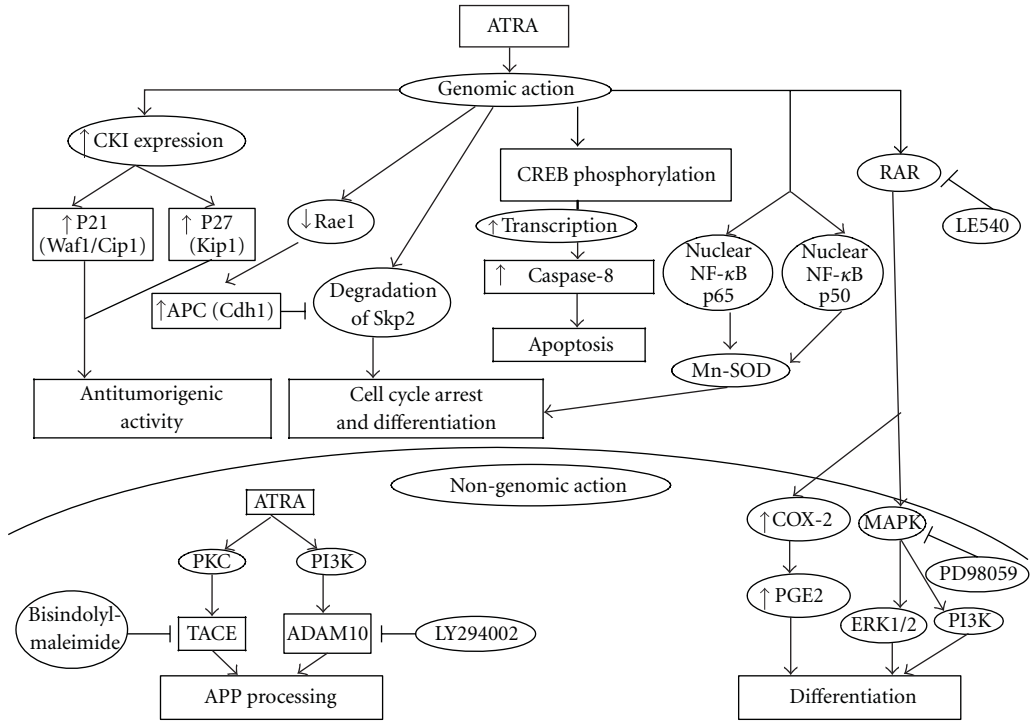

Figure 3: Genomic and non-genomic actions of all-*trans* retinoic acid (ATRA). Genomic action of ATRA includes caspase-8 transcription, cell cycle arrest, and apoptosis. Non-genomic action of ATRA involves induction of PI3K/Akt pathway for upregulation of secretase. ATRA activates retinoic acid receptor (RAR) and MAPK signaling pathway for contribution to differentiation in neuroblastoma.

Another reversible inhibitor of MetAP2 is A-357300, which is a promising therapeutic agent against neuroblastoma. A-357300 alone or in combination with cyclophosphamide significantly caused tumor regression and increased survival rate in CHP-134-derived neuroblastoma xenografts without any toxicity [79]. A-357300 induces cell cycle arrest G1 phase in neuroblastoma cells, and showed anti-angiogenic and anti-tumor potential in neuroblastoma murine models [80]. A800141, another orally active inhibitor of MetAP2, showed potent anti-angiogenic and anti-tumor activities in several tumor xenografts including neuroblastoma, B-cell lymphoma, and colon and prostrate carcinomas [81].

4.1.5. Endostatin for Inhibition of Angiogenesis in Neuroblastoma. Endostatin is a 20 kDa fragment of collagen XVIII that acts as an endogenous inhibitor of endothelial cell proliferation, tumor angiogenesis, and tumor growth. Recombinant human endostatin (rhEndostatin) worked as potent anti-angiogenic agent and was effective against human neuroblastoma xenograft model [82]. In another gene therapy approach, the angiogenesis inhibitor endostatin and the potent immunogen green fluorescent protein (GFP) were delivered to murine neuroblastoma cells prior to inoculation of tumor cells into syngeneic immunocompetent mice [54]. The combination of endostatin and GFP showed synergistic anti-tumor and immunogenic response suggesting that both anti-angiogenic and immunotherapeutic strategies worked to control neuroblastoma. Also, pre-existing primary neuroblastoma xenografts could hold back the growth of a new secondary subcutaneous tumor, inhibit angiogenesis, and induce apoptosis due to release of endostatin from the tumor [83].

4.1.6. Targeting HGF/c-Met for Inhibition of Angiogenesis in Neuroblastoma. One promising strategy to inhibit angiogenesis in neuroblastoma is to target the signaling pathway of HGF (also known as scatter factor, SF) along with its receptor HGFR (also known as c-Met) that plays important roles in mitogenic, motogenic, and morphogenic regulation of angiogenesis, tumor growth, and metastasis. Inhibitors of this signaling pathway have been shown to inhibit angiogenesis in multiple *in vitro* and *in vivo* models of cancers and may be used as an effective therapy to treat cancers. HGF and c-Met are upregulated in many human cancers including neuroblastoma [84] and highly responsible for neuroblastoma invasion *in vitro* and *in vivo*. Therefore, inhibitors targeting HGF/c-Met signaling may be an effective therapeutic approach to control angiogenesis and prevent tumor growth. The first strategy for inhibition may be blocking the binding of HGF to c-Met with the use of HGF antagonists and antibodies against HGF/c-Met. The HGF/c-Met antagonists NK4, uncleaved HGF, Sema, decoy Met, and recombinant variant Met were used previously. The antibodies targeting HGF are L2G7 (Galaxy Biotech), AMG102 (Amgen), OA-5D5 (Genentech), and CE-3556221 (Pfizer) that can inhibit tumor angiogenesis by blocking

the binding of HGF/c-Met [52]. In another study, it has been reported that inhibition of HGF activity by anti-HGF-antibodies or suppressing the function of c-Met by siRNA can repress the TrkB-induced invasiveness [51].

Next strategy is to inhibit the HGF/c-Met signaling pathway (a) by targeting Tyr kinase domain of c-Met for inhibiting tyrosine phosphorylation with inhibitors such as SU11274, PF2341066 (Pfizer), XL880, and XL184 (Exelixis) and (b) by targeting inhibitors of downstream effectors of HGF/c-Met signaling such as PI3K inhibitor LY294002 (to inhibit HGF/c-Met-induced cell motility) and MEK inhibitor PD98059 (to inhibit HGF/c-Met-mediated cell invasion). The other important inhibitors are Src inhibitor PD180970 and SU6656 that inhibit HGF/c-Met-induced Src and STAT3 activity [52]. Another inhibitor PHA665752 has been reported to suppress c-Met activity and block HGF-induced cell migration and proliferation of c-Met-positive neuroblastoma cells [85]. Thus, targeting HGF/c-Met signaling pathways could be a beneficial approach for controlling angiogenesis in neuroblastoma.

4.2. Combination Anti-Angiogenic Therapy to Treat Neuroblastoma. Despite multimodal myeloablative chemotherapy, this pediatric malignancy has a poor prognosis. Current therapeutic strategies including combination of novel targeted drugs such as signal transduction and angiogenesis inhibitor, differentiation and apoptosis inducers, and immunotherapeutic modulators can provide superior management and prevention of neuroblastoma. Combination of trichostatin A (TSA) and interferon-alpha (IFN-α) showed the most potent anti-angiogenic therapeutic strategy for devastating neuroblastoma [86]. Combination of TSA and IFN-α not only decreased endothelial cell migration, invasion, and capillary tubule formation but also inhibited expression of VEGF, HIF-1α, and MMP-9 in neuroblastoma cells [86]. Sunitinib (targeted against PDGFR and VEGFR) and rapamycin showed synergistic inhibition of tumor growth, angiogenesis, and anti-metastatic activity in both neuroblastoma cell culture and xenograft models suggesting the anti-cancer efficacy of this combination therapy [87]. The anti-VEGF antibody bevacizumab treatment in orthotopic neuroblastoma xenograft model causes normalization of the tumor vasculature due to VEGF blockade resulting in improved delivery and anti-tumor efficacy of chemotherapy [88]. Combination of the thrombospondin-1 peptide ABT-510 with valproic acid was highly effective to regress tumor growth and microvascular density in two different N-Myc-amplified cell lines-derived neuroblastoma xenografts when compared with monotherapy suggesting the potency of combination anti-angiogenic therapy for treatment of neuroblastoma [89]. Bortezomib (proteasome inhibitor) and 4-HPR showed synergistic anti-tumor and anti-angiogenic activities in neuroblastoma cell culture and xenograft models through involvement of endoplasmic reticulum stress response [90]. Anti-angiogenic agents (arginine deiminase, SU5416, and DC101) in combination with simultaneous irradiation inhibited *in vivo* growth of neuroblastomas with subsequent reduction in number of tumor vessels [91]. Valproic acid in combination with INF-α synergistically inhibited growth of malignant phenotype of human N-Myc-amplified neuroblastoma BE(2)-C cells [92]. This combination therapy caused marked downregulation of N-Myc, Bcl-2, and neural cell adhesion molecule with induction of differentiation in neuroblastoma [92].

4.3. Gene Therapy and Immunotherapy for Inhibition of Angiogenesis in Neuroblastoma. In gene therapy approach, genetic material encoding the therapeutic protein is transferred into the mammalian cells. Viral vector-mediated gene delivery of angiogenic inhibitors shows potent therapeutic promises against malignant neuroblastoma. Construction of retroviral and adenoviral vectors expressing the angiogenic inhibitor proteins is being tested in different neuroblastoma murine tumor models. In this approach, host cells are engineered to make the anti-angiogenic protein *in vivo* on long-term basis without daily administration of recombinant proteins. Single vector through the delivery of multiple genes can therefore target several angiogenic pathways. Gene therapy by retroviral vector for cell-mediated *in vivo* delivery of TIMP-3 could suppress tumor-induced angiogenesis and tumor growth [93]. Two replication-defective retroviral vectors were used to transduce murine neuroblastoma cells (NXS2). Single GFP expression was ineffective to reduce tumor growth whereas engineered expression of Flk-1 resulted in inhibition of endothelial cell proliferation and migration and also restriction of neuroblastoma growth [94]. Adeno-associated virus- (AAV-) mediated delivery of IFN-β with subsequent administration of TSA *in vitro* and *in vivo* in murine model of retroperitoneal neuroblastoma was highly effective, showing reduction in tumor cell count with elevated expression of p21 suggesting the potential of this therapy to treat this devastating disease [95]. AAV-mediated systemic delivery of human IFN-β (AAV-hIFN-β) in combination with low-dose cyclophosphamide showed anti-angiogenic activity with down regulation of VEGF and FGF-2 and also caused complete tumor regression in orthotopic retroperitoneal and disseminated models of neuroblastoma [95]. Triple combination of conditionally replicating oncolytic herpes simplex virus-1 vectors armed with IL-12, IL-18, or soluble B7-1 potentiated *in vivo* anti-tumor efficacy by augmenting T-cell-mediated response in neuroblastoma Neuro2a model [96]. Thus, insertion of immunostimulatory transgenes into viral genome could be a useful strategy for neuroblastoma therapy [96]. Neuroblastoma immunotherapy using cytokines may have direct anti-tumor and immunomodulatory effects. Co-transfection of IL-2 and IL-12 in Neuro2a cells down regulated *in vivo* tumorigenicity and provided CD4+ T cell and CD8+ T cell mediated immunotherapeutic responses in syngenic neuroblastoma model [97]. Immunogene therapy with IL-12- and IL-15-engineered neuroblastoma Neuro2a cells showed therapeutic efficacy by enhancement of CD8+ T-cell immunoresponses and potentiated the survivability of neuroblastoma-bearing syngenic mice [98]. Inhibition of angiogenesis by targeted immunotherapy may provide a novel treatment modality against neuroblastoma. Anti-angiogenic integrin α_v antagonist and antibody-IL-2 fusion protein induced tumor

regression and dramatically decreased tumor vessel density in syngenic neuroblastoma model [99].

