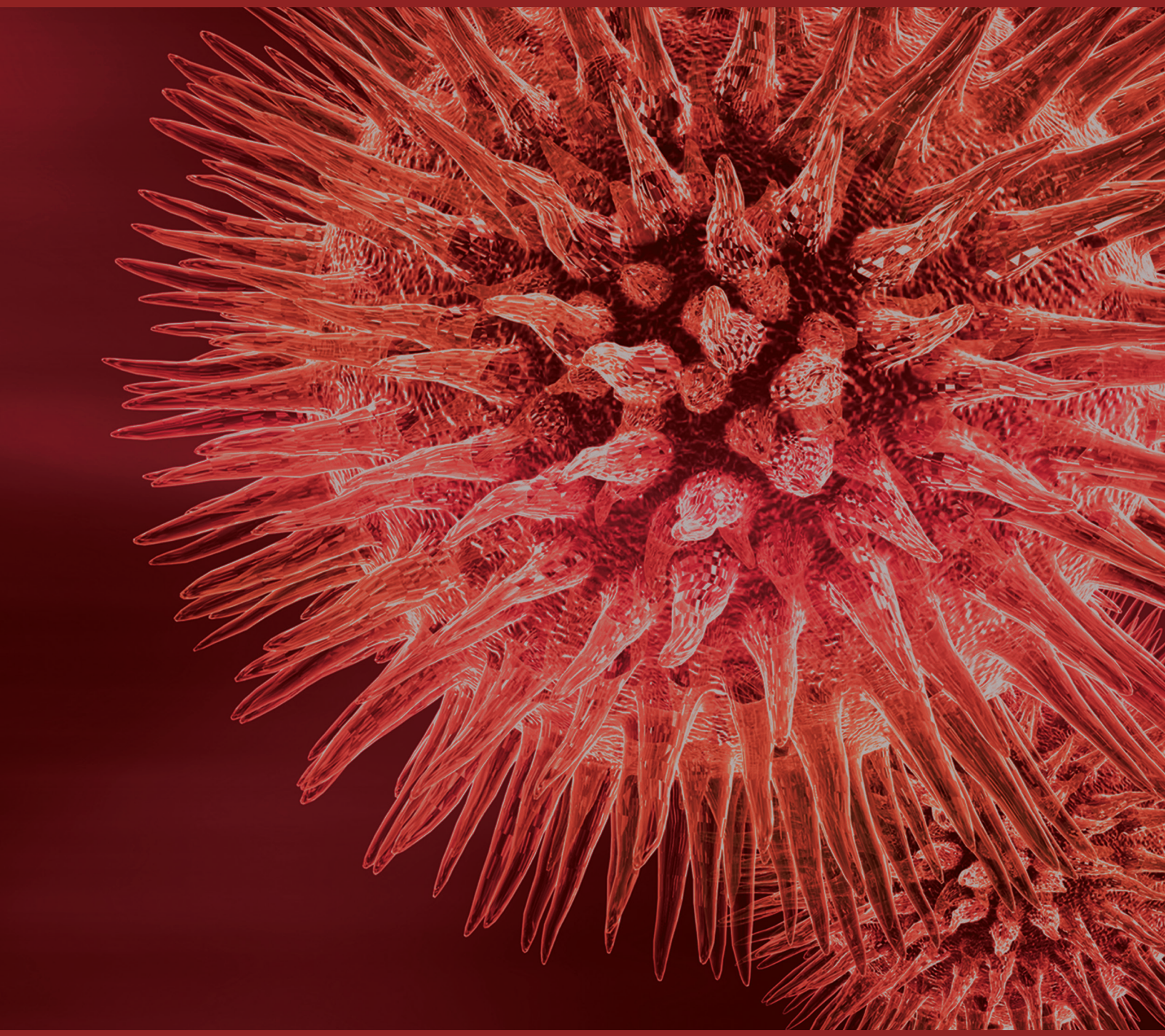
BioMed Research International

The Tumor Microenvironment and Cancer

Guest Editors: Zhen Chen, Zhiqiang Meng, Lijun Jia, and Rutao Cui



The Tumor Microenvironment and Cancer

BioMed Research International

The Tumor Microenvironment and Cancer

Guest Editors: Zhen Chen, Zhiqiang Meng, Lijun Jia, and Rutao Cui

Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 573947, 1 page
http://dx.doi.org/10.1155/2014/573947

Editorial

The Tumor Microenvironment and Cancer

Zhen Chen,[1,2] Zhiqiang Meng,[1,2] Lijun Jia,[2,3] and Rutao Cui[4]

[1] *Department of Integrative Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China*
[2] *Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China*
[3] *Cancer Institute, Fudan University Shanghai Cancer Center, Shanghai 200032, China*
[4] *Department of Dermatology, Boston University School of Medicine, Boston, MA 02118, USA*

Correspondence should be addressed to Zhen Chen; cz120@mail.sh.cn

Received 1 October 2014; Accepted 1 October 2014; Published 22 December 2014

Tumors are now recognized as structures of multiple cell types. It is increasingly appreciated that as the cancer progresses, the surrounding microenvironment is activated in support, coevolving through continuous paracrine communication and supporting carcinogenesis. Therefore, the characterization of these interactions, and the molecular identification of key mediators, will provide insights into tumor biology and suggest further novel therapeutic options.

In this special issue, C. Roma-Rodrigues et al. reviewed the role of exosome in tumor microenvironment, especially the cancer derived exosomes in the development and progression of cancer through modulation of intercellular communication within the tumor microenvironment by the transfer of protein, lipid, and RNA cargo. S. L. Schlereth et al. reviewed the crosstalk between cancer cells and tumor microenvironment that involved in the process of lymphangiogenesis. They showed that lymphangiogenesis is regulated by cells of the tumor microenvironment, including cancer-associated fibroblasts, mesenchymal stem cells, dendritic cells, or macrophages. Therefore, targeting pro-lymphangiogenic tumor microenvironment may represent a promising therapeutic option for cancer metastasis. J. Zhou et al. reviewed that chemoattractant receptors, a family of seven transmembrane G protein coupled receptors (GPCRs) combined with their ligands, are involved in almost every step of tumor development and progression such as increasing tumor cell migration, invasion, and metastasis. Bone metastases remain as the major problem for cancer patients. Bone metastases account for decreased quality of life and ultimately death of prostate, breast, and lung cancer patients. In this issue, D. Buenrostro et al. reviewed that the bone microenvironment cells including the fibroblasts, osteoblasts, osteoclasts, immune cells, and others may interact with the tumor cells and promote tumor cells to metastasize to the bone and other sites. While A. Romano et al.'s review focused on the role of immunological dysregulation in multiple myeloma progression and their potential clinical implications as novel therapeutic target.

We hope that this issue will help researchers understand the interactions between tumor cells and tumor microenvironment that mediate tumor progression and metastasis. We also hope that this special issue will initiate new discussions relating to the development of novel anticancer therapies or preventive strategies based on an understanding of the communication within the tumor microenvironment.

Zhen Chen
Zhiqiang Meng
Lijun Jia
Rutao Cui

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 639058, 14 pages
http://dx.doi.org/10.1155/2014/639058

Review Article

Impact of the Prolymphangiogenic Crosstalk in the Tumor Microenvironment on Lymphatic Cancer Metastasis

Simona L. Schlereth,[1] Nasrin Refaian,[1] Sandra Iden,[2] Claus Cursiefen,[1] and Ludwig M. Heindl[1]

[1] *Department of Ophthalmology, University of Cologne, Kerpener Straße 62, 50937 Cologne, Germany*
[2] *Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD) and Center for Molecular Medicine Cologne (CMMC), University of Cologne, Joseph-Stelzmann-Straße 26, 50931 Cologne, Germany*

Correspondence should be addressed to Simona L. Schlereth; simona.schlereth@uk-koeln.de

Received 11 April 2014; Accepted 14 August 2014; Published 1 September 2014

Academic Editor: Zhen Chen

Lymphangiogenesis is a very early step in lymphatic metastasis. It is regulated and promoted not only by the tumor cells themselves, but also by cells of the tumor microenvironment, including cancer associated fibroblasts, mesenchymal stem cells, dendritic cells, or macrophages. Even the extracellular matrix as well as cytokines and growth factors are involved in the process of lymphangiogenesis and metastasis. The cellular and noncellular components influence each other and can be influenced by the tumor cells. The knowledge about mechanisms behind lymphangiogenesis in the tumor microenvironmental crosstalk is growing and offers starting points for new therapeutic approaches.

1. Introduction

The spread of tumor cells via the lymphogen route into the draining lymph nodes is common in many malignant tumors, including malignant melanoma of the skin [1], head and neck squamous cell carcinoma [2], squamous cell carcinoma of the uterine cervix [3], colorectal carcinoma [4], breast cancer [5], and malignant melanoma of the conjunctiva [6–13]. Sentinel lymph node biopsy allows early detection of micrometastasis resulting in staging and treatment changes.