4.4. Vascular Targeting for Inhibition of Angiogenesis in Neuroblastoma. Vascular targeting has tremendous potential in neuroblastoma therapy. Vascular targeting with selective occlusion of pre-existing tumor blood vessels can induce tumor suppression by destruction of tumor vasculature and extensive necrosis. Two types of vascular targeting agents, small molecules (e.g., microtubule destabilizing agents, flavonoids), and ligand-based fusion proteins (e.g., immunotoxins, liposomally encapsulated drugs, antibodies conjugated with cytokines, and gene therapy) hold key role in tumor regression. Sterically stabilized immunoliposomal (SIL) therapy by targeted delivery of entrapped drugs to solid tumors can potentiate therapeutic efficacy. SIL loaded with doxorubicin (DXR) and targeted to the disialoganglioside receptor GD-2 in human neuroblastoma model can cause marked tumor regression. In neuroblastoma xenografts, the combined delivery of NGR peptide (targeting angiogenic endothelial cell marker aminopeptidase N) with DXR-loaded liposome showed dramatic tumor suppression by destruction of tumor vasculature [100]. Tumor vascular targeting by TNF-α with chemotherapeutic drug GNGRG peptide or coupling this peptide to the surface of liposomal DXR could augment the penetration of chemotherapeutic drugs in subcutaneous tumor models and therefore serve as a novel treatment strategy for neuroblastoma [101]. Immunotoxins- and antibody-based therapy showed promises in eradication of solid tumors. Cytokine gene transfection of tumor cells for induction of expression of major histocompatibility complex (MHC) class II in tumor endothelium was carried out in a neuroblastoma murine model [102]. The delivery of anti-class II-deglycosylated Ricin A chain caused destruction of IFN-γ-activated endothelial cells *in vitro* and complete thrombosis and decay of vasculature of neuroblastoma tumors *in viv*o suggesting that immunoconjugates attached with antibodies against tumor endothelium could offer extensive therapy to numerous solid tumors in humans [102].

5. Conclusion and Future Direction

Despite multimodal therapeutic approaches, the mortality rate is very high in patients with malignant neuroblastoma. Novel treatment strategies for improving patient survival and decreasing therapy-related toxicity are urgently warranted. Angiogenesis appears to be a primary requirement for growth, invasion, and metasatsis of malignant neuroblastomas. Inhibition of neovascularization by anti-angiogenic agent could provide promising therapeutic approach for treatment of neuroblastoma. Synergistic treatment modalities with the use of anti-angiogenic drugs in combination chemotherapy [103] and newer targeted therapy for inhibiting vascular sprouting and tumor vasculogenesis could provide effective tools for future therapies for this type of deadly cancer. Continuous low-dose chemotherapy or "metronomic dosing" could potentiate anti-angiogenic and apoptotic effects of cytotoxic agents on both proliferating endothelial cells and tumor cells. Metronomic scheduling of imatinib with the chemotherapeutic drug DXR showed anti-proliferative activity and apoptosis induction in both neuroblastoma cell lines and xenograft model [104]. Integrated treatment strategies for inducing tumor cell death, differentiation, and apoptosis, overcoming multiple drug resistance, and inhibiting angiogenesis with preclinical evaluation should provide justification for their future selection as clinical trials in high-risk neuroblastoma.

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Review Article

Targeting the Tumor Microenvironment: Focus on Angiogenesis

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Tumorigenesis is a complex multistep process involving not only genetic and epigenetic changes in the tumor cell but also selective supportive conditions of the deregulated tumor microenvironment. One key compartment of the microenvironment is the vascular niche. The role of angiogenesis in solid tumors but also in hematologic malignancies is now well established. Research on angiogenesis in general, and vascular endothelial growth factor in particular, is a major focus in biomedicine and has led to the clinical approval of several antiangiogenic agents including thalidomide, bevacizumab, sorafenib, sunitinib, pazopanib, temesirolimus, and everolimus. Indeed, antiangiogenic agents have significantly changed treatment strategies in solid tumors (colorectal cancer, renal cell carcinoma, and breast cancer) and multiple myeloma. Here we illustrate important aspects in the interrelationship between tumor cells and the microenvironment leading to tumor progression, with focus on angiogenesis, and summarize derived targeted therapies.

1. Introduction

Cancer research in both solid and hematologic malignancies until recently predominantly focused on the identification of genetic changes which are intimately associated with the induction and progression of tumors and metastasis [1]. A variety of multistep tumor models with accumulating somatic mutations has been proposed [2], most prominently the multistep colon cancer model of Dr. Vogelstein's group [3, 4]. In addition to focal genetic lesions (point mutations), chromosomal aberrations (e.g., aneuploidy, translocations, chromosomal deletions) as well as epigenetic alterations (e.g., DNA methylation, histone deacetylation, nucleosome remodeling, and RNA-associated silencing) induce deregulated expression of oncogenes and suppressor genes thereby leading to tumor cell proliferation, transformation and invasion [5, 6]. Recent studies add yet another facet to the complex multistep model of tumorigenesis by demonstrating that tumor cells carrying genomic and epigenomic abnormalities also trigger changes in their microenvironment. In turn, these changes enable the formation of a selective supportive "tumor microenvironment" [7, 8].

The cellular tumor microenvironment that is, the bone marrow microenviroment is composed of *nonhematopoietic cells* including endothelial cells (ECs); cancer-associated fibroblasts (CAFs); and cells involved in bone homeostasis including chondroblasts, osteoclasts, and osteoblasts; and *hematopoietic cells* including immune cells (including natural killer cells (NK) cells, tumor-associated macrophages (TAMs), T lymphocytes, monocytes); erythrocytes; megakaryocytes and platelets; stem cells; progenitor and precursor cells; and circulating endothelial precursors (CEPs). The noncellular microenvironment is composed of the *extracellular matrix* (*ECM)* proteins including fibronectin, laminin, collagen, osteopontin, proteoglycans, and glycosaminoglycans—and the *liquid milieu* (cytokines and growth factors, proteases) (Table 1). Tumor cell-induced disruption of the microenvironment homeostasis between the highly organized cellular and extracellular compartments support sustained proliferative signaling, evade growth suppressors, resist cell death, enable replicative immortality, activate invasion and metastasis, reprogram energy metabolism, evade immune destruction, and induce drug resistance and angiogenesis. Based on our enhanced understanding of the functional importance of the tumor microenvironment and tumor angiogenesis, in particular, new molecular targets have been identified.

Table 1: Tumor microenvironment and its compartments.

Microenvironment	Tumor entities	
	Epithelial solid tumors	Hematological tumors
	For example, Breast Cancer	For example, multiple myeloma
Extracellular matrix (ECM)	fibronectin, laminin, collagen, proteoglycans, thrombospondin, fibrinogen, elastin, fibrin, tenascin, tetranectin	fibronectin, laminin, collagen, proteoglycans, glycosaminoglycans
Cellular	Hematopoietic: TAM, T and B lymphocytes, neutrophils, NK cells, mesenchymal stem cells	Hematopoietic: HSCs, BM-derived CEPs, hematopoietic and mesenchymal progenitor and precursor cells, NK cells, NKT cells, macrophages, T and B lymphocytes, DCs, monocytes, platelets, megakaryocytes, erythrocytes
	nonhematopoietic: CAFS, myoepithelial cells, ECs, pericytes	nonhematopoietic: fibroblasts/BMSCs, chondrocytes, OCs, OBs, ECs
Liquid	Hormones: estrogen, progesterone	
	cytokines and growth factors: VEGF, HGF/SF, bFGF, PDGFα/β, TGFα/β, IL-1, IL-6, TNFα, GM-CSF, CSF-1, IGF-1/2, EGF, SDF-1	Cytokines and growth factors: VEGF, IGFs, TNFα, CD40, IL-1, IL-6, IL-10, IL-11, IL-15, IL-21, HGF, bFGF, SDF-1, TGFβ, LIF, OSM, MIP-1α, Wnts
	proteases: cathepsin B and D, elastase, uPA, plasmin, MMPs (e.g., MMP-1, -2, -3, -9)	proteases: uPA, plasmin, MMPs (e.g., MMP-2, -9)

TAM: tumor-associated macrophage; NK: nature killer; CAFS: cancer-associated fibroblasts; EC: endothelial cell; HSC: hematopoietic stem cells; CEP: circulating endothelial precursor; NKT: nature killer T; DC: dendritic cell; BMSC: bone marrow stromal cell; OC: osteoclast; OB: osteoblast; VEGF: vascular endothelial growth factor; HGF/SF: hepatocyte growth factor/scatter factor; bFGF: basic Fibroblast Growth Factors; PDGF: platelet-derived growth factor; TGF: transforming growth factor; TNF: tumor necrosis factor; IL: interleukin; GM-CSF: granulocyte macrophage colony stimulating factor; CSF: colony stimulating factor; EGF: epidermal growth factor; SDF: stromal cell-derived factor; uPA: urokinase plasminogen activator; MMP: matrix metalloproteinase; IGF: Insulin-like growth factor; LIF: leukaemia inhibitory factor; OSM: oncostatin M; MIP-1α: macrophage inflammatory protein 1α.
References for breast cancer: [8–11].
References for multiple myeloma: [12].

This paper aims to illustrate important aspects in the interrelationship between tumor cells and the tumor microenvironment, tumor angiogenesis in particular, in tumor progression. Four tumor entities, in which antiangiogenic agents have already significantly changed treatment strategies, are taken as examples: colorectal cancer (CRC), renal cell carcinoma (RCC), and breast cancer (BC), as well as multiple myeloma (MM).

2. Tumor Angiogenesis

Research on tumor angiogenesis is a major focus in biomedicine. Historically, Dr. Virchow was the first to identify a huge number of blood vessels in tumors in 1863 [13]. Few decades later in 1907, Goldman was the first to describe tumor vascularization in carcinomas of the stomach, the liver, and other organs [14]. In 1913, Murphy reported about the angiogenic response induced by Jensen rat sarcoma cells in the chick chorioallantoic membrane (CAM) [15]. The term "angiogenesis" was first used in 1935 and described the formation of new blood vessels in the placenta [16] and four years later in wound healing and tumor growth [17]. However, it was not until 1971 when Folkman hypothesized that inhibition of angiogenesis may be a potential way to inhibit cancer progression [18]. Subsequently, independent studies by Senger and Dvorak, Ferrara and Henzel, as well as Connolly and colleagues led to the purification, identification and cloning of vascular endothelial growth factor (VEGF), the key proangiogenic factor [19–23]. Since then, our knowledge of molecular mechanisms to tumor angiogenesis continually increased leading to the discovery of promising antiangiogenic therapies for tumor patients [24, 25]. Specifically, the impact of the tumor microenvironment, and tumor angiogenesis in particular, has been studied in greater detail in three types of solid cancer (CRC, RCC, BC)—and MM. These diseases serve as paradigm diseases for ongoing studies also in other tumor entities.

During tumorigenesis, the appropriate balance between proangiogenic and antiangiogenic molecules which arise from cancer cells and stromal cells in response to direct cell-cell, cell-ECM binding as well as to autocrine and paracrine growth factor stimulation, is lost [26, 27]. The "angiogenic switch", a rapid increase of blood vessel formation to support tumor growth, is triggered by (1) oncogene-mediated tumor expression of angiogenic proteins including VEGF, fibroblast growth factor (FGF), platelet derived growth factor (PDGF), endothelial growth factor (EGF), lysophosphatic acid (LPA), and angiopoietin (Ang), (2) metabolic and/or mechanical stress, (3) genetic mutations, (4) the immune response, and maybe most prominently (5) hypoxia. Tumor-angiogenesis therefore depends on tumor type, site, growth, and stage of disease and contributes to tumor growth, invasion, and metastasis.

The main mechanism of tumor angiogenesis is endothelial sprouting which crucially depends on VEGF upregulation and the interaction between ECs, pericytes, stroma cells as well as their association with the ECM [28, 29]. Specifically, VEGF and angiopoietin activate matrix degrading enzymes including the plasminogen activator (PA) and matrix metalloproteinases (MMPs) to loosen the matrix and favor EC migration [30]. VEGF and angiopoietin-2 (Ang-2)/type I tyrosine kinase receptor 2 (TIE 2) system then induce the detachment of pericytes and thereby increase vessel porosity. Plasma proteins are exuded and provide a gradient for EC migration [31–34]. Mechanistically, vessel sprouting is mediated by specialized ECs: tip cells lead the new sprout; stalk cells trail behind the pioneering tip cell, proliferate to form an elongating, stalk and create a lumen; and endothelial nonproliferating phalanx cells sense and regulate perfusion in the persistent sprout. Functionally, VEGF induces both NOTCH 1-mediated proliferation in stalk cells as well as directed migration of delta-like 4 (DLL 4)-expressing tip cells towards the sources of angiogenic factors. Endothelial cell-derived factor epidermal growth factor-like domain multiple 7 (Egfl 7) and components of the ECM then regulate vascular lumen formation [35]. Finally, PDGF produced by ECs then recruits pericytes, which surround and stabilize new vessels.

Besides sprouting, the formation of the endothelial lining of tumor vessels is promoted by cooption of neighboring preexisting vessels [36], intussusception (insertion of connective tissue columns into vessel lumen), glomeruloid angiogenesis, as well as VEGF-induced recruitment of highly proliferative circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) from the BM, hematopoietic stem cells (HSCs), progenitor cells, monocytes, and macrophages [37]. In addition, tumor cells themselves act as ECs to form functional avascular blood conduits or mosaic blood vessels [38–42].