The outgrowth of new lymphatic vessels from preexisting lymphatic vessels (lymphangiogenesis) has recently gained much interest in tumor research since it is the initial step in lymphogenic metastasis [14]. Although the role of intratumoral versus peritumoral lymphangiogenesis is still debated, its role as a decisive risk factor for tumor metastasis is now established.

Lymphangiogenesis is mediated by binding of the lymphangiogenic growth factors vascular endothelial growth factor- (VEGF-) C and VEGF-D to their specific lymphatic receptor, VEGF receptor 3 [15]. VEGF-C and VEGF-D can be released by a variety of tumor cells or by peritumoral nonmalignant cells of the tumor microenvironment [16–19], thus explaining the occurrence of tumor-associated lymphangiogenesis.

The cellular crosstalk in the tumor microenvironment is likely to play a role in promoting lymphangiogenesis and thus lymphatic metastasis. A variety of factors in the tumor microenvironment, including extracellular matrix (ECM) with cancer-associated fibroblasts (CAFs) and mesenchymal stem cells (MSCs), cells of the innate and adaptive immune system (dendritic cells, macrophages, and T- and B-cells) as well as cytokines and growth factors produced by the tumor and stromal cells [20, 21], has been considered to contribute to this process.

This review focuses on the role of tumor microenvironmental components in tumor-associated lymphangiogenesis and therefore the lymphatic metastasis cascade. Better understanding of these mechanisms is required to improve future therapeutic strategies aiming at minimizing the lymphatic spread of the tumor to the regional lymph nodes in order to the prolong survival of cancer patients.

2. Cytokines and Growth Factors Control Lymphangiogenesis

Growth factors of the vascular endothelial growth factor (VEGF) family are well understood in lymphangiogenesis. VEGF is the target of one of the first therapeutics: VEGF blocking antibody bevacizumab is used in colon cancer [22].

VEGF-D has been shown to induce the formation of blood and lymphatic vessels in tumors and VEGF-D expression on tumor cells led to increased lymphatic metastasis [23]. However, other authors emphasize the tissue specific effects on blood or lymph endothelial growth of VEGF-D [24]. In many forms of human cancer, a correlation of VEGF-C expression within the primary tumor and lymph node metastasis has been observed [25–30]. VEGF-C overexpression in breast cancer increased intratumoral lymphangiogenesis and was associated with enhanced metastasis into draining lymph nodes and lungs [31]. This might be caused by a tumor secreted VEGF-C dependent increase of matrix metalloproteinase- (MMP-) 9 production, followed by an increased matrix degradation and migration [32]. Other studies conclude that tumor derived VEGF-C draining to the regional lymph nodes may promote the outgrowth of lymph node metastasis [33].

Controversy exists whether VEGF-A is able to induce lymphangiogenesis. Recent studies indicate that the VEGF-A/VEGF-R2 signaling pathway is involved in lymphangiogenesis [14, 34]. Hirakawa et al. detected that VEGF-A overexpressing primary tumors can induce lymph node lymphangiogenesis and were associated with increased lymph node metastasis [35]. Lymph node lymphangiogenesis per se is thought to actively promote metastasis [36] and can also be induced by tumor cells [37].

Beside the VEGF family, the angiopoietins- (Ang-) 1 and Ang-2 are important in tumor angiogenesis. They bind to their receptors Tie 1 and Tie 2 on vascular endothelial cells and are involved in lymphangiogenesis and metastasis [38–42]. Ang-2 is upregulated by different factors including VEGF-A or insulin like growth factor 1 and induces angiogenesis in the presence of VEGF-A [39]. A reduced prognosis has been shown for different tumors overexpressing Ang-2 [39]. Ang-2 seems to have a destabilizing effect on blood vessels, an early step in neovascularization [43], whereas Ang-1 expressed by pericytes and others promotes stability of vessels [38]. In pancreatic cancer, elevated circulating Ang-2 was correlated with the extent of lymphatic metastasis and therefore seems to participate in the control of lymphatic metastasis [44].

Other factors that are involved in lymphangiogenesis are platelet derived growth factor- (PDGF-) BB [45], fibroblast growth factor- (FGF-) 2 [46], sphingosine 1 phosphate (S1P) [47], and hepatocyte growth factor (HGF) [48].

Lymphatic endothelium cells express different markers, including lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), podoplanin D2-40, prospero homeobox transcription factor 1 (prox1), and VEGF-R3 [49]. Lately, besides the significant correlation of lymphatic markers LYVE-1 and podoplanin D2-40 [50] in many forms of cancer and their negative correlation to prognosis mentioned above, prox1 and forkhead box (FOX) C2, regulators of angiogenesis and lymphangiogenesis, came into focus of cancer research. Sasahira et al. report that prox1 expression correlated with progression, lymphatic vessel density, metastasis, and worse prognosis [51]. Prox1 activated VEGF-C expression in vitro, whereas FOXC2 enhances prox1 and VEGF-A expression [51].

Chemokines are important signal proteins, involved in cell migration and chemotaxis. Chemokine ligands bind to their specific receptors. Metastatic cells seem to adopt this mechanism and express analogue receptors to improve their migration to distinct tissues [52]. Many different chemokine pathways are known and their role in cancer has been completely and well reviewed by others [53]; therefore, in this review we focus on chemokine axes involved in lymphatic metastasis: the chemokine receptor 7 (CCR7) with its ligands CCL19 and 21 and the CXCR4/CXCR12 axis. The CCR7 axis is a very important physiological axis for migration of immune cells and CCL21 regulates the homing to the lymphoid tissues [54].

Chemokines have been shown to be involved in tumor lymphangiogenesis and metastasis; for example, VEGF-C upregulated chemokine ligand 21 (CCL21) on lymphatic endothelium, whereby CCR7 expressing tumor cells were attracted towards the lymphatic vessels [32].

Many studies show that primary tumor cells and metastatic cells express CCR7 in the draining lymph node and that there is a significant correlation between lymph node metastasis and CCR7 expression in many tumor entities [55–58]. In one study the authors suggest that CCR7 enhances metastasis by upregulating MMP-9 expression [59]. Li et al. showed that hypoxia may induce CCR7 expression on tumor cells to stimulate migration and invasion of lung cancer cells, using the HIF1α and HIF2α pathway [60].

Other chemokines such as CXC chemokine type 2 (CXCR2) seem to be involved in lymphangiogenesis, as a high expression of CXCR2 is associated with increased lymph node metastases and a reduced prognosis in resected esophageal carcinoma [61].

Chemokine receptor CXCR4 is involved in metastasis of multiple cancer entities, including breast cancer [62], gastric cancer [63], prostate cancer [64], melanoma [65], uveal melanoma [66], or glioblastoma [67], to name just a few.

CXCR4 is upregulated in metastatic breast cancer cell lines and lymph node metastasis [62], and cells expressing CXCR4 predominantly migrate to tissues that express the ligand CXCL12 [62]. These tissues include the common sides of breast cancer metastasis, including lung, lymph node, brain, and bone marrow [5, 62]. Interestingly, in vivo inhibition of the CXCR4/CXCL12 axis reduced lymph node and lung metastasis [62]. Others showed that the de novo