Oxygen tension is the key regulator of VEGF expression, predominantly via the hypoxia-inducible factor (HIF)/von Hippel-Lindau tumor suppressor gene (VHL) pathway. Under normoxic conditions, prolyl hydroxylase domain (PHD) proteins hydroxylate prolyl residues on HIF, which are recognized by VHL, polyubiquitinated, and undergo proteasomal degradation. Tumor growth is often accompanied by a decrease in oxygen tension due to insufficient vascularization [43]. In turn, the process of tumor angiogenesis gets initiated and blood vessels supply nutrients and oxygen for the tumors that reach a hypoxic and necrotic area [43]. Under hypoxic conditions, PHD proteins are inactive, and nonhydroxylated HIF accumulates, translocates to the nucleus and binds to hypoxia-response elements (HRE) thereby initiating transcription of various genes that play a central part in angiogenesis. Genes induced by HIF include VEGF, PDGF, transforming growth factor-β (TGF-β), TGFα, epidermal growth factor receptor (EGFR), insulin-like growth factor 2 (IGF2), MMP1, stromal cell-derived factor 1 (SDF1), glucose transporter 1 (involved in glucose metabolism), as well as carbonic anhydrase 9 (CAIX), and activin B [44–47]. Factor inhibiting HIF (FIH) modulates interaction of HIF with the coactivators CBP/p300 [48]. HIF is also regulated by oxygen-independent pathways via growth-factor receptors or other signaling molecules. Specifically, growth factors, signaling molecules, and loss of function mutations of molecules such as VHL, p53, and PTEN, trigger HIF-1α synthesis. HIF expression is also controlled by specific microRNAs. A recent study identified a unique microRNA in hypoxic endothelia cells, miR424, that promotes HIF-1 stabilization and angiogenesis [49, 50]. Importantly, besides being a key regulator of angiogenesis, HIF activity is required for tumor cell survival and proliferation, migration, invasion, pH regulation, metabolism, drug and radiation resistance, immune evasion, and genetic stability [51, 52].

3. Colorectal Cancer

Major improvements in the therapy of CRC have been made during the last decades. These improvements are based on our increased knowledge of the role of the tumor microenvironment, and angiogenesis in particular, in CRC tumorigenesis. In the late 1980s, Dr. Vogelstein postulated a paradigm of multistep carcinogenesis in CRC involving a progressive series of specific and well-defined genetic alterations in tumor suppressor genes (APC, p53, or DCC) and in oncogenes (K-Ras), which render normal mucosa to carcinoma [53, 54]. Besides inducing tumor cell proliferation, survival, migration, and drug resistance, these alterations trigger changes in the tumor microenvironment, tumor angiogenesis in particular, *via* upregulation of VEGF as well as deregulation of other molecules including EGFR and COX2. Increased levels of VEGF and EGFR expression have been found in patients with localized as well as metastatic CRC [55–60]. Based on successful clinical phase III trials both VEGF inhibitors (e.g., bevacizumab) as well as EGFR inhibitors (e.g., cetuximab, panitumumab) have been approved and incorporated into novel treatment regimens of progressed CRC.

Metabolic products of cyclooxygenase 2 (COX2), prostaglandins in particular, contribute to neovascularisation and support vasculature-dependent growth of CRC, invasion, and metastasis [31, 61, 62]. COX2 is upregulated in approximately 50% of adenomas and 85% of adenocarcinomas [63, 64] and associated with worse survival among CRC patients [65]. Genetic deletion of COX2 dramatically reduces intestinal polyp formation supporting a key function of COX2 in CRC tumorigenesis [66]. Functionally, COX2 triggers secretion of MMP2 and MMP9 and enhances the expression of proangiogenic growth factors including VEGF and bFGF. It therefore contributes to the dissolution of the collagen matrix, EC migration, and formation of tubular networks [67–70]. COX2 inhibitors suppress VEGF and bFGF expression and thereby block angiogenesis [71–73]. Indeed, both aspirin and nonaspirin-NSAIDs given daily reduce the incidence of CRC significantly [74, 75].

Another potential therapeutic target is endoglin, a membrane-steady TGFβ coreceptor regulating tumorangiogenesis in CRC [76, 77]. High levels of soluble Endoglin have been found in CRC and BC patients [78] where it contributes to EC dysfunction [79, 80]. However, exact mechanism

of soluble endoglin on tumor angiogenesis remain to be identified.

In summary, inhibitors of growth factors contributing to tumor angiogenesis such as VEGF, EGF, and also COX2 have already been incorporated into novel treatment regimens and maintenance therapies in CRC. Promising future therapeutic targets include endoglin.

4. Renal Cell Carcinoma

Renal cell carcinoma/hypernephrom accounts for 2-3% of all cancer cases in adults. It is the seventh most common cancer in men and the ninth most common in women [81]. While localized RCC has a 5-year survival rate of 60–70%, metastatic RCC is the most lethal of all urological cancers [82]. Resistant to chemotherapy [81], only immunotherapy with IL-2 and interferon α (IFN α) has been utilized for systemic RCC therapy until most recently [83]. The introduction of antiangiogenic agents has dramatically improved treatment options in metastatic RCC. Indeed, an unprecedented six antiangiogenic agents have been approved for RCC treatment during the last 5 years including sunitinib, temsirolimus, everolimus, pazopanib, bevacizumab, and sorafenib. These agents improve progression-free survival. However, improvements of overall survival have not been demonstrated yet.

The evaluation of antiangiogenic agents for treatment of RCC has been triggered by the finding that RCC is a highly vascular tumor and that increased microvessel density (MVD) correlates with increased risk of metastasis, recurrence and adverse prognosis. High expression of VEGF and other angiogenic factors are predominantly triggered by the inactivation of the VHL tumor suppressor gene due to the loss of 3p [84–88]. Consequently, HIF is not degraded even under normoxic conditions [85]. Furthermore, VHL has many functions that are independent of HIF [89]. For example, inactivated VHL cannot interact with fibronectin and hydroxylated collagen IV. It thereby leads to impaired ECM organization invasion and angiogenesis in RCC [90, 91].

Besides VHL/HIF signaling, other signaling pathways may also participate in the regulation of secreted angiogenic factors in RCC. For example, in VHL-defective RCC cells, oncoprotein HDM2 not only affects constitutively expressed HIFα, but also directly regulates protein levels of HIF angiogenic targets (e.g., VEGF, PA inhibitor-1 (PAI-1), and endothelin-1 (ET-1)) [92].

RCC is one of the most immunogenic tumors [93]. Importantly, besides its effects on angiogenesis VEGF modulates immune tolerance in the tumor microenvironment by attenuating dendritic cell differentiation [94], and increasing secretion of immunosuppressive cytokines [95]. Anti-RCC activity of VEGF-inhibitors may therefore, at least in part, also be mediated *via* modulation of the antitumor immunity.

5. Breast Cancer

In 2010, BC was the cancer with the most new cases (207,090 women) of females in the USA and forth highest death rate (39,840 women) [96]. As in CRC and RCC, VEGF expression is also upregulated in BC. Moreover, angiogenesis represents a major independent prognostic factor in BC [97]. VEGF production and secretion within the BC microenvironment is triggered by a number of stimuli including growth factors, cytokines, hormones, loss of p53 function, RAS and SRC mutations, hypoxia as well as overexpression of HER2 (HER2/neu, ErbB2) [98–100]. Moreover, high levels of MMP-9 are produced and secreted by BC cells [101] and release sequestered VEGF from the adjacent ECM [102]. Importantly, VEGF levels are higher in premenopausal patients than in postmenopausal patients indicating that steroid hormones increase VEGF expression [103]. Indeed, upregulation of VEGF in tumor cell lines is triggered by the interaction of the ERα/estradiol-complex with an imperfect estrogen response element located 1.5 kb upstream of the VEGF transcription start site [104, 105].

HER2 is a member of the EGFR family encoded by the ERB2 gene. In human BC, the HER2 gene is amplified in 20–30% of all BC [106, 107]. Phosphorylation of the tyrosine kinase domain results in tumor cell and EC proliferation and survival *via* PI3K- and Ras/MEK/MAPK-signaling pathways [57, 108, 109]. In addition to phosphorylation, cleavage of the extracellular domain of HER2 generates an intracellular domain (p95) which activates these signaling pathways.

Another regulator of angiogenesis in BC is osteoprotegerin (OPG), a glycoprotein belonging to the TNF receptor (TNFR) superfamily whose production is triggered by direct cell-EC contact [110]. High levels of OPG are present in tumor ECs and correlate with tumor grade in BC [111].

Similarly, the transcription factor HOXB9 is overexpressed in 42% of patients with BC. It induces production of TGF-β, ErbB ligands, and several angiogenic factors (VEGF, bFGF, IL-8, and ANGPTL-2) thereby resulting in the induction of mesenchymal cell fate, invasion, as well as angiogenesis [112].

Finally, *fes* proto-oncogene (also known as *fps*) which encodes a Src homology 2 (SH2) domain-containing cytoplasmic PTK mediates tumor angiogenesis and metastasis [113]. Indeed the tumor microenvironment in Fes-deficient mice showed reduced vascularity and fewer tumor-associated macrophages indicating a therapeutic role for fes-inhibition [114].

In addition to bevacizumab, a variety of additional antiangiogenic agents is under clinical investigation for treatment of BC in the palliative as well as in the adjuvant setting. Importantly, also the anti-BC activity of tamoxifen is, at least in part, due to its antiangiogenic effect [115–117].

6. Multiple Myeloma

MM is a B-cell neoplasm characterized by excess clonal proliferation of malignant plasma cells in the bone marrow, elevated serum and urine monoclonal protein, osteolytic bone lesions, renal disease, and immunodeficiency. MM is the second most frequent malignancy of the blood in the USA. It causes about 1% of neoplastic diseases and 13% of hematological malignancies [118, 119]. The development of MM involves both early and late genetic changes in the

tumor cell as well as selective supportive conditions by the bone marrow (BM) microenvironment, BM angiogenesis in particular [120]. It is suggested that MGUS and nonactive MM in which the tumor growth is arrested are "avascular phases" of plasma cell tumors, while the active MM is the "vascular phase", which is associated with clonal expansion and epigenetic modifications of the microenvironment as well as the "angiogenic switch" [121, 122]. Importantly, these findings correlate with disease progression and poor prognosis. Moreover, BM MVD at the time of initial diagnosis is an important prognostic factor for median overall survival (OS) and median progression-free survival (PFS) in patients undergoing autologous transplantation as frontline therapy for MM [123].

VEGF within the MM BM microenvironment induces growth, survival as well as migration of MM cells in an autocrine manner via VEGFR-1 and triggers angiogenesis via VEGF-2 in ECs [122–127]. Recent studies suggest the existence of MM-specific ECs (MMECs) which produce growth and invasive factors for plasma cells, including VEGF, FGF-2, MMP-2 as well as MMP-9. Compared to healthy human umbilical vein EC (HUVEC), MMECs secrete higher amounts of the CXC chemokines (e.g., IL8, SDF1-α, MCP-1), which act in a paracrine manner to mediate plasma cell proliferation and chemotaxis [120–123, 126, 128, 129]. In turn, MM cells and stromal cells prolong survival of ECs both by increased secretion of EC survival factors, such as VEGF, and by decreased secretion of antiangiogenic factors [123, 130, 131].

Based on the enhanced understanding of the functional importance of the MM BM microenvironment and its interrelation with the MM cell resulting in homing, seeding, proliferation and survival, new molecular targets have been identified and derived treatment regimens in MM have already changed fundamentally during recent years. The anti-MM activity of thalidomide, bortezomib, and lenalidomide is mediated, at least in part, also via antiangiogenic effects [132]. For the treatment of MM, additional antiangiogenic therapies are therefore being evaluated in combination with conventional or novel anti-MM therapies [12, 127].

7. Inhibitors of Angiogenesis (Table 2)

7.1. Thalidomide and the IMiDs (Lenalidomide/Revlimid, Pomalidomide/Actimid). In 1994, D'Amato et al. studied the mechanism of thalidomide's teratogenicity and found that thalidomide (Celgene) is a potential inhibitor of angiogenesis [133]. Based on this finding and the discovery that bone marrow MVD plays a key role in MM pathogenesis, thalidomide was used empirically to treat patients with refractory relapsed MM in the late 90s. Remarkable clinical responses rendered thalidomide to be the first antiangiogenic agent for cancer treatment [134]. Currently, thalidomide is not only used in patients with refractory/relapsed but also with newly diagnosed MM.