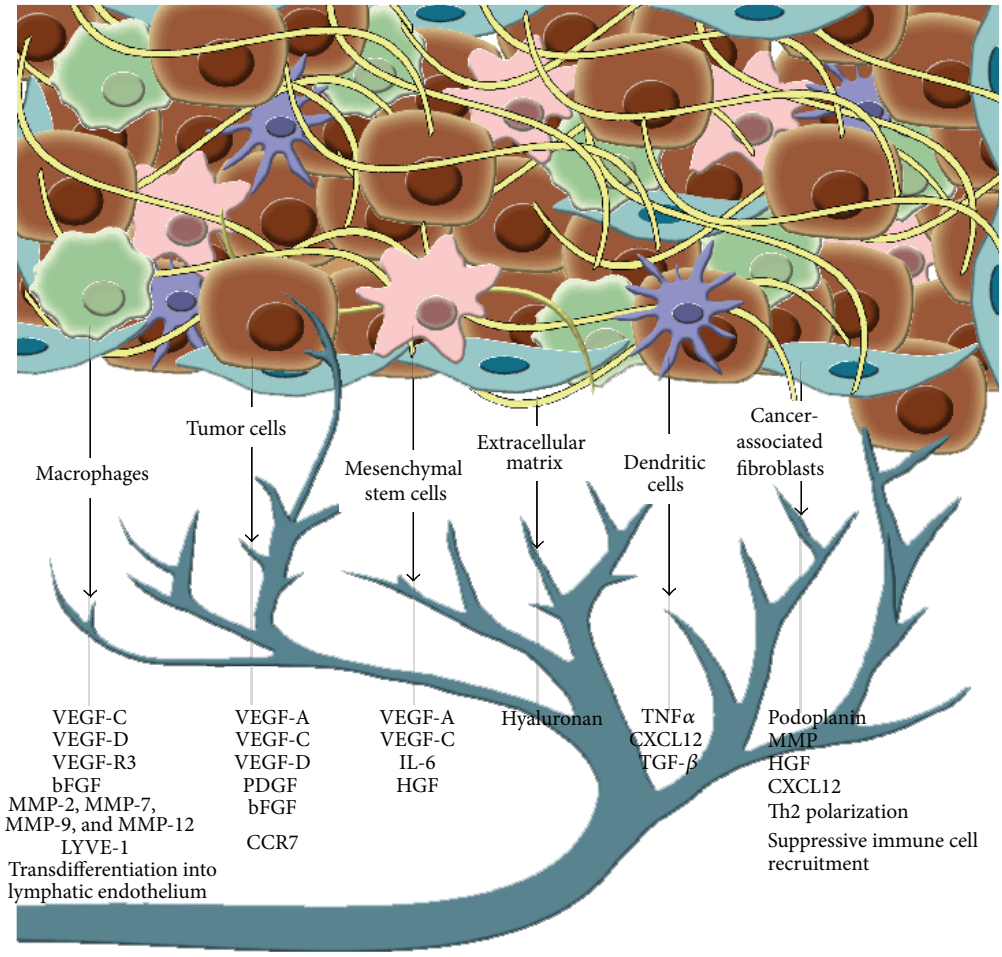


Figure 1: The prolymphangiogenic crosstalk of the tumor microenvironment: tumor cells as well as macrophages, dendritic cells, the extracellular matrix, cancer-associated fibroblasts, and mesenchymal stem cells can promote lymphangiogenesis by secretion or expression of different factors.

expression of CXCR4 is sufficient for metastasis to occur, shown by the B16 melanoma cell line transfected with CXCR4 [68]. In gastric cancer, CXCR4 expression is involved in lymph node metastasis [63, 69].

In prostate cancer, CXCR4 expression has been shown to increase tumor invasion and metastasis [64]. It may therefore serve as a prognostic marker in prostate cancer [70].

One last example is the CXCR3-CXCL9 axis: CXCR3 expression has been detected in several human melanoma cell lines and the mouse melanoma cell line B16F10. The loss of CXCR3 expression reduces lymph node metastasis in a murine melanoma model [71].

To summarize, the best-studied group of growth factors is the VEGF family, whereby in many forms of cancer an association between VEGF-C and metastasis has been recognized. Within the chemokines in cancer, the CXCR4/CXCL12 axis is currently best characterized and CXCR4 is a ubiquitously expressed receptor on tumor cells. Chemokine expression is often associated with elevated lymphatic metastasis. Tumor cells seem to have adopted these migration paths for facilitated access into lymphatic vessels and towards the draining lymph nodes. Cytokines and growth factors involved in lymphangiogenesis are summarized in Figure 1 and explained in detail in the following sections.

3. Senescence and Senescence-Associated Secretory Phenotype: Cell Autonomous and Nonautonomous Roles

Most mammalian cells have a limited proliferative capacity, and after various rounds of proliferation accompanied by telomere shortening, cells undergo permanent cell cycle arrest and enter a state called cellular senescence. Senescent cells remain viable and metabolic active and thereby further contribute to tissue homeostasis. Senescence can be prematurely induced by stress factors and DNA damage, for example, upon oncogene expression or UV irradiation, and is mediated by activation of the Arf/p53/p21 and/or p16/pRb pathways [72]. Senescence has been observed in various cell types of the tumor microenvironment including fibroblasts and immune cells and is considered a physiological tumor-suppressive mechanism in human cancers as it counteracts proliferation of premalignant cells [73, 74]. Human nevi, for

instance, are frequently positive for activating BRafV600E mutations; however, these cells bear a senescent phenotype [75]. Abrogation of such oncogene-induced senescence by PI3K activation allows for melanoma formation [76]. Induction of cellular senescence therefore has been recognized as promising therapeutic approach to prevent the proliferation of cancer cells. Recently, however, it became apparent that senescence in surrounding tissue cells might have both tumor-suppressive as well as promoting consequences [77]. Senescent cells secrete a variety of growth and regeneration promoting cytokines, chemokines, growth factors, and proteases, a phenomenon called senescence-associated secretory phenotype (SASP). SASP has recently been described for a variety of cancers and is considered to significantly modulate the properties of the specific tumor microenvironment. SASP factors can induce recruitment of immune cells like NK-cells and T-cells that help eliminate premalignant cells, and such NK-cell recruitment appears to be critical for tumor regression in vivo [78]. On the contrary, senescent tissue cells via SASP factors can directly promote tumor cell proliferation, invasion, and immune-editing to escape elimination by the immune system, thus overall providing a tumor-permissive micromilieu. Importantly, induction of senescence in NK cells has recently been reported to promote vascular remodeling and angiogenesis [79], opening the possibility that senescence and SASP may also contribute to tumor-associated lymphangiogenesis, although this remains to be demonstrated experimentally.

In the case of SASP of senescent fibroblasts in the tumor microenvironment, a significant overlap of its expression profile with that of cancer-associated fibroblasts (CAFs, see below) has been reported. For instance, upregulation of IL-6, IL-8, various CXCLs, and MMP-3 constitutes a common signature of CAFs and SASP [80]. Together, though induction of permanent cell cycle arrest within tumor cells is a desirable feature to suppress tumorigenesis, senescence of immune and surrounding tissue cells may have opposing outcome on tumor progression and angiogenesis, underscoring the importance of a better understanding of the cross-talk between tumor cells and their particular microenvironment.

4. Extracellular Matrix (ECM) with Cancer-Associated Fibroblasts (CAFs) and Mesenchymal Stem Cells (MSCs) Promote Lymphangiogenesis

Tumor-associated lymphangiogenesis may arise in the tumor microenvironment. The tumor microenvironment is mainly composed of the extracellular matrix (ECM) enriched with nonmalignant stroma cells, such as cancer-associated fibroblasts (CAFs) and mesenchymal stem cells (MSCs).

4.1. Extracellular Matrix (ECM). The ECM is a complex three-dimensional network made of fibrous proteins, such as collagen and fibronectin, and nonfibrous proteins, namely, glycosaminoglycans, proteoglycans, and glycoproteins. Located between cell clusters in all tissues, it strengthens the tissues, provides a channel for communication and migration within the tissue and under physiologic conditions, and acts as scaffold to keep growth factors insoluble [81–83]. Cancer cells may stimulate the tumor microenvironment by producing growth factors, including PDGF, transforming growth factor- (TGF-) β, VEGF, basic fibroblast growth factor (bFGF), and interleukins [83]. The altered expression of such mediators by tumor cells, which also have autocrine effects, often leads to production of proteolytic enzymes by the tumor cells [84, 85]. They may also stimulate stromal cells, for example, fibroblasts, to secrete molecules with a similar proteolytic effect on the ECM [86].

Therefore, not only tumor cells, but also stroma cell activation may modify the ECM towards an environment that promotes microinvasion of tumor cells [87]. Major components digesting ECM and cell surface proteins include MMPs, bone morphogenic protein 1 (BMP1), tissue serine proteinases, and adamalysin-related membrane proteinases [88]. Remodeling the ECM can significantly modulate migratory and angiogenic properties, for instance by release of cryptic protein sites and specific new molecule fragments [81]. Cryptic protein domains in ECM components such as fibronectin are typically masked in a folded structure and are thereby not accessible. Proteolytic enzymes can release these domains and open new integrin binding sites and antiangiogenic sequences [81] or activate latent TGF-β by proteolytic cleavage [89].

Hyaluronan, an important mucopolysaccharide of the ECM, provides an environment of proliferation and migration [90]. Interestingly, lymphatic endothelial cells exclusively express a hyaluronan receptor, known as LYVE-1 [91]. The functional impact of LYVE-1 receptors on tumor-associated lymphangiogenesis is not fully understood. However, it was demonstrated recently that low molecular weight hyaluronan promoted lymphatic endothelial cell (LEC) proliferation, migration, and tube formation, mediated via binding to LYVE-1 [92]. Therefore, in the tumor context hyaluronan seems to promote hem- and lymphangiogenesis.

In summary, dynamic remodeling of the ECM and cell-substrate interactions display one important feature in tumor-mediated lymphangiogenesis.