Subsequently, a series of thalidomide-derived immunomodulatory drugs (IMiDs) including lenalidomide (Revlimid) and pomalidomide (Actimid) have been developed [135]. A phase I dose-escalation trial using lenalidomide in patients with relapsed and refractory MM demonstrated either response or stabilization of disease in 79% cases [136]. Two clinical phase II trials confirmed these data and achieved complete responses with favorable side effect profiles; two clinical phase III trials comparing lenalidomide to dexamethasone/lenalidomide treatment of relapsed MM provided the basic for its FDA approval in 2006. In the relapsed/refractory setting an overall response of 30% was achieved by the new IMID pomalidomide, alone or in combination with dexamethasone. More than 100 clinical studies with thalidomide or lenalidomide combined with other agents are currently recruiting or ongoing.

Adverse side effects of thalidomide and the IMiDs include polyneuropathy, fatigue, skin rash, and venous thromboembolism (VTE), or blood clots, which could lead to stroke or myocardial infarction. Both thalidomide and the IMiDs overcome the growth and survival advantage conferred by the BM milieu, at least in part by downregulating VEGF [137, 138], and inhibition of proliferation and capillarogenesis of MMECs [128].

7.2. Bevacizumab (Avastin). Bevacizumab (Genetech) [139] binds biologically active forms of VEGF and prevents its interaction with VEGF receptors (VEGFR-1 and VEGFR-2), thereby inhibiting endothelial cell proliferation and angiogenesis. In preclinical studies bevacizumab reduced microvascular growth and inhibited metastasis of colon growth in nude mice [140–142].

When tested in patients with metastatic CRC bevacizumab in combination with conventional chemotherapy demonstrated significant survival benefits. Based on this finding, the US FDA approved bevacizumab in February 2004, followed by the EMEA approval in January 2005, as first-line treatment of metastatic CRC in combination with 5-fluorouracil-(FU-) based chemotherapy regimens. In 2006, bevacizumab in combination with 5-FU was also approved for second-line treatment of CRC. In contrast, the use of bevacizumab in the adjuvant setting cannot be recommended [143–145]. Bevacizumab is therefore the first VEGF-targeting agent approved both by the US FDA as well as the EMEA for cancer treatment [146].

Since its initial approval as first-line treatment in metastatic CRC in 2004, bevacizumab has been approved for use in combination with other chemotherapeutics in four other tumor types: in 2009 (US) and 2007 (EU) for advanced RCC, in 2008 for metastatic HER2-negative BC, in 2009 for glioblastoma, and in 2004 for non-small cell lung cancer (NSCLC) [147–150].

Specifically, the E2100 study was the first Phase III study using bevacizumab in metastatic BC as first-line treatment. Bevacizumab was investigated in combination with and without paclitaxel. In combination with bevacizumab, progression-free survival was doubled (5.8 months to 11.3 months). The overall response rate increased from 22 to 50%. Because of this study, bevacizumab was approved for metastatic BC [151]. But as the overall survival did not show any benefit, Fojo and Wilkerson [152] believe that the E2100 trial overestimated the benefit of bevacizumab and

Table 2: Summary of drugs, their revealed targets and indications in clinical trails. Drugs without a single treatment trial are marked with a "*".

Drug (brand name, company)	Target	Approved	Clinical trials with single treatment	Indication
Bevacizumab (Avastin, Genentech/Roche)	Monoclonal antibody against VEGFA	mCRC, mRCC, NSCLC, metastatic HER2-negative breast cancer, glioblastoma	Phase I, II	Multiple solid tumors (e.g., RCC, BC, pancreatic, prostate, ovarian, brain cancers) and hematologic malignancies (e.g., MM)
Sunitinib, SU11248 (Sutent, Pfizer)	TKI of VEGFR 1–3, PDGFRα/β, c-Kit, Flt3, RET, CSF-1R	mRCC, GIST	Phase I	Multiple solid tumors (e.g., RCC, BC, melanoma, lung)
Pazopanib (Votrient, GlaxoSmithKline)	TKI of VEGFR 1–3, PDGFRα/β, c-kit tyrosine kinases	mRCC	Phase I, II	Multiple solid tumors (e.g., BC, RCC, ovarian, lung) and others (e.g., lymphoma)
Sorafenib, BAY43-9006 (Nexavar, Bayer)	TKI of Multiple cell surface kinases (VEGFR 1–3, RET, PDGFRβ, Flt-3, c-Kit, CSF-1) and intracellular kinases (CRAF, BRAF, mutant BRAF)	mRCC, unresectable hepatocellular carcinoma	Phase I, II	Multiple solid tumors (e.g., RCC, BC, melanoma, lung cancers) and hematologic malignancies (e.g., MM)
Vandetanib, ZD6474 (Zactima, AstraZeneca)	TKI of VEGFR, EGFR and RET	Metastatic medullary thyroid cancer	Phase I, II	NSCLC, RCC, glioblastoma
Bortezomib, PS-341 (Velcade, Millennium Pharmaceuticals)	26S proteasome inhibitor	MM, relapsed mantle cell lymphoma	Phase I, II	MM, lymphoma, leukemia and multiple solid tumors (e.g., RCC, BC, lung, prostate)
Temsirolimus (Torisel, Wyeth)	mTOR inhibitor	mRCC	Phase I, II	Multiple solid tumors (e.g., RCC, BC, melanoma, prostate, liver cancers) and hematologic malignancies (e.g., lymphoma)
Everolimus, RAD001 (Afinitor, Novartis)	mTOR inhibitor	Advanced renal cell carcinoma	Phase I, II	Multiple solid tumors (e.g., BC, pancreatic, gastric cancers) and lymphoma
Thalidomide (Thalomid, Celgene)	Angiogenesis inhibitor, multiple	MM	*	MM
Lenalidomide, CC-5013 (Revlimid, Celgene)	Angiogenesis inhibitor, Thalidomide derivative	MM	Phase I, II	MM, lymphoma, chronic lymphocytic leukemia, and multiple solid tumors (e.g., CRC, ovarian)
Pomalidomide, CC-4047 (Actimid, Celgene)	Angiogenesis inhibitor, Thalidomide derivative	No yet approved	Phase I	MM, Lymphoma
Aflibercept, VEGF-trap (ZALTRAP, Sanofi-Aventis and Regeneron)	Decoy receptor for all VEGF-A isoforms	No yet approved	*	mCRC, RCC, Ovarian, NSCLC, prostate cancers, lymphoma, leukemia
Axitinib, AG-013736 (Pfizer)	TKI of VEGFR 1–3, PDGFRβ, c-KIT and CSF-1	No yet approved	Phase I	mRCC, BC, NSCLC, metastatic pancreatic cancer, GIST, lung cancer, thyroid cancer
Icrucumab, IMC-18F1 (ImClone)	Monoclonal antibody against VEGFR-1	No yet approved	Phase I	Advanced solid tumors, (e.g., CRC, BC, carcinoma of urinary tract)
Ramucirumab, IMC-1121b (ImClone)	Monoclonal antibody against VEGFR-2	No yet approved	*	CRC, BC, mRCC, Advanced liver, gastric, prostate, ovarian, and NSCL cancers, melanoma
Vatalanib, PTK787 (Novartis)	TKI of VEGFR 1–3, PDGFRα/β, and c-KIT	Not yet approved	*	Multiple solid tumors (e.g., CRC, glioblastoma, NSCLCs) and hematologic malignancies (e.g., leukemia)

Table 2: Continued.

Drug (brand name, company)	Target	Approved	Clinical trials with single treatment	Indication
Enzastaurin, LY317615.HCl (Eli Lilly)	PKC inhibitor	Not yet approved	*	BC, mCRC, Brain tumor, advanced NSCL, glioblastoma, lymphoma
Cediranib, AZD2171 (Recentin, AstraZeneca)	TKI of VEGFR 1–3	Not yet approved	Phase I	RCC, CRC, BC, ovarian, prostate cancer, lung, brain, head and neck cancers, glioblastoma, melanoma
Vectibix, panitumumab (Amgen)	EGFR	mCRC	Phase I, II	mCRC, pancreatic, HNSCC, NSCLC, lung
Erbitux, cetuximab, (Imclone, Bristol-Myers Squibb)	EGFR	mCRC	Phase I, II	mCRC, HSNCC, brain, MM, lung, pancreatic, liver
Trastuzumab, herceptin (Genentech)	HER2 receptor	Gastric cancer, HER2 positive BC	Phase I, II	BC, gastric
Tykerb, lapatinib (GlaxoSmithKline)	EGFR and HER2 receptor	BC	Phase I, II	BC, CRC, lung, HNSCC, pancreatic, melanoma
Tamoxifen, Novadex, Istubal, Valodex (AstraZeneca)	Estrogen receptor	BC	Phase I, II	BC, bladder, melanoma, prostate

TKI: tyrosine kinase inhibitors; mCRC: metastatic colorectal cancer; NSCLC: nonsmall cell lung cancer; mRCC: metastatic renal cell carcinoma; GIST: gastrointestinal stroma tumor after progression; MM: multiple myeloma; BC: breast cancer; HNSCC: head and neck sequmous cell carcinoma.

that further studies need to target the VEGF polymorphism of VEGF in order to identify the patients that derive true benefit from bevacizumab [153]. Based on two double-blind studies (AVADO and RIBBON-1) showing high toxicity without significant improvements of progression-free survival [154–156], the use of bevacizumab as first-line therapy in progressed Her2-negative BC has been removed by the US FDA in 2010. In a meta-analysis, Ranpura et al. report that addition of bevacizumab to systemic antineoplastic therapy is associated with a significantly increased risk (relative risk of 1.46; incidence, 2.5% versus 1.7%) of fatal adverse events (FAEs), in BC patients [157, 158]. However, clinical studies evaluating bevacizumab in combination with conventional therapies both in Her2-negative and also Her2-positive patients are ongoing. It may be possible to focus bevacizumab treatment in patients most likely to benefit, and avoid treatment of patients unlikely to benefit or more likely to experience toxic effects [157].

Although generally well tolerated, side effects of bevacizumab treatment include minor (hypertension, proteinuria, nosebleed, upper respiratory infection, gastrointestinal symptoms, and headache) and rarely serious (gastrointestinal perforations, hemorrhage, and thrombolysis) adverse effects.

7.3. Cetuximab (Erbitux). Cetuximab (Merck, ImClone, Briston-Myers-Squibb) is a recombinant, human-IgG1/mouse chimeric monoclonal antibody which blocks phosphorylation and activation of receptor-associated kinases by binding to the receptor. Erbitux is single-used or used in combination with other therapies to treat CRC. The US FDA used three separate clinical trials as a base to approve Erbitux for treatment of EGFR-expressing, recurrent metastatic CRC in patients who are intolerant to irinotecan-based chemotherapy in 2004. In 2007, the US FDA expanded labeling and granted regular approval for single-agent cetuximab for the treatment of patients with EGFR-expressing metastatic CRC after failure of both irinotecan- and oxaliplatin-based chemotherapy regimens (http://www.cancer.gov/).

Known side-effects are rash, asthenia/malaise, diarrhea, nausea, abdominal pain, vomiting, fever, and infusion reaction [159–162].

7.4. Panitumumab (Vectibix). Panitumumab (Amgen), a recombinant, human IgG2 kappa monoclonal antibody, binds specifically to the extracellular domain of EGFR and thereby prevents its activation and downstream signaling sequeale [163–166]. In 2006, panitumumab was approved by the US FDA for treatment of EGFR-expressing metastatic CRC with disease progression despite prior treatment; in 2008 by the EMEA for the treatment of refractory EGFR-expressing metastatic CRC in patients with nonmutated K-Ras.

Known side-effects include dermatological toxicities, ocular toxicities, hypomagnesemia, fatigue, abdominal pain, nausea, diarrhea and constipation.

7.5. VEGF-Trap (ZALTRAP, Aflibercept), HuMV833, and Other Monoclonal Antibodies Targeting VEGF. VEGF-trap (Sanofi-Aventis and Regeneron) is a soluble decoy receptor protein consisting of a hybrid Fc construct in which domain 2 of VEGFR-1 is fused to domain 3 of the VEGFR-2 [167, 168]. VEGF-trap is known to have high affinity

to all isoforms of VEGF-A. It caused vessel-regression of coopted vessels in a model of neuroblastoma [169]. Several clinical phase II/III trials testing the VEGF-trap in solid and hematologic malignancies including CRC, MM, pancreatic cancer, prostate cancer, NSCLC are ongoing (http://clinicaltrials.gov/). On April 26, 2011, Sanofi-Aventis and Regeneron reported about the positive phase III results with VEGF-trap in second-line mCRC. The VELOUR study evaluates ZALTRAP in combination with FOLFIRI chemotherapy *versus* FOLFORI plus placebo. Exact results are eagerly awaited for the second half of 2011.