4.2. Cancer-Associated Fibroblasts (CAFs). Recently, there is increasing evidence that fibroblasts are a prominent modifier of cancer progression [93, 94]. CAFs are tall spindle shaped mesenchymal cells that share characteristics with smooth muscle cells and fibroblasts [83]. They can immunohistochemically be identified with a combination of different markers, since they show an elevated expression of α-smooth muscle actin, vimentin, desmin, and fibroblast-activating protein (FAP) compared to normal stromal fibroblasts [95, 96]. CAFs can promote tumor growth and progression but also influence the stromal microenvironment by producing large amounts of growth factors, cytokines and extracellular matrix proteins (e.g., collagen and fibronectin), and MMPs [21, 97, 98]. Bauer et al. showed an increased expression of TGF-β2, insulin-like growth factor-binding protein 2, tumor necrosis factor (ligand) superfamily member 4, and heparin-binding EGF-like growth factor in CAFs compared

to regular fibroblasts [99]. Moreover, CAFs secrete growth factors such as HGF or TGF-β but also ECM glycoproteins such as Tenascin-C (TNC) [100]. Tumor cells on the other hand secrete TGF-β or platelet-derived growth factor (PDGF), which are important factors for interactions between tumor cells and fibroblasts. TGF-β modulates fibroblasts and myofibroblasts towards CAFs [101, 102]. CAFs support tumor growth and metastasis indirectly through recruitment of immune cells such as tumor-associated macrophages (TAMs), myeloid suppressor cells (MDSCs), or regulatory T-cells (T_{regs}). All these cells are influencing the tumor microenvironment towards an immune suppressive environment and are thereby protecting the tumor. In vivo experiments showed that elimination of CAFs favors a Th1 over a Th2 polarization in the tumor microenvironment of a murine breast cancer model [103].

The modulation of the tumor microenvironment induces angiogenesis and lymphangiogenesis. CAFs secrete the stromal cell-derived factor 1 (SDF1), also known as CX chemokine ligand 12 (CXCL12), which is important in recruitment of endothelial progenitor cells (EPCs) in tumors. CXCL12 itself stimulates the tumor growth directly, via the CXC-chemokine receptor 4 (CXCR4), expressed among others on human breast carcinoma cells [104]. An elevated amount of CAFs significantly correlated with an increased lymphatic vessel density in ovarian cancer [105]. Another study showed that CAFs express podoplanin in the context of different tumors. The podoplanin expression of CAFs positively correlated with the VEGF-C expression of the tumor cell and the intratumoral amount of CD31+ blood vessels. In contrast, the increased expression of podoplanin in CAFs negatively correlated with peritumoral microvessels and LYVE-1 positive lymphatic vessels. The expression of podoplanin in CAFs did not correlate with the VEGF-A or VEGF-D expression in tumor cells [106].

Summarizing, CAFs seem to be important in the tumor microenvironment, where they indirectly contribute to lymphangiogenesis and metastasis by the induction of Th2 T-cells, recruitment of suppressive immune cells, and secretion of growth factors. However, the exact mechanisms are not fully understood.

4.3. Mesenchymal Stem Cells (MSCs). MSCs are non-hematopoietic multipotent cells that are able to differentiate into bone, fat, or cartilage tissue. They are involved in tissue repair and maintenance and have a tropism to wounded tissue [107]. In the context of trauma or tumor, they are capable of migrating towards these tissue sides, induced by chemokines or inflammatory factors [108]. MSCs show a specific migration to growth factors such as PDGF, EGF, and VEGF and a reduced migration in the presence of specific inhibitors, such as Glivec, Erbitux, and Avastin [109]. (More to therapeutic approaches is listed below; see point 5.) MSC themselves produce an amount of tumor promoting factors, including IL-6 [86], TGF-β, VEGF, and HGF [107, 110].

Using these factors, MSCs are capable of enhancing lymphangiogenesis and lymphatic metastasis. LECs express an HGF receptor (also known as c-Met or MET) and HGF promotes lymphatic vessel function and formation [111]. In vitro cocultures of MSCs and endothelial progenitor cells (EPCs) revealed that MSCs secreted VEGF-A in bioavailable amounts (350 pg/mL), despite the secretion of VEGF inhibitors (sVEGF-R1/sVEGF-R2) by EPCs [110]. Moreover, in a syngeneic mouse model, subcutaneous coinjection of MSCs and EPCs in Matrigel induced both blood- and lymphangiogenesis [110], highlighting the proangiogenic effect of MSCs in vivo.

VEGF-A can induce proliferation and migration of lymphatic endothelial cells (LECs). Dellinger and Brekken showed that VEGF-R2 acts as the primary receptor controlling VEGF-A induced lymphangiogenesis in an ERK1/2 and Akt-dependent manner [112]. In inflammatory neovascularization, VEGF-A stimulates LECs and lymphangiogenesis indirectly via macrophage recruitment [18].

As mentioned above, MSCs are capable of secreting IL-6. IL-6 and a proinflammatory cytokine is upregulated in different cancer entities. For example, a significant correlation between IL-6 protein and VEGF-C mRNA with lymph node metastasis in human oral squamous cell carcinoma has been demonstrated [113]. Moreover, in vitro experiments revealed that IL-6 induces VEGF-C expression in human oral squamous cell carcinoma cell line [113] and VEGF-C expression in IL-6 treated murine LECs [114].

MSCs are able to express a lymphatic phenotype, when cultured in lymphatic induced medium and VEGF-C [115]. Vice versa, tumor cells secrete growth factors, cytokines, and chemokines to promote the migration and survival of MSCs [108]. Karnoub et al. reported that MSC infiltration into tumor stroma promotes metastasis in breast cancer [116].

In conclusion, MSCs seem to have direct and indirect effect on lymphangiogenesis and lymphatic metastasis, mainly via VEGF-A, VEGF-C, HGF, and IL-6.

5. Immune Cells (Dendritic Cells, Macrophages) Control Lymphangiogenesis

Tumor-associated lymphangiogenesis is under the influence of innate immune cells of the tumor microenvironment, especially dendritic cells and macrophages.

5.1. Dendritic Cells (DCs). DCs are the most potent antigen presenting cells of the human body. They can be subdivided into different subsets and to fully understand their functions in the tumor microenvironment, DC subsets should be examined individually with regard to influences on their behavior, dependent on different local factors. DCs are involved in tumor immunology and angiogenesis by stimulating inflammation or inducting tolerance. They can internalize tumor antigen and cross-present it to T-cells within the draining lymph node. This is an important step towards an antitumoral immune reaction [117]. However, controversial data exists on their role in the tumor microenvironment, DC activation, or tolerance induction. On the one hand, DNA derived from necrotized tumor cells may be involved in the DC activation [118]. On the other hand, tumor cells have been shown to inhibit DC maturation through the secretion of IL-10 [119].

Within DCs, two major subsets can be differentiated: the myeloid DCs (mDCs, also known as conventional DCs) and the plasmacytoid DCs (pDCs) [120]. Both can be induced towards a tumor promoting state in the tumor microenvironment: mDCs contribute to the survival of multiple melanoma cells [121]. pDCs seem to have immunoregulatory properties in the tumor microenvironment and induce T_{regs} in the human ovarian carcinoma [122]. pDCs also contribute to angiogenesis by producing proangiogenic cytokines, such as IL-8 and tumor necrosis factor alpha (TNFα) in the ovarian carcinoma [123]. pDCs were detected in solid tumor tissue and metastatic cervical lymph nodes in head and neck squamous cell carcinoma [124]. In breast cancer, pDCs infiltration into the primary tumor was associated with shorter overall survival [125].

In general, tumor-associated DCs (TADCs) can secrete different proangiogenic factors, such as TGF-β, granulocyte macrophage colony-stimulating factor (GM-CSF), CXCL12, or TNFα [126, 127]. TADCs are able to differentiate into endothelial-like cells under tumor specific culture conditions [128] and CD34− CD11c+ immature DCs cocultured with tumor-cell conditioned media showed an endothelial-like differentiation [129]. Whether or not DCs participate in lymphangiogenesis is still a topic of ongoing research.

DCs can be influenced by VEGFs. VEGF has been shown to inhibit DC maturation by blocking NF-κB transcription [130]. In the cornea, VEGF-R3 blocking antibody reduced the DC migration towards the draining lymph nodes [131].

DCs as antigen presenting cells are able to take up antigen and migrate to the draining lymph node, guided through a CCL21 gradient. Interestingly, some tumor cells express the CCL21 receptor CCR7, thereby enabling them to access lymphatic vessels [62, 64, 132].

Regarding surface receptors of dendritic cells, programmed cell death ligand 1, PD-L1, came into focus of interest (see therapeutic approaches below). Also known as B7 homolog 1, this transmembrane protein seems to play a major role in suppressing the immune system. PD-1 and its ligand function as a complex transmit an inhibitory signal which downregulates T-cell activation and proliferation. The ligand PD-L1 is expressed on antigen presenting cells, whereas the receptor PD-1 has been found on activated T- or B-cells, macrophages, and myeloid cells as well as multiple tumor cells [133–136] and in vitro cell lines of uveal melanoma and cutaneous melanoma [137].

In breast cancer, sentinel lymph nodes with metastasis were associated with fewer mature dendritic cells within the lymph node [138]. Similarly, immature DCs have been detected in melanoma metastasis [139], but also the presence of mature DCs within the tumor tissue correlated with lymph node metastasis [125]. High mobility group box 1 (HMGB1) secreted by tumor cells induced the suppression of DCs and is associated with lymph node metastasis in human colon cancer [140]. Similarly, lymph node metastasis significantly correlated with number of DC expression in gastric cancer [141].

Recently a distinct population of DCs, namely the 6-sulfo LacNAc(+) DCs (slanDCs) were detected in metastatic tumor draining lymph nodes. Here, slanDCs surrounded the cancer cells, while being absent at the primary tumor side [142].

Taken together, DCs are able to secrete proangiogenic factors and induce an immune tolerant milieu in the tumor microenvironment. VEGF secreted by tumor cells or tumor-associated macrophages inhibits DC maturation, and a reduced number of mature dendritic cells can be associated with elevated lymph node metastasis in breast cancer [138]. However, due to their various subgroups, further studies are needed to fully understand their impact on lymphangiogenesis and metastasis.

5.2. Macrophages. Macrophages play an essential role in driving tumor hem- and lymphangiogenesis [143]. Known as tumor-associated macrophages (TAMs), they may sense hypoxia in tumor tissue and secrete VEGFs, basic fibroblast growth factor (bFGF), thymidine phosphorylase (TP), MMP-2, MPP-7, MPP-9 and MPP-12 [144], and urokinase type plasminogen activator (uPA) [145] to induce both hem- and lymphangiogenesis. TAMs do not only express prolymphangiogenic factors VEGF-C, VEGF-D, and VEGF-R3 [17], but they can also transdifferentiate into lymphatic endothelium [146]. TAMs have also been shown to express LYVE-1 [147, 148] and F4/80+ LYVE-1 + macrophages integrated into peritumoral lymphatic vessels [148]. TAMs are often regulated towards an M2 phenotype. In uveal melanoma, these M2 macrophages were found to be mainly CD68+ CD163+ and high amounts of these cells were associated with a poorer prognosis [149]. This observation has also been made in a variety of other tumor entities, for example, breast cancer [150], glioma [151], or melanoma [152]. In cutaneous squamous cell carcinoma, elevated VEGF-C levels derived from TAMs were associated with increased peritumoral lymphatic vessel density [153] and may thereby coordinate metastasis [154]. Depleting macrophages during tumor induction reduced incidence of ocular tumors and improved survival in mice [149, 155, 156]. VEGF-A and VEGF-C as well as MMP-9, secreted by TAMs and tumor cells, have been shown to induce peritumoral lymphangiogenesis [17, 157]. VEGF-A hereby may stimulate the upregulation of VEGF-C expression or through binding on VEGF-R2 expressed on lymphatic endothelium [42].

Other proangiogenic effects of TAMs can also be achieved indirectly, for example, via inhibition of DC maturation, and thereby contributing to an immune tolerant status [158]. An increased amount of immature DCs within tumor tissue was associated with elevated tumor vascularization [158]. This inhibitory and immune-suppressive effect is mainly achieved by interleukin 10, prostaglandin E2 (PGE2), and TGF-β secretion of TAMs [144].

To summarize, macrophages in the tumor microenvironment are a major source of proangiogenic growth factors. Not only do they stimulate lymphangiogenesis through activation of endothelial cells, but they can also participate in this process by expressing LYVE-1 or becoming integral components of lymphatic vessels [146].

5.3. T-Cells. Recent research has led to a better understanding of the role of adaptive immune cells in the tumor

microenvironment and first therapeutic options interfering with T-cell functions have successfully been US Food and Drug Administration (FDA) approved for antibody-based treatments in patients with advanced melanoma, for example, ipilimumab (see therapeutic approaches below).

A major attempt in development of immunological treatment strategies focuses on the identification of tumor-cell specific markers that may serve as therapy targets. Antigen recognition involves CD8+ T-cells recognizing tumor antigen [118]. Within the tumor microenvironment, two categories of CD8+ T-cells have been described. Some tumor tissues contain tumor-infiltrating T-cells, which secrete IFN-γ, whereas others lack T-cell infiltration and signs of inflammation. Whereas in the first group the tumor most likely inhibits the immune response, in the second group the immune system seems to ignore the ongoing tumor process (immune ignorance). The T-cell infiltrating phenotype has been shown for different types of cancer, including colorectal cancer [159, 160], renal cell carcinoma, melanoma, and ovarian cancer [161–164], and may have a positive prognostic value. A very good clinical outcome could be demonstrated for a high CD8+ T-cell to Fop3+ T_{regs} ratio in the ovarian cancer tumor microenvironment [165]. However, some melanomas still progress despite a T-cell infiltration, possibly related to a regress of the effectiveness of T-cells against tumor cells. This reduced effectiveness might be induced by the immunosuppressive tumor microenvironment [166]. The second group lacking tumor-infiltrating CD8+ T-cells was associated with an increased risk for metastasis into draining lymph nodes and decreased survival in dermal melanoma [161, 167].

Currently, the impact of T-cells on lymphangiogenesis and whether VEGF is involved in this context are mainly unknown. One study revealed that T-cells migrate responding to VEGF and that activated T-cells can express VEGF-R1 on their surface. Moreover, VEGF increased IL-10 secretion of these cells and might therefore direct chemotaxis and immune modulation of T-cells in tumor tissues [168].

5.4. B-Cells. Similar to T-cells, our knowledge on a potential role of B-cells in lymphangiogenesis is limited. Ruddell et al. reported B-cell accumulation in tumor draining lymph nodes, which induced lymphangiogenesis and increased lymphatic flow in Eμ-*c-Myc* transgenic mice [169]. These mice exhibited increased lymphatic metastasis of lymphoma and melanoma [170]. Harrell et al. made similar observations. In a melanoma mouse model, B-cells were important for lymphangiogenesis and increased lymphatic flow through tumor draining lymph nodes [171]. However, the underlying mechanisms still need to be investigated.

6. Future Immunotherapeutic Strategies to Block Lymphangiogenesis and Prevent Lymphatic Metastasis

New therapeutic approaches have made it into clinical treatment to some extent. Although many of them are interfering with immune cell function, a secondary effect on lymphangiogenesis is expected, as immune activation induces lymphangiogenesis by different factors mentioned above. Below the latest therapeutic approaches or ideas are listed which may be used on a regular basis in the future to improve cancer treatment.

VEGF Inhibiting Antibodies. One of the first antibodies interfering with VEGF function was bevacizumab (Avastin), which was FDA approved for treatment of metastatic colorectal carcinoma [22]. Only a few studies have been performed with explicit focus on lymphangiogenesis. Sunitinib, a small molecule interfering with VEGF-R1 and VEGF-R2, PDGF α and PDGF β, KIT receptor, and Flt3 receptor [172], was FDA approved in 2006 and is currently used for the treatment of metastatic renal cell carcinoma and gastrointestinal stromal tumors [173]. A similar molecule is sorafenib, interfering with VEGF-R2 and VEGF-R3, PDGF receptor β, and c-KIT receptor [174], and is FDA approved for advanced renal cell carcinoma [175]. Studies in mice revealed that, by using RNA interference to inhibit VEGF-C expression, the lymphangiogenesis, the number of lymph node metastasis has been reduced and the survival prolonged [176]. Interfering with VEGF-R3 resulted in similar observations [177]. A VEGF-D blocking antibody reduced lymphatic metastasis in mice [23].

In human colorectal cancer, it has been shown that cyclooxygenase 2 (COX2) (involved in production of prostaglandins [178]) and VEGF-C are coexpressed [179]. In a mice lung cancer model celecoxib, a selective COX2 inhibitor reduced lymphangiogenesis and lymph node metastasis [180] indicating that VEGF expression and thereby lymphangiogenesis might be associated with prostaglandins.

Due to the increasing number of new therapeutics interfering with VEGF function, we refer to excellent reviews which address VEGF inhibitors in depth, for example, that by Takahashi [181].

Programmed Death Ligand 1. PDL1 is an immune regulator, expressed on APCs and in 20–50% on human cancer cells [133, 137]. Tumor-induced PDL1 inhibits T-cell function and induces immune tolerance but also apoptosis of T-cells [182]. In contrast, it induces the expansion of T_{regs} [183]. Therefore, blocking this ligand on the tumor cells and on antigen presenting cells improves tumor defense and T-cells with anticancer properties restore their effector function [184]. However, severe side effects have been reported when interfering with the immune system [185].

Cancer Immunotherapy Using Dendritic Cells. Targeting DCs and performance of an adoptive transfer, for example, with antigen loaded DCs, may improve immunotherapy in the future. There are different ways to vaccine DCs by using tumor lysate, viral vectors, DNA plasmids, or antigen peptides [186]. The optimized vaccination and administration approaches (intralymphatic, intravenous, or intradermal, etc.) are subject of ongoing research to improve clinical outcomes [186]. Furthermore, the procedure is restricted by the DC maturation state and dose finding [186]. It is possible that DCs in vivo might become suppressive DCs, thus counteracting antitumor immune responses. Despite its

pros and cons, DC vaccination is a promising field for future improvements in cancer therapy.