Similarly, HuMV833, a humanized monoclonal IgG antibody-binding VEGF-A isoforms (VEGF121 and VEGF165), demonstrated antitumor effects in a variety of human tumor xenograft models [170, 171].

Additionally, antibodies against VEGFR-1 or VEGFR-2 (IMC-18F1, IMC-1121B, ImClone) are under preclinical and clinical investigation. IMC-18F1 is a fully human, high affinity neutralizing antibody that specifically blocks VEGFR-1 activation, which has demonstrated preclinical activity in BC [172]. IMC-1121B (ramucirumab), a fully human monoclonal IgG1 antibody against the extracellular domain of VEGFR-2, is currently under evaluation in various entities including advanced liver, kidney, prostate, ovarian, colorectal, melanoma, BC, and NSCL cancer [173, 174].

7.6. Trastuzumab (Herceptin). Trastuzumab (Genentech) is a recombinant humanized monoclonal antibody which binds to the extracellular domain of the HER2 receptor and inhibits the intracellular tyrosine kinase activity. In addition, it blocks cleavage of HER2 and thereby the production of p95, interferes with either homodimerization or heterodimerization of HER2 with itself or other HER receptors, and recruits Fc-competent immune effector cells and other components of antibody-dependent cell-mediated cell cytotoxicity (ADCC). In 1998, trastuzumab was FDA approved for treatment of patients with HER2-positive metastatic BC in combination with paclitaxel. In 2006, FDA approval of trastuzumab was expanded for the adjuvant setting in combination with chemotherapy regimens containing doxorubicin, cyclophosphamide, and paclitaxel. In January 2008, FDA approval was revised to include the use of trastuzumab also as a single agent in the adjuvant setting [175].

7.7. Small Molecule Inhibitors. Although Avastin is an effective medication and studies testing the VEGF-trap or VEGFR-targeting antibodies are promising, drug resistance always develops likely due to targeting a single tumorigenic pathway. Indeed extended blockade of VEGF alone results in tumor revascularization, dependent on other proangiogenic factors such as FGF [176]. Small-molecule inhibitors have the advantage of being orally available as well as more promiscuous in target inhibition and also less expensive [177, 178]. Based on these therapeutic advantages, many tyrosine kinase inhibitors (TKIs) have been developed and subjected to clinical trials. Indeed, the second-generation multi-targeted receptor kinase inhibitors (RTKIs) sorafenib, sunitinib, and pazopanib have now been approved for the treatment of advanced RCC and gastrointestinal stroma tumor (GIST), hepatocellular carcinoma (HCC). Moreover, preliminary data in other malignancies, most prominently including CRC and BC are promising.

7.7.1. Sorafenib (Nexavar). Sorafenib (Bayer HealthCare Pharmaceuticals and Onyx Pharmaceuticals) [179, 180] is a RTK inhibitor which targets VEGFR2, VEGFR-3, Raf, PDGFRβ, Flt3, and c-Kit. It was approved for the treatment of advanced RCC in 2005 and for the treatment of unresectable HCC in 2007. Advanced clinical studies in NSCLC and melanoma are ongoing.

7.7.2. Sunitinib (Sutent). Sunitinib (Pfizer) is another multi-targeted TKI which targets VEGFR2, PDGFRα/β, c-Kit, Flt3, RET [181–184]. Based on a phase III clinical trial, in which sunitinib demonstrated improvements in progression-free survival when compared to IFNα, it was approved for first-line and second-line therapy of metastatic RCC [185, 186]. In addition, sunitinib was also approved for treatment of GIST in 2006 [187]. Advanced clinical studies are ongoing in breast, colorectal, and lung cancer. Both sorafenib and sunitinib alone or in combination therapy are under clinical evaluation in MM.

7.7.3. Temsirolimus (Torisel) and Everolimus (Afinitor). Temsirolimus (Wyeth Pharmaceuticals), a derivative of rapamycin, is a specific inhibitor of the mammalian target of rapamycin (mTOR). mTOR pathway has an important role in regulating the synthesis of HIF and proteins that control cell proliferation, such as c-myc and cyclin D1. Therefore, inhibiting mTOR in RCC downregulates HIF activity and stops the production of cell-cycle regulators [188, 189]. In 2007, Temsirolimus was approved for the treatment of advanced RCC. As compared with IFNα, temsirolimus improved overall survival among patients with mRCC and poor prognosis [190].

Everolimus (Novartis), another rapamycin analogue was approved for treatment of patients with mRCC whose disease had progressed despite prior treatment with sunitinib, sorafenib, or both in 2009 [191, 192].

Clinical studies which evaluate the activity of temsirolimus and everolimus in other tumor entities including BC, gastric cancer, HCC, MM, and lymphoma are ongoing.

7.7.4. Pazopanib (Votrient). Pazopanib (GlaxoSmithKline) is a novel orally available, small-molecule tyrosine kinase inhibitor of VEGF-receptor-1, -2, -3 with IC50's of 10, 30, and 47 nM, respectively. In 2010, pazopanib was approved as the third TKI and the last among the six treatments for mRCC (sorafenib, sunitinib, temsirolimus, everolimus, bevacizumab) approved by the FDA during the last 5 years. The basis for this approval was a randomized, double-blind, placebo-controlled phase III study evaluating the efficacy and safety of pazopanib in 435 patients with locally advanced and/or mRCC. The median PFS for the pazopanib was 9.2 months compared with 4.2 months for the placebo in overall population ($P < 0.001$) [193]. Moreover, the combination of

pazopanib with lapatinib was effective in patients with BC, and preclinical data in MM were promising [194]. Clinical studies which evaluate the activity of pazopanib in other tumor entities are ongoing.

7.7.5. Axitinib. Axitinib (Pfizer) is an oral, potent, and selective inhibitor of VEGFR 1–3, PDGFRβ, and c-KIT. Promising data from a clinical phase I study [195] prompted the clinical evaluation of Axitinib in a variety of malignancies. Excitingly, clinical activity has now been demonstrated in sorafenib-refractory metastatic RCC [196] and patients with advanced NSCLC [197]. Moreover, a clinical phase III trial in patients with unresectable, locally advanced, or metastatic pancreatic cancer treated with gemcitabine plus axitinib is now ongoing to verify a small gain in overall survival observed in a clinical phase II trial [198]. Clinical trials in mCRC showed no benefit of axitinib in first- and second-line combination therapies with oxaliplatin-containing chemotherapies in comparison to bevacizumab [199].

Additional clinical studies are ongoing in GIST, lung cancer, thyroid cancer, and breast cancer. Dose-limiting toxicities primarily seen at higher dose levels included hypertension, hemoptysis, and stomatitis. The observed hypertension was manageable with medication. Stomatitis was generally tolerable and managed by dose reduction or drug holidays.

7.7.6. Lapatinib (Tyverb). Lapatinib (GlaxoSmithKline) is another orally available TKI inhibiting both EGFR and HER2 receptors [200–202]. It was FDA approved in 2007 for combination therapy for triple-positive BC patients already treated with capecitabine or which have progressed on trastuzumab. In 2010, lapatinib additionally received accelerated approval as front-line therapy in this patient cohort.

Side effects of Tyverb include diarrhea, palmar-plantar erythrodysesthesia, nausea, rash, vomiting, muscular inflammation, stomatitis, pain in extremities, dyspnea, and fatigue [203–206].

8. Discussion

Recent studies delineate a key role for the tumor microenvironment in tumorigenesis. Investigating the complex functional interrelation between the cellular and noncellular compartments of the tumor microenvironment has already led to the identification of new therapeutic targets. One pivotal compartment within the microenvironment is the vascular niche. Indeed, 40 years after Dr. Folkman's seminal postulation in 1971 that angiogenesis is required for tumor growth and progression and may therefore represent a new target for cancer therapy [18], it is well established that angiogenesis plays an important role in solid as well as in hematologic malignancies. Tumor angiogenesis is now recognized to be a hallmark of cancer, initiated by enhanced tumor/tumor-stroma cell-specific production of proangiogenic molecules, and/or suppression of antiangiogenic factors (angiogenic switch) as well as via tumor-associated hypoxia. The introduction of antiangiogenic agents into clinical practice was a milestone event in cancer therapy during the last decade.

VEGF, EGF, and PDGF represent key factors in tumor angiogenesis. Blocking BM angiogenesis in MM with thalidomide; and VEGF with the first-in-class antiangiogenic drug bevacizumab; or EGFR with cetuximab in CRC have become established anticancer strategies. Following the introduction of bevacizumab, efforts focused on the identification of compounds targeting VEGF signaling sequelae that can be given orally. Several second-generation orally available small-molecule antiangiogenic drugs have now been identified including sunitinib, pazopanib, and sorafenib and have recently been approved for treatment of cancers including CRC, BC, RCC, and MM. However the optimal use of antiangiogenics is tumor- and stage-dependent. Moreover, although antiangiogenic antibodies as well as small molecules targeting VEGF and EGF signaling pathways significantly prolong overall survival of cancer patients, resistance always develops and disease relapse is inevitable. Recent molecular mechanistic studies may explain the disappointing results of previous clinical studies using VEGF inhibitors *alone* either in early or *refractory/progressive* disease. Modest, though significant, survival benefits were observed in patients with *advanced* tumors treated with bevacizumab and other antiangiogenics *even when combined* with conventional chemotherapies. Further studies are needed to increase our understanding of tumor angiogenesis and of how resistance against antiangiogenic agents develops. Potential mechanisms of evasive resistance include the redundancy of proangiogenic signals in later disease stages; recruitment of vascular progenitor cells and proangiogenic monocytes from the bone marrow, increased and tight pericyte coverage, or increased capabilities for invasion and metastasis; preexisting inflammatory cell-mediated vascular protection; hypovascularity; invasive and metastatic cooption of normal vessels; and mutational alteration of genes within endothelial cells [207]. Therapeutic benefits may be achieved by initiating treatment with VEGF-inhibitors early: by using antiangiogenic cocktails, which not only target VEGF both in patients with early and late-stage disease, as well as metronomic therapy [208].

Novel approaches to improve antiangiogenic therapy include strategies to target the angiopoietin-TIE system, Hif-1, endothelial-specific integrin/survival signaling (e.g., by cilengitide) as well as the use of vascular-disrupting agents (VDAs), which selectively disrupt already existing tumor vessels by targeting dysmorphic endothelial cells. Given the benefits of combination therapy, it is also crucial to optimize existing or identify new treatment regimens in order to reduce drug-associated toxic side effects.

In summary, antiangiogenic compounds like thalidomide, bevacizumab, sorafenib, sunitinib, and pazopanib, temsirolimus and everolimus have already demonstrated activity in a variety of cancers most prominently including BC, CRC, RCC, and MM. However, with the increase of our knowledge of the complexity of molecular mechanisms contributing to tumor angiogenesis in general, and MM BM angiogenesis in particular, we aim to identify additional

therapeutic targets, to further optimize treatment regimens; and to reduce mechanisms leading to antiangiogenic drug-resistance in order to further improve patient outcome and reduce drug toxicity.

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F. Fan and A. Schimming contributed equally to this paper.

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Review Article

Antiangiogenic Therapy for Patients with Recurrent and Newly Diagnosed Malignant Gliomas

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Malignant gliomas have a poor prognosis despite advances in diagnosis and therapy. Although postoperative temozolomide and radiotherapy improve overall survival in glioblastoma patients, most patients experience a recurrence. The prognosis of recurrent malignant gliomas is dismal, and more effective therapeutic strategies are clearly needed. Antiangiogenesis is currently considered an attractive targeting therapy for malignant gliomas due to its important role in tumor growth. Clinical trials using bevacizumab have been performed for recurrent glioblastoma, and these studies have shown promising response rates along with progression-free survival. Based on the encouraging results, bevacizumab was approved by the FDA for the treatment of recurrent glioblastoma. In addition, bevacizumab has shown to be effective for recurrent anaplastic gliomas. Large phase III studies are currently ongoing to demonstrate the efficacy and safety of the addition of bevacizumab to temozolomide and radiotherapy for newly diagnosed glioblastoma. In contrast, several other antiangiogenic drugs have also been used in clinical trials. However, previous studies have not shown whether antiangiogenesis improves the overall survival of malignant gliomas. Specific severe side effects, difficult assessment of response, and lack of rational predictive markers are challenging problems. Further studies are warranted to establish the optimized antiangiogenesis therapy for malignant gliomas.