Genetically Modified Autologous T-Cells. Lately, there were first reports of patients who have been treated with an adoptive transfer of genetically modified autologous T-cells, which could improve certain B-cell malignancies [187, 188] or chronic lymphoid leukemia [189]. Thereby the T-cell antigen receptor was modified to target CD19 (expressed on B-cells) and a T-cell signaling molecule. First cases treated with these T-cells revealed a complete remission, although accompanied by adverse events during treatment [187–189]. Taken together, these first studies are showing promising results from autologous T-cell transfers and might improve cancer treatment in the future.

Anti-CTLA4 Antibodies. In physiological conditions, T-cells are stimulated via CD28, which interacts with B7.1 and B7.2 on dendritic cells. Besides the "on button" CD28, T-cells express CTLA4, which can be regarded as "off button." CTLA4 serves as a coinhibitor on activated T-cells to regulate their immune response [183]. Anti-CTLA4 antibodies such as ipilimumab are immune modulatory biologics and are regarded as a milestone in the treatment of metastatic melanoma [183]. Ipilimumab was FDA approved in 2011. It is able to block the major inhibitor of activated T-cells CTLA4 and blocks the interaction to its ligand B7.1 and B7.2 expressed on antigen presenting cells [190]. T-cells are thereby effectively and long-term activated to fight against tumor cells. However, immune modulatory biologics may have severe side effects, due to excessive and autoaggressive effects of the immune system [191]. CTLA4 deficient mice die early as a result of an uncontrolled lymphocyte proliferation that leads to multiorgan destruction [192].

Interfering with CTLA4 can also induce immune suppressive and immune tolerance: the antibody CTLA4-Ig-RFP occupies the B7.1 and B7.2 receptor on DCs and thereby blocks its interaction with CD28 [193].

CCR7-CCL19/21. Interfering with the CCL21-CCR7 axis to reduce immune cell or tumor cell migration has been tried in different approaches. Antagonists of CCL21 seem to prevent the development of chronic graft versus host disease [194] or reduced allergic conjunctivitis by blocking CCR7 in mice [195]. Obstructing CCR7 expression at mRNA level in a murine tumor model inhibited lymph node metastasis and lymphangiogenesis [196]. Pretreatment with an allogenic melanoma-derived cell lysate was capable of upregulating CCR7 expression on therapeutic human tumor presenting DCs and inducing migration to the lymph node [197]. This knowledge might be used for future improvement of immunotherapy. However, all studies interfering with the CCR7 axis in humans to treat cancer and metastasis are still in very early stages.

7. Conclusions

Lymphangiogenesis is a very early step in lymphatic metastasis. It is regulated and promoted not only by the tumor cells themselves, but also by cells of the tumor microenvironment, including cancer-associated fibroblasts, mesenchymal stem cells, dendritic cells, or macrophages. Even the extracellular matrix as well as cytokines and growth factors are involved in the process of lymphangiogenesis and metastasis. Many mechanisms behind lymphangiogenesis in the tumor microenvironmental crosstalk are still incompletely understood. A better insight of the underlying mechanisms might improve future therapeutics to reduce lymphatic spread of cancer cells to the draining lymph nodes in order to increase the survival of cancer patients. A personalized and thereby optimized therapy interfering with the affected parts of the tumor microenvironment is a promising approach for future treatment of lymphatic metastasis and thus tumor related death.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Simona L. Schlereth and Nasrin Refaian contributed equally to this work.

Acknowledgments

This work was supported by the German Research Foundation (HE 6743/2-1 to Ludwig M. Heindl; Priority Research Project SFB 643: B10, CU 47/6-1, CU 47/4-1 to Claus Cursiefen; CRC829 and CRC832 to Sandra Iden), the European Union (FP7 STRONG to Claus Cursiefen), German Cancer Aid (to Ludwig M. Heindl and Claus Cursiefen, emerging field of the Center of Integrated Oncology Cologne-Bonn), and the University of Cologne (GEROK Program to Simona L. Schlereth and Ludwig M. Heindl).

References

[1] J. D. Shields, M. Borsetti, H. Rigby et al., "Lymphatic density and metastatic spread in human malignant melanoma," *British Journal of Cancer*, vol. 90, no. 3, pp. 693–700, 2004.

[2] N. J. P. Beasley, R. Prevo, S. Banerji et al., "Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer," *Cancer Research*, vol. 62, no. 5, pp. 1315–1320, 2002.

[3] M. W. Beckmann, G. Mehlhorn, F. Thiel, C. Breuel, P. A. Fasching, and S. Ackermann, "Therapiefortschritte beim primären Zervixkarzinom," *Deutsches Ärzteblatt*, vol. 102, pp. A979–A986, 2005.

[4] E. Sundlisæter, A. Dicko, P. Ø. Sakariassen, K. Sondenaa, P. Ø. Enger, and R. Bjerkvig, "Lymphangiogenesis in colorectal cancer—prognostic and therapeutic aspects," *International Journal of Cancer*, vol. 121, no. 7, pp. 1401–1409, 2007.

[5] J. Bruce, D. C. Carter, and J. Fraser, "Patterns of recurrent disease in breast cancer," *The Lancet*, vol. 1, no. 7644, pp. 433–435, 1970.

[6] L. M. Heindl, C. Hofmann-Rummelt, W. Adler et al., "Prognostic significance of tumor-associated lymphangiogenesis in malignant melanomas of the conjunctiva," *Ophthalmology*, vol. 118, no. 12, pp. 2351–2360, 2011.

[7] L. M. Heindl, C. Hofmann-Rummelt, W. Adler et al., "Tumor-associated lymphangiogenesis in the development of conjunctival melanoma," *Investigative Ophthalmology & Visual Science*, vol. 52, no. 10, pp. 7074–7083, 2011.

[8] L. M. Heindl, C. Hofmann-Rummelt, W. Adler et al., "Tumor-associated lymphangiogenesis in the development of conjunctival squamous cell carcinoma," *Ophthalmology*, vol. 117, no. 4, pp. 649–658, 2010.

[9] L. M. Heindl, F. Bucher, G. O. Naumann, and C. Cursiefen, "Lack of ciliary body lymphatics in iridociliary melanocytoma," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 252, pp. 169–171, 2014.

[10] L. M. Heindl, T. N. Hofmann, W. Adler et al., "Intraocular tumor-associated lymphangiogenesis: a novel prognostic factor for ciliary body melanomas with extraocular extension?" *Ophthalmology*, vol. 117, no. 2, pp. 334–342, 2010.

[11] L. M. Heindl, T. N. Hofmann, H. L. J. Knorr et al., "Intraocular lymphangiogenesis in malignant melanomas of the ciliary body with extraocular extension," *Investigative Ophthalmology & Visual Science*, vol. 50, no. 5, pp. 1988–1995, 2009.

[12] L. M. Heindl, T. N. Hofmann, F. Schrödl, L. M. Holbach, F. E. Kruse, and C. Cursiefen, "Intraocular lymphatics in ciliary body melanomas with extraocular extension: functional for lymphatic spread?" *Archives of Ophthalmology*, vol. 128, no. 8, pp. 1001–1008, 2010.

[13] L. M. Heindl, F. Schrödl, E. Lütjen-Drecoll, and C. Cursiefen, "Ciliary body lymphangiogenesis," *Ophthalmology*, vol. 120, no. 7, pp. e41–e42, 2013.

[14] M. Nagahashi, S. Ramachandran, O. M. Rashid, and K. Takabe, "Lymphangiogenesis: a new player in cancer progression," *World Journal of Gastroenterology*, vol. 16, no. 32, pp. 4003–4012, 2010.

[15] V. Joukov, K. Pajusola, A. Kaipainen et al., "A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases," *The EMBO Journal*, vol. 15, no. 2, pp. 290–298, 1996.

[16] M. J. Karkkainen and T. V. Petrova, "Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis," *Oncogene*, vol. 19, no. 49, pp. 5598–5605, 2000.

[17] S. F. Schoppmann, P. Birner, J. Stöckl et al., "Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis," *The American Journal of Pathology*, vol. 161, no. 3, pp. 947–956, 2002.

[18] C. Cursiefen, L. Chen, L. P. Borges et al., "VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment," *Journal of Clinical Investigation*, vol. 113, no. 7, pp. 1040–1050, 2004.

[19] K. Maruyama, M. Ii, C. Cursiefen et al., "Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages," *The Journal of Clinical Investigation*, vol. 115, no. 9, pp. 2363–2372, 2005.

[20] J. C. Becker, M. H. Andersen, D. Schrama, and P. Thor Straten, "Immune-suppressive properties of the tumor microenvironment," *Cancer Immunology, Immunotherapy*, vol. 62, no. 7, pp. 1137–1148, 2013.

[21] H. Ungefroren, S. Sebens, D. Seidl, H. Lehnert, and R. Hass, "Interaction of tumor cells with the microenvironment," *Cell Communication and Signaling*, vol. 9, article 18, 2011.

[22] N. Ferrara, K. J. Hillan, H.-P. Gerber, and W. Novotny, "Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer," *Nature Reviews Drug Discovery*, vol. 3, no. 5, pp. 391–400, 2004.

[23] S. A. Stacker, C. Caesar, M. E. Baldwin et al., "VEGF-D promotes the metastatic spread of tumor cells via the lymphatics," *Nature Medicine*, vol. 7, no. 2, pp. 186–191, 2001.

[24] T. V. Byzova, C. K. Goldman, J. Jankau et al., "Adenovirus encoding vascular endothelial growth factor-D induces tissue-specific vascular patterns in vivo," *Blood*, vol. 99, no. 12, pp. 4434–4442, 2002.

[25] T. Tsurusaki, S. Kanda, H. Sakai et al., "Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis," *British Journal of Cancer*, vol. 80, no. 1-2, pp. 309–313, 1999.

[26] Y. Yonemura, Y. Endo, H. Fujita et al., "Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer," *Clinical Cancer Research*, vol. 5, no. 7, pp. 1823–1829, 1999.

[27] K. Akagi, Y. Ikeda, M. Miyazaki et al., "Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues," *The British Journal of Cancer*, vol. 83, no. 7, pp. 887–891, 2000.

[28] T. Niki, S. Iba, M. Tokunou, T. Yamada, Y. Matsuno, and S. Hirohashi, "Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma," *Clinical Cancer Research*, vol. 6, no. 6, pp. 2431–2439, 2000.

[29] I. Hashimoto, J. Kodama, N. Seki et al., "Vascular endothelial growth factor-C expression and its relationship to pelvic lymph node status in invasive cervical cancer," *British Journal of Cancer*, vol. 85, no. 1, pp. 93–97, 2001.

[30] C. Schietroma, F. Cianfarani, P. M. Lacal et al., "Vascular endothelial growth factor-C expression correlates with lymph node localization of human melanoma metastases," *Cancer*, vol. 98, no. 4, pp. 789–797, 2003.

[31] M. Skobe, T. Hawighorst, D. G. Jackson et al., "Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis," *Nature Medicine*, vol. 7, no. 2, pp. 192–198, 2001.

[32] A. Issa, T. X. Le, A. N. Shoushtari, J. D. Shields, and M. A. Swartz, "Vascular endothelial growth factor-C and C-C chemokine receptor 7 in tumor cell-lymphatic cross-talk promote invasive phenotype," *Cancer Research*, vol. 69, no. 1, pp. 349–357, 2009.

[33] L. Quagliata, S. Klusmeier, N. Cremers et al., "Inhibition of VEGFR-3 activation in tumor-draining lymph nodes suppresses the outgrowth of lymph node metastases in the MT-450 syngeneic rat breast cancer model," *Clinical & Experimental Metastasis*, vol. 31, pp. 351–365, 2014.

[34] R. S. Saad, L. Kordunsky, Y. L. Liu, K. L. Denning, H. A. Kandil, and J. F. Silverman, "Lymphatic microvessel density as prognostic marker in colorectal cancer," *Modern Pathology*, vol. 19, no. 10, pp. 1317–1323, 2006.

[35] S. Hirakawa, S. Kodama, R. Kunstfeld, K. Kajiya, L. F. Brown, and M. Detmar, "VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis," *Journal of Experimental Medicine*, vol. 201, no. 7, pp. 1089–1099, 2005.

[36] T. Tammela and K. Alitalo, "Lymphangiogenesis: molecular mechanisms and future promise," *Cell*, vol. 140, no. 4, pp. 460–476, 2010.

[37] S. S. Dadras, B. Lange-Asschenfeldt, P. Velasco et al., "Tumor lymphangiogenesis predicts melanoma metastasis to sentinel lymph nodes," *Modern Pathology*, vol. 18, no. 9, pp. 1232–1242, 2005.

[38] J. Holash, P. C. Maisonpierre, D. Compton et al., "Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF," *Science*, vol. 284, no. 5422, pp. 1994–1998, 1999.

[39] H. Huang, A. Bhat, G. Woodnutt, and R. Lappe, "Targeting the ANGPT-TIE2 pathway in malignancy," *Nature Reviews Cancer*, vol. 10, no. 8, pp. 575–585, 2010.

[40] W. Sun, "Angiogenesis in metastatic colorectal cancer and the benefits of targeted therapy," *Journal of Hematology & Oncology*, vol. 5, article 63, 2012.

[41] S. Davis, T. H. Aldrich, P. F. Jones et al., "Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning," *Cell*, vol. 87, no. 7, pp. 1161–1169, 1996.

[42] C. Scavelli, A. Vacca, G. Di Pietro, F. Dammacco, and D. Ribatti, "Crosstalk between angiogenesis and lymphangiogenesis in tumor progression," *Leukemia*, vol. 18, no. 6, pp. 1054–1058, 2004.

[43] P. C. Maisonpierre, C. Suri, P. F. Jones et al., "Angiopoietin-2, a natural antagonist for Tie_2 that disrupts in vivo angiogenesis," *Science*, vol. 277, no. 5322, pp. 55–60, 1997.

[44] P. Schulz, C. Fischer, K. M. Detjen et al., "Angiopoietin-2 drives lymphatic metastasis of pancreatic cancer," *FASEB Journal*, vol. 25, no. 10, pp. 3325–3335, 2011.

[45] R. Cao, M. A. Björndahl, P. Religa et al., "PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis," *Cancer Cell*, vol. 6, no. 4, pp. 333–345, 2006.

[46] L. K. Chang, G. Garcia-Cardeña, F. Farnebo et al., "Dose-dependent response of FGF-2 for lymphangiogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 32, pp. 11658–11663, 2004.

[47] C. M. Yoon, B. S. Hong, H. G. Moon et al., "Sphingosine-1-phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/PLC/Ca^{2+} signaling pathways," *Blood*, vol. 112, no. 4, pp. 1129–1138, 2008.

[48] R. Cao, M. A. Björndahl, M. I. Gallego et al., "Hepatocyte growth factor is a lymphangiogenic factor with an indirect mechanism of action," *Blood*, vol. 107, no. 9, pp. 3531–3536, 2006.

[49] S. Podgrabinska, P. Braun, P. Velasco et al., "Molecular characterization of lymphatic endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 25, pp. 16069–16074, 2002.

[50] M. Miyahara, J.-I. Tanuma, K. Sugihara, and I. Semba, "Tumor lymphangiogenesis correlates with lymph node metastasis and clinicopathologic parameters in oral squamous cell carcinoma," *Cancer*, vol. 110, no. 6, pp. 1287–1294, 2007.

[51] T. Sasahira, N. Ueda, K. Yamamoto et al., "Prox1 and FOXC2 act as regulators of lymphangiogenesis and angiogenesis in oral squamous cell carcinoma," *PloS ONE*, vol. 9, Article ID e92534, 2014.

[52] J. D. Shields, M. E. Fleury, C. Yong, A. A. Tomei, G. J. Randolph, and M. A. Swartz, "Autologous chemotaxis as a mechanism of tumor cell homing to lymphatics via interstitial flow and autocrine CCR7 signaling," *Cancer Cell*, vol. 11, no. 6, pp. 526–538, 2007.

[53] P. J. Sarvaiya, D. Guo, I. Ulasov, P. Gabikian, and M. S. Lesniak, "Chemokines in tumor progression and metastasis," *Oncotarget*, vol. 4, pp. 2171–2185, 2013.

[54] R. M. Steinman, M. Pack, and K. Inaba, "Dendritic cells in the T-cell areas of lymphoid organs," *Immunological Reviews*, vol. 156, pp. 25–37, 1997.

[55] Y. Ding, Y. Shimada, M. Maeda et al., "Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma," *Clinical Cancer Research*, vol. 9, no. 9, pp. 3406–3412, 2003.

[56] H. Takeuchi, A. Fujimoto, M. Tanaka, T. Yamano, E. Hsueh, and D. S. B. Hoon, "CCL21 chemokine regulates chemokine receptor CCR7 bearing malignant melanoma cells," *Clinical Cancer Research*, vol. 10, no. 7, pp. 2351–2358, 2004.

[57] K. Mashino, N. Sadanaga, H. Yamaguchi et al., "Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma," *Cancer Research*, vol. 62, no. 10, pp. 2937–2941, 2002.

[58] S. López-Giral, N. E. Quintana, M. Cabrerizo et al., "Chemokine receptors that mediate B cell homing to secondary lymphoid tissues are highly expressed in B cell chronic lymphocytic leukemia and non-Hodgkin lymphomas with widespread nodular dissemination," *Journal of Leukocyte Biology*, vol. 76, no. 2, pp. 462–471, 2004.