1. Introduction

Malignant gliomas such as glioblastoma and anaplastic gliomas are the most common primary brain tumors in adults [1]. Temozolomide and radiotherapy have been demonstrated to improve overall survival in glioblastoma patients [2–4]. Despite advances in diagnosis and therapy, prognosis remains poor with a median overall survival of 12 to 15 months in glioblastoma due to the resistance to radiotherapy and chemotherapy. Although anaplastic gliomas tend to respond well to these treatments, the median survival time is 2 to 3 years [5, 6]. The prognosis of recurrent malignant gliomas is dismal with the median overall survival and progression-free survival (PFS) of 7.5 months and 2.5 months, respectively [7]. More effective therapeutic strategies are needed for these patients.

Malignant gliomas are characterized by vascular proliferation or angiogenesis [8, 9]. Vascular endothelial growth factor (VEGF) is highly expressed in glioblastoma and has been shown to regulate tumor angiogenesis [10]. Bevacizumab was developed as a humanized monoclonal antibody against VEGF. Clinical trials of recurrent glioblastoma showed benefits of bevacizumab in response rate and PFS [11–13]. Based on these favorable results, bevacizumab was approved by the US Food and Drug Administration (FDA) for recurrent glioblastoma. For newly diagnosed glioblastoma, phase II trials showed that the addition of bevacizumab to temozolomide and radiotherapy improves PFS [14, 15]. Other antiangiogenic drugs have also been investigated and used in several clinical studies [16]. In this paper, we focus on biological and clinical findings of antiangiogenesis therapy for malignant gliomas.

2. Biological Aspects of Antiangiogenic Therapy for Glioblastoma

Advances in molecular biology have provided pathogenesis of malignant gliomas. Several clinical and preclinical studies

proposed that tumor-related blood vessel, called "angiogenesis", is required for solid tumor growth, including malignant gliomas [10, 16]. Endothelial proliferation is a marker of histological grading systems for malignant gliomas because of an association between a degree of microvascularity and biologic aggressiveness [17]. Glioblastoma is particularly characterized by vascular proliferation and the extent of necrosis. These findings indicate that tumor antiangiogenesis is a promising candidate to inhibit the growth of malignant gliomas. VEGF, a critical mediator of angiogenesis, has emerged as a novel target of antiangiogenic therapy. Glioblastoma cells have been shown to secrete VEGF, resulting in the endothelial proliferation and tumor survival *in vivo* [18]. VEGF is expressed in malignant gliomas and is associated with tumor grade and vascularity [19, 20]. Therefore, it is postulated that antiangiogenesis suppresses blood flow and inhibits the tumor growth. Monoclonal antibodies against VEGF were shown to inhibit the growth of glioma cells *in vivo* [21]. A VEGF inhibitor directly affects glioma stem cells that are more resistant to chemotherapy and radiotherapy [22]. Furthermore, antiangiogenesis can normalize tumor vasculature and decrease interstitial fluid pressure, providing an improved delivery of chemotherapeutics and oxygen. Consequently, antiangiogenesis is expected to work synergistically with radiotherapy and chemotherapy [23, 24]. Given these findings, VEGF inhibitors are expected to be a novel antiangiogenic therapy for malignant gliomas.

3. The Efficacy and Safety of Bevacizumab for Recurrent Malignant Gliomas

3.1. Bevacizumab for Recurrent Glioblastoma. Bevacizumab was developed as a humanized monoclonal antibody to bind VEGF-A, preventing the interaction and activation of VEGF receptor tyrosine kinases [25, 26]. This drug is approved by the FDA and is in clinical use for the treatment of colorectal cancer, nonsmall cell lung cancer, breast cancer, renal cell carcinoma, and glioblastoma [27]. Table 1 shows several clinical studies of bevacizumab for recurrent malignant glioma patients.

The first phase II trial for 35 recurrent glioblastoma was performed to investigate the efficacy of intravenous administration of bevacizumab and irinotecan, a topoisomerase 1 inhibitor [11]. The 6-month PFS was 46%, and median overall survival was 10.5 months, respectively. At least a partial response was observed in 57% of patients. A larger, randomized, noncomparative phase II study, called the BRAIN study, was performed using bevacizumab with or without irinotecan for 167 recurrent glioblastoma. In this study, the response rates were 28.2% and 37.8%, and 6-month PFS were 42.6% and 50.3% in bevacizumab alone and bevacizumab plus irinotecan groups, respectively [12]. Another phase II study of bevacizumab alone for 48 recurrent glioblastomas showed that response rate was 35% and 6-month PFS was 29%, respectively [13]. These results were more favorable than a previous database of 8 negative trials having 6-month PFS of 15% for recurrent glioblastoma patients [7]. Furthermore, an additional advantage of bevacizumab is its ability to decrease tumor edema and to reduce steroid dose. Given the efficacy of bevacizumab for recurrent glioblastoma in the clinical setting, bevacizumab monotherapy has since been approved by the FDA.

3.2. Toxicity Profile of Bevacizumab. Since VEGF plays an important role in vascular function and physiological angiogenesis, its inhibition via bevacizumab has been reported to cause serious adverse events [34]. The first phase II study of recurrent glioblastoma treated by bevacizumab and irinotecan reported that five patients (14%) discontinued treatment due to central nervous system (CNS) hemorrhage, deep venous thrombosis, and pulmonary emboli [11]. No fatal adverse events were reported in this study. BRAIN study reported the detailed information on adverse events of bevacizumab [12]. Grade ≥ 3 adverse events were observed in 65.8% and 46.4% of bevacizumab plus irinotecan and bevacizumab alone groups, respectively. The most common causes of grade ≥ 3 adverse events were convulsion, hypertension, neutropenia, and fatigue. CNS hemorrhages of any grades were observed in five patients (3.0%). There were one fatal adverse event (1.3%) in bevacizumab plus irinotecan group and two (2.3%) in bevacizumab alone group, respectively. Selecting appropriate patients, early assessment of toxicity, and adequate management should be required to reduce the serious adverse events of bevacizumab.

Arterial and venous thromboses are generally reported in treatment of antiangiogenesis therapy, although the inherent risk of these thromboses is higher among malignant glioma patients. A retrospective study of 9849 patients with malignant gliomas showed that 2-year cumulative incidence of symptomatic venous thromboembolism was 7.5% [35]. Further studies are necessary to evaluate the additional risk of thrombosis in malignant glioma patients treated by bevacizumab.

Recently, a meta-analysis of randomized control trials in several tumor types showed that bevacizumab in combination with chemotherapy increases fatal adverse events when compared with chemotherapy alone [36]. The overall incidence of fatal adverse events was 2.5% in bevacizumab therapy with the common causes being hemorrhage, neutropenia, and gastrointestinal tract perforation. Interestingly, the type of chemotherapeutic agents was significantly associated with relative risk of fatal adverse events. The addition of bevacizumab was associated with increased fatal adverse events in patients receiving taxanes or platinum agents (3.3% versus 1.0%) but not in those receiving other agents (0.8% versus 0.9%). In clinical trials of malignant gliomas, the addition of irinotecan or temozolomide to bevacizumab has often been performed and may be associated with lower fatal adverse events. However, this meta-analysis did not include the trials of brain tumors, and further investigations are required to evaluate the fatal adverse events of bevacizumab and chemotherapy in malignant gliomas.

3.3. Bevacizumab for Recurrent Anaplastic Gliomas. Anaplastic gliomas have a slightly better prognosis than glioblastoma [37], and the median survival time is 2 to 3 years. However, there are no standard treatments for progression or recurrence of anaplastic gliomas, and a novel treatment

Table 1: Bevacizumab for recurrent or newly malignant gliomas.

Study	Agents	Patients	RR	MPFS	6-PFS	MST	Ref.
Phase II	Bevacizumab + irinotecan	35 recurrent GBM	57%	6 months	46%	10.5 months	[11]
Phase II	Bevacizumab	85 recurrent GBM	28%	4.2 months	43%	9.2 months	[12]
	Bevacizumab + irinotecan	82 recurrent GBM	38%	5.6 months	50%	8.7 months	
Phase II	Bevacizumab	48 recurrent GBM	35%	4 months	29%	7.7 months	[13]
Phase II	Bevacizumab + irinotecan	23 recurrent GBM	61%	5.0 months	30%	10 months	[28]
		9 recurrent AG	67%	7.5 months	56%	Not reached	
Phase II	Bevacizumab + irinotecan	33 recurrent AG	61%	7.5 months	55%	16.3 months	[29]
Retrospective	Bevacizumab + SRT	20 recurrent GBM	50%	7.3 months	65%	12.5 months	[30]
		5 recurrent AG	60%	7.5 months	60%	16.5 months	
Retrospective	SRS + bevacizumab	49 recurrent GBM	—	5.2 months	—	11.2 months	[31]
	SRS + other drugs		—	2.1 months	—	3.9 months	
Phase II	Bevacizumab + erlotinib	25 recurrent GBM	48%	4.5 months	28%	10.5 months	[32]
		32 recurrent AG	31%	5.9 months	44%	17.8 months	
Phase II	Bevacizumab + RT/TMZ	70 newly diagnosed GBM	—	13.6 months	88%	19.6 months	[14]
Phase II	Bevacizumab + RT/TMZ	125 newly diagnosed GBM	—	13.8 months	87%	—	[15]
Phase II	Adjuvant bevacizumab + irinotecan + TMZ	125 newly diagnosed GBM	—	13.8 months	—	21.3 months	[33]

RR: response rate; MPFS: median progression-free survival; 6-PFS: 6-month progression-free survival; MST: median overall survival time; GBM: glioblastoma multiforme; AG: anaplastic gliomas; SRT: stereotactic radiotherapy; SRS: stereotactic radiosurgery; RT: radiotherapy; TMZ: temozolomide.

strategy is needed. Recent studies have shed light on the antiangiogenic therapy in recurrent anaplastic gliomas. A phase II study of bevacizumab and irinotecan was performed in 23 glioblastoma and 9 anaplastic gliomas [28]. The response rates were 61% and 67%, and 6-month PFS were 30% and 56% in glioblastoma and anaplastic gliomas, respectively. Another phase II trial was conducted for 33 anaplastic gliomas treated by bevacizumab and irinotecan [29]. This study included 25 anaplastic astrocytomas and 8 anaplastic oligodendrogliomas. The 6-month PFS and overall survivals were 55% and 79%, respectively. At least a partial response was observed in 61% of patients, and dose of dexamethasone was decreased in 67%. These findings indicate that bevacizumab and irinotecan can be an active regimen for recurrent anaplastic gliomas.

3.4. Additional Treatment to Bevacizumab for Recurrent Malignant Gliomas. The addition of targeting therapy or radiotherapy to bevacizumab has been performed for recurrent malignant gliomas. Gutin et al. retrospectively analyzed bevacizumab and stereotactic radiotherapy (30 Gy in 5 fractions) for 25 recurrent malignant gliomas [30]. There were 20 glioblastomas and 5 anaplastic gliomas in this study with all patients receiving prior radiotherapy. Response rate was 50%, and 6-month PFS was 65% in glioblastoma patients. Three patients (12%) discontinued treatment due to tumor hemorrhage, wound dehiscence, and bowel perforation, although no radiation necrosis was detected. The authors concluded that treatment was well tolerated and beneficial for recurrent malignant gliomas. Cuneo et al. retrospectively evaluated the efficacy and safety of stereotactic radiosurgery and adjuvant bevacizumab for recurrent malignant gliomas [31]. Median PFS was 5.2 months, and 1-year overall survival was 50% in glioblastoma patients treated by radiosurgery and adjuvant bevacizumab. These results were significantly better than radiosurgery and other drugs. The authors concluded that salvage radiosurgery and bevacizumab improve outcomes in recurrent malignant gliomas.

A phase II study of bevacizumab plus erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, was performed for patients with recurrent malignant gliomas [32]. This study included 25 glioblastomas and 32 anaplastic gliomas. The response rate and 6-month PFS were 48% and 28% for glioblastoma and 31% and 44% for anaplastic gliomas, respectively. Grade 1 or 2 rash, mucositis, diarrhea, and fatigue were the most common adverse events. The authors concluded that treatment was tolerated, but the additional benefits of erlotinib were unclear when compared with historical bevacizumab-containing regimens. These studies indicate that additional therapy to bevacizumab can be promising strategy, although it is still unclear which agent has the efficacy in combination with bevacizumab for malignant gliomas. Further studies are required to establish the additional agents to bevacizumab.

4. Resistance to Bevacizumab

In the maintenance of bevacizumab, patients with malignant gliomas inevitably experience tumor recurrence. Furthermore, recurrent tumors after bevacizumab failure are

reported to be more aggressive with rebound edema [38]. Although additional agents to bevacizumab have been attempted for patients after bevacizumab failure, disease prognosis was extremely poor with median PFS of 37.5 days and 6-month PFS of 2%, respectively [39]. The authors concluded that alternative strategies should be considered for these patients. De Groot et al. showed that bevacizumab induced a particularly invasive tumor phenotype expressing insulin-like growth factor binding protein-2 and matrix metalloprotease-2 in glioblastoma [40].

Preclinical studies indicated that alternative pro-angiogenic signaling pathways are upregulated in resistance to antiangiogenic therapies [41]. These other angiogenic factors such as fibroblast growth factors and platelet-derived growth factors (PDGF) can compensate for the loss of VEGF activity under bevacizumab treatment [42]. Additional agents inhibiting other antiangiogenic pathways may suppress these resistances, and further clinical and animal studies are clearly required to overcome the resistance of bevacizumab.

5. Imaging of Response to Antiangiogenic Therapy

Most studies defined partial and complete responses as radiological objective response according to McDonald criteria that are based on contrast-enhanced CT or MRI [43]. However, an accurate assessment of tumor response by conventional modality is limited, since bevacizumab directly alters tumor blood vessels [44]. As a result of this, response rate and 6-month PFS are debatable as a measure of antitumor activity [44]. Norden et al. reported that bevacizumab suppressed enhancing tumor recurrence, but not nonenhancing and infiltrative tumor growth, indicating that bevacizumab may change the recurrence patterns of malignant gliomas [45]. Iwamoto et al. reported that contrast-enhanced MRI did not adequately evaluate disease status, whereas nonenhancing tumor recurrence was significantly associated with overall survival in recurrent glioblastoma treated by bevacizumab [46]. Given these findings, The Response Assessment in Neuro-Oncology Working Group was established to develop the new response criteria for clinical trials of brain tumors [47]. They proposed to incorporate T2 and fluid-attenuated inversion recovery (FLAIR) changes on MRI to assess the infiltrative pattern progression of malignant gliomas.

Other studies have looked into establishing a reliable radiological modality in antiangiogenesis therapy. Positron emission tomography (PET) using [^{18}F] fluorothymidine (FLT) offers noninvasive assessment of cell proliferation [48]. The response measured by FLT-PET significantly predicted the overall survival in recurrent glioblastoma treated by bevacizumab ($P = 0.061$) [49]. Recently, Ellingson et al. reported that relative nonenhancing tumor ratio, the ratio of FLAIR to contrast-enhancing volume, was predictive for overall survival and PFS in the treatment of bevacizumab for recurrent glioblastoma [50]. Further studies are warranted to establish the imaging modality to evaluate the response to antiangiogenic therapy and to predict the prognosis.

6. Biological Markers Predicting Response

A variety of biomarkers predicting the efficacy of bevacizumab have been reported in several tumor types including malignant gliomas [51]. These predictive biomarkers are expected to lead to a personalized therapy that selects patients who can benefit from bevacizumab. Sathornsumetee et al. examined several biological markers in recurrent malignant gliomas treated by bevacizumab and irinotecan [52]. High VEGF expression was significantly associated with higher radiographic response ($P = 0.024$), and high carbonic anhydrase 9 expression predicted poor overall survival ($P = 0.016$). Higher hypoxia-inducible factor-2 alpha and VEGF receptor-2 expressions were also reported to be associated with poor survival in recurrent malignant gliomas treated by bevacizumab and erlotinib [32].

Recently, circulating VEGF concentrations are reported to predict the prognosis in solid tumors treated by bevacizumab [51, 53]. The measurement of circulating proteins is an attractive strategy since blood is easily accessible and the assay is inexpensive. Circulating VEGF concentrations are expected to reflect VEGF-dependent angiogenesis and to predict the benefit from bevacizumab [51]. Gururangan et al. examined the VEGFR-2 phosphorylation in peripheral blood mononuclear cells in recurrent malignant gliomas and diffuse brainstem glioma treated by bevacizumab [54]. They showed that circulating VEGFR-2 was inhibited by bevacizumab, but they did not show information on whether it is a prognostic biomarker. These clinical trials have provided some potential predictive markers (e.g. tumor VEGF expression or circulating markers), which require a phase III study for proper evaluation [55].

7. Addition of Bevacizumab to Temozolomide and Radiotherapy for Newly Diagnosed Glioblastoma

Several clinical studies have been performed to evaluate the safety and efficacy of the addition of bevacizumab for newly diagnosed glioblastoma (Table 1). Lai et al. reported a phase II study of the addition of bevacizumab to the standard treatment of temozolomide and radiotherapy for 70 newly diagnosed glioblastomas [14]. Bevacizumab was intravenously administered every 2 weeks from the first day of treatment. The median overall survival and PFS were 19.6 and 13.6 months, respectively. The authors concluded that the addition of bevacizumab improved PFS but not overall survival when compared with a control group treated with first-line temozolomide and radiotherapy who had mostly received bevacizumab at recurrence. Another phase II study also reported preliminary results on the addition of bevacizumab to the standard temozolomide and radiotherapy regimen in 125 newly diagnosed glioblastomas [15]. In this study, toxicity was minimal, and most patients (90%) continued treatment, with median PFS of 13.8 months. Recently, Desjardins et al. reported a phase II study of bevacizumab in combination with temozolomide plus radiotherapy followed by bevacizumab, temozolomide, and irinotecan for 125 newly diagnosed glioblastomas at the

Society for Neuro-Oncology (SNO) annual meeting in 2010 [33]. This study had median overall survival of 21.3 months and PFS of 13.8 months, respectively. These studies showed encouraging results; however, it is still unclear whether the addition of bevacizumab to standard temozolomide and radiotherapy can improve the overall survival.

Currently, two randomized phase III trials, ROTG 0825 and AVAGLIO, are ongoing for newly diagnosed glioblastoma treated by temozolomide and radiotherapy with or without bevacizumab [56, 57]. These studies will show the role of bevacizumab in frontline treatment in glioblastoma patients.

8. The Effect of Bevacizumab on Radiation Adverse Events

Bevacizumab has been reported to affect the specific adverse events of radiotherapy [58, 59]. Sherman et al. reported that six glioblastoma patients developed severe radiation optic neuropathy following bevacizumab [58]. All of them received 60 Gy in 30 fractions in the initial treatment. Patients received a median of 7.5 doses of bevacizumab followed by onset of visual symptoms. Although the detailed mechanism remains unclear, the authors indicated that bevacizumab decreases optic nerve tolerance to radiation. Another case series study reported that bevacizumab induced optic neuropathy and Brown-Sequard syndrome after irradiation [59]. The authors hypothesized that bevacizumab following radiotherapy inhibits VEGF-dependent repair of normal neural tissue.

In contrast, bevacizumab has been reported to be effective for the management of radiation necrosis and retinopathy [60, 61]. Radiation necrosis is a serious complication of radiotherapy and includes extended edema. Pathological findings show that endothelial cell dysfunction causes tissue hypoxia and necrosis with the local cytokine release, including VEGF [62, 63]. Corticosteroids, surgery, anticoagulation, and hyperbaric oxygen have been performed, although there is no evidence to support routine use in clinical practice [64]. Retrospective studies have shown that bevacizumab decreased the edema and improved the clinical outcome in patients with radiation necrosis [60, 65–67]. Interestingly, a small randomized trial was recently performed to demonstrate this effect [68]. Patients having radiation necrosis with progressive neurologic symptoms were assigned to bevacizumab ($n = 14$) and placebo groups ($n = 7$). Bevacizumab was intravenously administered every 3 weeks for 12 weeks. Radiological response and improvement of neurological symptoms were observed in the bevacizumab treated group but not in placebo group. The authors concluded that the class I evidence of bevacizumab efficacy for radiation necrosis was shown in this study.

Radiation retinopathy is a chronic and progressive condition that results from radiation exposure. Retinal vascular endothelial cell damage causes microaneurysms, telangiectasias, neovascularization, vitreous hemorrhage, macular edema, and tractional retinal detachment. Radiation retinopathy has been treated by laser photocoagulation, corticosteroids, and anticoagulation, although the management is still challenging [61]. Bevacizumab has been expected to be a therapeutic modality for radiation retinopathy. Finger reported that intravitreal injection of bevacizumab was effective for retinal hemorrhage, exudation, and edema, which improved visual acuity of patients [69]. There were no ocular and systemic side effects by bevacizumab. Furthermore, the authors recently showed that intravitreal bevacizumab was effective for radiation optic neuropathy [70].

Although these results indicate that some radiation vasculopathies are potentially treatable by bevacizumab, exacerbation of radiation necrosis by this drug was also reported [71]. It is still unclear how bevacizumab affects the radiation adverse events. Meticulous followup is required when bevacizumab is administered after radiotherapy. Animal models of radiation necrosis are needed to investigate the mechanism of bevacizumab.

9. Other Antiangiogenic Drugs for Malignant Gliomas

VEGF has been shown to be the main player in tumor angiogenesis, and its inhibitor, bevacizumab, has been thoroughly investigated in clinical and animal studies. Other drugs such as pan VEGF receptor tyrosine kinase inhibitors have also been reported to inhibit VEGF pathways. Several biological pathways including integrin, fibroblast growth factor, and PDGF also are associated with the angiogenesis. Currently, several types of antiangiogenic drugs have been investigated and used in clinical trials for recurrent as well as newly diagnosed glioblastoma [72]. In this section, we review these drugs and the results of clinical trials (Table 2).

9.1. Cilengitide. Cilengitide competitively binds $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptors that are expressed on tumor cells and activated endothelial cells during angiogenesis. Cilengitide can directly inhibit the growth of integrin-expressing tumor cells and indirectly act as an antiangiogenesis agent [87, 88]. Glioblastoma cells express integrin receptors, and cilengitide has shown an antitumor effect in glioblastoma xenografts *in vivo* [89, 90]. A randomized phase II study of 81 recurrent glioblastoma was performed to determine the efficacy and safety of cilengitide [73]. The patients were randomly assigned to receive either 500 or 2000 mg of cilengitide twice weekly. Patients treated with 2000 mg showed a trend toward better results with 6-month PFS of 15%. The treatment was well tolerated, and significant hematologic toxicity was uncommon. A phase I/IIa study of cilengitide combined with temozolomide and radiotherapy for 52 newly diagnosed glioblastoma patients was conducted [74]. This combination therapy was well tolerated without additional toxicity, and median overall survival was 16.1 months. The authors concluded that this regimen showed promising activity against newly diagnosed glioblastoma when compared with historical controls. Based on these results, two randomized trials, CENTRIC and CORE, are currently ongoing to determine the efficacy of cilengitide for newly diagnosed glioblastoma with or without a methylated

Table 2: Other antiangiogenesis drugs for recurrent or newly diagnosed malignant gliomas.

Target	Study	Agent	Patients	RR	MPFS	6-PFS	MST	Ref.
Integrin	II	Cilengitide (500 mg/day)	41 recurrent GBM	5%	7.9 months	10%	6.5 months	[73]
		(2000 mg/day)	40 recurrent GBM	13%	8.1 months	15%	9.9 months	
Integrin	I/IIa	Cilengitide + RT/TMZ	52 Newly diagnosed GBM	—	8.0 months	69%	16.1 months	[74]
bFGF	II	Thalidomide	39 recurrent MG	6%	2.5 months	—	7.0 months	[75]
bFGF	II	Thalidomide + carmustine	40 recurrent MG	24%	3.3 months	28%	—	[76]
bFGF	II	Thalidomide + irinotecan	32 recurrent GBM	6%	3.3 months	25%	9.0 months	[77]
bFGF	I	Lenalidomide	24 recurrent GBM	0%	1.8 months	13%	6.0 months	[78]
VEGFR	II	Cediranib (45 mg/day)	31 recurrent GBM	27%	3.9 months	26%	7.6 months	[79]
VEGFR	III	Cediranib (30 mg/day)	325 recurrent GBM	—	—	16%	—	[33]
		Cediranib (20 mg/day) + lomustine		—	—	35%	—	
		Lomustine + placebo		—	—	26%	—	
VEGFR	II	Adjuvant sorafenib + TMZ	47 newly diagnosed GBM	13%	6.0 months	50%	12 months	[80]
VEGFR	II	Sunitinib	21 recurrent MG	0%	1.6 months	—	3.8 months	[81]
VEGFR	I	Vatalanib + RT/TMZ	19 newly diagnosed GBM	13%	7.2 months	—	16.2 months	[82]
VEGFR	II	Pazopanib	35 recurrent GBM	6%	3.0 months	3%	8.8 months	[83]
PDGFR	II	Imatinib	31 recurrent GBM	6%	1.7 months	16%	5.2 months	[84]
PDGFR	III	Imatinib	120 recurrent GBM	—	1.5 months	7%	5.3 months	[85]
		Imatinib + hydroxyurea	120 recurrent GBM	—	1.5 months	5%	4.8 months	
PDGFR	R	Dasatinib	14 recurrent GBM	0%	0.9 months	0%	2.6 months	[86]

RR: response rate; MPFS: median progression-free survival; 6-PFS: 6-month progression-free survival; MST: median overall survival time; GBM: glioblastoma multiforme; RT: radiotherapy; TMZ: temozolomide; bFGF: basic fibroblast growth factor; MG: malignant gliomas; VEGFR: vascular endothelial growth factor receptor; PDGFR: platelet-derived growth factor receptor; R: retrospective.

O^6-methylguanine-DNA methyltransferase (MGMT) promoter [91, 92].

9.2. Thalidomide and Lenalidomide. Thalidomide was developed as a sedative drug in 1950s and was withdrawn due to teratogenic effects. However, thalidomide was recently reported to have an antiangiogenic activity by inhibiting basic fibroblast growth factor (bFGF) [93], which can be exploited as an antitumor drug. Several clinical trials have been performed to assess the efficacy and safety of thalidomide for vascular tumors including malignant gliomas. This drug has since been approved by the FDA for the treatment of malignant myeloma [94]. Fine et al. showed a phase II study of thalidomide alone for 39 patients with recurrent malignant gliomas [75]. Thalidomide was well tolerated with modest sedation and constipation, although median PFS and overall survival were 2.5 months and 7.0 months, respectively. Another phase II study of thalidomide combined with carmustine was performed for 40 recurrent malignant gliomas [76]. Although the addition of carmustine seemed to improve the prognosis, the response rate and median PFS of combination group were 24% and 3.3 months, respectively. Puduvalli et al. reported a phase II trial of thalidomide and irinotecan for 32 recurrent glioblastomas [77]. The combination therapy was well tolerated with mild myelosuppression and sedation. At least a partial response was detected in two patients (6%), and 6-month PFS was 25%, respectively. These results indicate that thalidomide plus cytotoxic agents seem to have a mild antitumor activity for recurrent malignant gliomas patients when compared with thalidomide alone.

Lenalidomide, a potent structural and functional thalidomide analog, has antiangiogenic, anti-inflammatory, and immunomodulatory activities in preclinical studies [95, 96]. This drug is approved by the FDA for myelodysplastic syndrome with chromosome 5q deletion and multiple myeloma. Recently, lenalidomide has been performed for recurrent brain tumors in clinical trials [78, 97]. Fine et al. reported that lenalidomide was well tolerated; however, no objective responses were seen in a phase I study [78]. Median 6-month PFS was 12.5% in recurrent glioblastoma patients. Warren et al. conducted a phase I study of lenalidomide for pediatric patients with recurrent or progressive brain tumors [97]. This treatment was well tolerated with the primary toxicity being myelosuppression. Partial responses were seen in two patients (4%) with low-grade gliomas. Because these studies were phase I trials, further investigations are required to evaluate the antitumor activity for malignant gliomas.

9.3. VEGF Receptor Tyrosine Kinase Inhibitors (Cediranib, Sorafenib, Sunitinib, Vatalanib, and Pazopanib). Currently, VEGF receptor tyrosine kinase inhibitors are viewed as

promising antiangiogenic agents in the setting of malignant gliomas. Cediranib was developed as an oral pan-VEGF receptor tyrosine kinase inhibitor. Preclinical studies showed that cediranib normalized tumor vasculature and decreased the edema in glioblastoma, improving the prognosis without inhibition of tumor growth [98, 99]. Batchelor et al. conducted a phase II study of cediranib for 31 recurrent glioblastoma patients [79]. Patients were administered a 45 mg/day dose of cediranib. Partial response according to the MacDonald criteria was observed in 26.6% of patients, and 6-month PFS was 25.8%. Corticosteroids were reduced or discontinued in 27% of patients. Toxicities were manageable, and common Grade 3 to 4 toxicities were fatigue, hypertension, and diarrhea. Furthermore, they showed the changes of growth factors in plasma after cediranib (e.g., bFGF, VEGF receptor 1, and matrix metalloproteinase-2), which were associated with treatment response or survival in this therapy. Based on these promising results, the authors conducted a phase III study of cediranib for 325 patients with recurrent glioblastoma, and the preliminary results were reported at the 2010 SNO annual meeting [33]. Patients were assigned on a 2 : 2 : 1 ratio to cediranib monotherapy 30 mg/day, combination of cediranib 20 mg/day plus lomustine, and lomustine monotherapy plus placebo groups. The 6-month PFS was 16% in cediranib monotherapy, 34.5% in the combination, and 25.8% in lomustine plus placebo groups, respectively, although the results were not significantly different between these groups. The efficacy of cediranib monotherapy seems to be less than the initial phase II study, and the possible reason for this discrepancy is that different doses of cediranib were used between two studies.

Sorafenib and sunitinib are inhibitors of multiple receptor tyrosine kinases including VEGF receptor. Sorafenib was approved by the FDA for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma [100]. Hainsworth et al. conducted a phase II trial of concurrent radiotherapy and temozolomide followed by adjuvant sorafenib and temozolomide for 47 newly diagnosed glioblastomas [80]. This regimen was well tolerated without significant grade 3 or 4 toxicities, although median overall survival and PFS were 12 months and 6 months, respectively. The authors concluded that the addition of sorafenib did not appear to improve the prognosis of these patients. Sunitinib was reported in a phase II study of 21 recurrent malignant gliomas [81]. No objective responses were detected, and median overall survival and PFS were 3.8 and 1.6 months, respectively. This study showed that single-agent sunitinib had insufficient activity for recurrent malignant gliomas.

Vatalanib is a small molecule inhibitor of VEGF receptor, PDGF receptor, and c-kit. In a phase I trial, vatalanib was added to the standard regimen of temozolomide and radiotherapy for 19 newly diagnosed glioblastomas [82]. Response rate was 13%, and median overall survival was 16.2 months, respectively. Pazopanib is a multitargeted tyrosine kinase inhibitor, including VEGF receptor-1, -2, and -3. A phase II trial of pazopanib was performed for recurrent glioblastoma [83]. However, this drug did not have enough antitumor activity with response rate of 5.7% and median PFS of 3.0 months.

Despite several trials of VEGF receptor tyrosine kinase inhibitors, the efficacy has not been established. In a retrospective study of glioblastoma patients who failed VEGF receptor tyrosine kinase inhibitors, bevacizumab salvage therapy still provided benefits with response rate of 21% and 6-month PFS of 12.5%, respectively [101]. Although there are no comparative studies, VEGF receptor inhibition therapy may be less effective for malignant gliomas when compared with bevacizumab [102].

9.4. PDGF Receptor Tyrosine Kinase Inhibitor (Imatinib, Dasatinib, and Tandutinib). The PDGF pathway also plays a role in angiogenesis [103]. PDGF receptor inhibitors (e.g., imatinib, dasatinib, and tandutinib) have been performed in clinical trials of malignant gliomas. Imatinib is a multitargeted tyrosine kinase inhibitor and blocks PDGF receptor α, PDGF receptor β, and c-KIT receptor. Preclinical study has demonstrated the antitumor effect of imatinib on glioblastoma cell lines [104]. A phase II study of imatinib was performed for 112 recurrent gliomas [84]. The 6-month PFS was 16% in glioblastoma, 4.0% in pure/mixed anaplastic oligodendrogliomas, and 9% in low-grade or anaplastic astrocytoma. In 31 glioblastoma patients, response rate was 6%, and median survival was 5.2 months, respectively. This study indicated that single agent imatinib was well tolerated but had limited antitumor activity. A randomized phase III study was conducted for 240 recurrent glioblastoma patients treated by hydroxyurea with or without imatinib [85]. The results from the two arms were very similar, and 6-month PFS was 5% in the combination arm and 7% in the imatinib alone arm, respectively. The authors concluded that there were no clinical benefits from the addition of imatinib. Taken together, these results suggest that imatinib is discouraged in recurrent glioblastoma patients.

Dasatinib and tandutinib are oral molecule inhibitors of several targets, including PDGF and c-kit. Dasatinib was approved by FDA for the treatment of chronic myelogenous leukemia [105]. A retrospective study reported the efficacy of dasatinib for 14 recurrent glioblastomas who failed bevacizumab therapy [86]. However, objective response rate was 0%, and 6-month PFS was 0%, respectively. Currently, a phase II trial of dasatinib (RTOG 0627) is ongoing to evaluate the efficacy and safety for recurrent glioblastoma or gliosarcoma [106]. Combined treatments with tandutinib and bevacizumab are being performed in a phase II study for recurrent malignant gliomas [107]. Preliminary results cautioned that neuromuscular junction dysfunction was observed in this regimen.

Although PDGF receptor inhibitors are effective in preclinical studies, it is still unclear whether these drugs have an antitumor effect in malignant glioma patients. One possible reason for the limited antitumor effect is that PDGF receptor inhibitor such as imatinib cannot cross the blood-brain barrier via the P-glycoprotein efflux pump [108].

10. Summary and Perspectives

Despite advances in treatment therapeutics, patients with malignant gliomas still have poor prognosis. A better

understanding of tumor angiogenesis has allowed us to target VEGF in antiangiogenic therapy. Bevacizumab is considered as a well-established antiangiogenic therapy in several solid tumors. A phase II trials of recurrent glioblastoma showed favorable response rates (28% to 57%) and 6-month PFS (29% to 50.3%) [11–13]. Based on these promising results, bevacizumab was approved by the FDA for the recurrent glioblastoma. Regarding recurrent anaplastic gliomas, bevacizumab has been reported to be effective as well [28, 29]. Additional therapies (e.g., chemotherapy, targeting therapy, and radiotherapy) to bevacizumab have been reported for recurrent malignant gliomas, and these results were encouraging. However, the timing, dosing, and the ideal treatment partners of bevacizumab have remained controversial. Further investigations are warranted to establish an antiangiogenic treatment for recurrent malignant gliomas.

Bevacizumab is expected to be on the frontline treatment of patients with glioblastoma. Phase II trials have reported the addition of bevacizumab to standard temozolomide and radiotherapy regimen for newly diagnosed glioblastoma [14, 15]. However, the authors concluded that this regimen improved PFS but not overall survival when compared with control group [14]. Currently two randomized phase III trials, RTOG 0825 and AVAGLIO, are ongoing to demonstrate the efficacy and safety of combined therapy of bevacizumab, temozolomide, and radiotherapy for newly diagnosed glioblastoma [56, 57]. These studies will show the role of bevacizumab in the first-line treatment of newly diagnosed glioblastoma.

Many other antiangiogenic therapies (e.g., cilengitide and cediranib) have also been performed in clinical trials. These studies showed encouraging results and are expected to improve the prognosis of malignant gliomas. However, some phase II trials have several limitations such as small sample size, possible enrollment bias, patient selection, and reliance on historical control data. These limitations are associated with a high false-positive rate, and the results from phase II studies are often not validated in phase III studies [109]. Phase II studies must be appropriately planned to have the greatest potential for informing the design of phase III trials [109].

Antiangiogenic therapies provide favorable results and seem to be attractive strategy in malignant gliomas. However, several problems such as including severe toxicities, resistance, evaluation of response, and lack of predictive biomarkers still remain. The unique severe adverse effects related to bevacizumab have been reported, such as CNS hemorrhage, deep venous thrombosis, and pulmonary emboli [11–13]. BRAIN study reported that Grade ≥ 3 adverse events were observed 65.8% and 46.4% in bevacizumab plus irinotecan and bevacizumab alone groups, respectively [13]. To reduce the serious adverse events associated with bevacizumab, selecting appropriate patients, early assessment of toxicity, and adequate management should be required.

Malignant glioma patients maintained on bevacizumab inevitably experience the treatment failure. Recurrent tumors following bevacizumab failure appear to be more aggressive with rebound edema [38]. Preclinical study showed that other angiogenic factors, such as fibroblast growth factors and PDGF, can compensate for the loss of VEGF activity under bevacizumab treatment [42]. A novel therapeutic strategy is required to overcome the resistance to bevacizumab of malignant gliomas.

An accurate assessment of tumor response by conventional modality is limited in antiangiogenic therapy due to alterations in tumor blood vessels [44]. The Response Assessment in Neuro-Oncology Working Group proposed that T2 and FLAIR changes on MRI should include the response criteria [47]. FDG-FLT is also expected to accurately evaluate the treatment response in bevacizumab due to its ability to detect cell proliferation [48].

Tumor VEGF expressions or circulating markers potentially predict the prognosis in malignant glioma treated by bevacizumab, although the rational biomarker has not been established. Novel biological markers are required to investigate, providing a personalized treatment that selects the patients who can benefit from bevacizumab.

Although several limitations on antiangiogenic therapy have been reported, this treatment is expected to improve the prognosis of malignant gliomas. Further investigation is warranted to establish the safe and effective antiangiogenic therapy for malignant gliomas.

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