[59] P. Li, F. Liu, L. Sun et al., "Chemokine receptor 7 promotes cell migration and adhesion in metastatic squamous cell carcinoma of the head and neck by activating integrin $\alpha v\beta 3$," *International Journal of Molecular Medicine*, vol. 27, no. 5, pp. 679–687, 2011.

[60] Y. Li, X. Qiu, S. Zhang, Q. Zhang, and E. Wang, "Hypoxia induced CCR7 expression via HIF-1α and HIF-2α correlates with migration and invasion in lung cancer cells," *Cancer Biology and Therapy*, vol. 8, no. 4, pp. 322–330, 2009.

[61] P. Sui, P. Hu, T. Zhang, X. Zhang, Q. Liu, and J. Du, "High expression of CXCR-2 correlates with lymph node metastasis and predicts unfavorable prognosis in resected esophageal carcinoma," *Medical Oncology*, vol. 31, article 809, 2014.

[62] A. Müller, B. Homey, H. Soto et al., "Involvement of chemokine receptors in breast cancer metastasis," *Nature*, vol. 410, no. 6824, pp. 50–56, 2001.

[63] J. Ying, Q. Xu, G. Zhang, B. Liu, and L. Zhu, "The expression of CXCL12 and CXCR4 in gastric cancer and their correlation to lymph node metastasis," *Medical Oncology*, vol. 29, no. 3, pp. 1716–1722, 2012.

[64] M. Arya, H. R. H. Patel, C. McGurk et al., "The importance of the CXCL12-CXCR4 chemokine ligand-receptor interaction in prostate cancer metastasis," *Journal of Experimental Therapeutics and Oncology*, vol. 4, no. 4, pp. 291–303, 2004.

[65] S. Scala, P. Giuliano, P. A. Ascierto et al., "Human melanoma metastases express functional CXCR4," *Clinical Cancer Research*, vol. 12, no. 8, pp. 2427–2433, 2006.

[66] R. Franco, G. Botti, M. Mascolo et al., "CXCR4-CXCL12 and VEGF correlate to uveal melanoma progression," *Frontiers in Bioscience—Elite*, vol. 2, no. 1, pp. 13–21, 2010.

[67] C. Savarin-Vuaillat and R. M. Ransohoff, "Chemokines and chemokine receptors in neurological disease: raise, retain, or reduce?" *Neurotherapeutics*, vol. 4, no. 4, pp. 590–601, 2007.

[68] T. Murakami, W. Maki, A. R. Cardones et al., "Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells," *Cancer Research*, vol. 62, no. 24, pp. 7328–7334, 2002.

[69] B.-C. Zhao, Z.-J. Wang, W.-Z. Mao et al., "CXCR4/SDF-1 axis is involved in lymph node metastasis of gastric carcinoma," *World Journal of Gastroenterology*, vol. 17, no. 19, pp. 2389–2396, 2011.

[70] Y. X. Sun, J. Wang, C. E. Shelburne et al., "Expression of CXCR4 and CXCL12 (SDF-1) in human prostate cancers (PCa) in vivo," *Journal of Cellular Biochemistry*, vol. 89, no. 3, pp. 462–473, 2003.

[71] K. Kawada, M. Sonoshita, H. Sakashita et al., "Pivotal role of CXCR3 in melanoma cell metastasis to lymph nodes," *Cancer Research*, vol. 64, no. 11, pp. 4010–4017, 2004.

[72] J. Campisi, "Aging, cellular senescence, and cancer," *Annual Review of Physiology*, vol. 75, pp. 685–705, 2013.

[73] P. Castro, D. Giri, D. Lamb, and M. Ittmann, "Cellular senescence in the pathogenesis of benign prostatic hyperplasia," *The Prostate*, vol. 55, no. 1, pp. 30–38, 2003.

[74] M. Collado and M. Serrano, "Senescence in tumours: evidence from mice and humans," *Nature Reviews Cancer*, vol. 10, no. 1, pp. 51–57, 2010.

[75] C. Michaloglou, L. C. W. Vredeveld, M. S. Soengas et al., "BRAFE600-associated senescence-like cell cycle arrest of human naevi," *Nature*, vol. 436, no. 7051, pp. 720–724, 2005.

[76] L. C. W. Vredeveld, P. A. Possik, M. A. Smit et al., "Abrogation of BRAFV600E-induced senescence by PI3K pathway activation contributes to melanomagenesis," *Genes and Development*, vol. 26, no. 10, pp. 1055–1069, 2012.

[77] M. C. Velarde, M. Demaria, and J. Campisi, "Senescent cells and their secretory phenotype as targets for cancer therapy," *Interdisciplinary Topics in Gerontology*, vol. 38, pp. 17–27, 2013.

[78] W. Xue, L. Zender, C. Miething et al., "Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas," *Nature*, vol. 445, no. 7128, pp. 656–660, 2007.

[79] S. Rajagopalan and E. O. Long, "Cellular senescence induced by CD158d reprograms natural killer cells to promote vascular remodeling.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 50, pp. 20596–20601, 2012.

[80] E. Alspach, Y. Fu, and S. A. Stewart, "Senescence and the pro-tumorigenic stroma," *Critical Reviews in Oncogenesis*, vol. 18, pp. 549–558, 2013.

[81] R. Kalluri, "Basement membranes: structure, assembly and role in tumour angiogenesis," *Nature Reviews Cancer*, vol. 3, no. 6, pp. 422–433, 2003.

[82] R. Kalluri and M. Zeisberg, "Fibroblasts in cancer," *Nature Reviews Cancer*, vol. 6, no. 5, pp. 392–401, 2006.

[83] M. M. Mueller and N. E. Fusenig, "Friends or foes—bipolar effects of the tumour stroma in cancer," *Nature Reviews Cancer*, vol. 4, no. 11, pp. 839–849, 2004.

[84] M. M. Mueller, T. Werbowetski, and R. F. Del Maestro, "Soluble factors involved in glioma invasion," *Acta Neurochirurgica*, vol. 145, no. 11, pp. 999–1008, 2003.

[85] W. G. Stetler-Stevenson and A. E. Yu, "Proteases in invasion: matrix metalloproteinases," *Seminars in Cancer Biology*, vol. 11, no. 2, pp. 143–152, 2001.

[86] F. Mbeunkui and D. J. Johann Jr., "Cancer and the tumor microenvironment: a review of an essential relationship," *Cancer Chemotherapy and Pharmacology*, vol. 63, no. 4, pp. 571–582, 2009.

[87] L. F. Brown, A. J. Guidi, S. J. Schnitt et al., "Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast," *Clinical Cancer Research*, vol. 5, no. 5, pp. 1041–1056, 1999.

[88] Z. Werb, "ECM and cell surface proteolysis: regulating cellular ecology," *Cell*, vol. 91, no. 4, pp. 439–442, 1997.

[89] J. Krstic and J. F. Santibanez, "Transforming growth factor-beta and matrix metalloproteinases: functional interactions in tumor stroma-infiltrating myeloid cells," *The Scientific World Journal*, vol. 2014, Article ID 521754, 14 pages, 2014.

[90] T. C. Laurent and J. R. E. Fraser, "Hyaluronan," *The FASEB Journal*, vol. 6, no. 7, pp. 2397–2404, 1992.

[91] S. Banerji, J. Ni, S.-X. Wang et al., "LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan," *Journal of Cell Biology*, vol. 144, no. 4, pp. 789–801, 1999.

[92] N. Itano, L. Zhuo, and K. Kimata, "Impact of the hyaluronan-rich tumor microenvironment on cancer initiation and progression," *Cancer Science*, vol. 99, no. 9, pp. 1720–1725, 2008.

[93] T. D. Tlsty and P. W. Hein, "Know thy neighbor: stromal cells can contribute oncogenic signals," *Current Opinion in Genetics and Development*, vol. 11, no. 1, pp. 54–59, 2001.

[94] B. Elenbaas and R. A. Weinberg, "Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation," *Experimental Cell Research*, vol. 264, no. 1, pp. 169–184, 2001.

[95] P. Garin-Chesa, L. J. Old, and W. J. Rettig, "Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 18, pp. 7235–7239, 1990.

[96] D. Lazard, X. Sastre, M. G. Frid, M. A. Glukhova, J.-P. Thiery, and V. E. Koteliansky, "Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 3, pp. 999–1003, 1993.

[97] H. Y. Chang, J.-T. Chi, S. Dudoit et al., "Diversity, topographic differentiation, and positional memory in human fibroblasts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 20, pp. 12877–12882, 2002.

[98] M. Shimoda, K. T. Mellody, and A. Orimo, "Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression," *Seminars in Cell and Developmental Biology*, vol. 21, no. 1, pp. 19–25, 2010.

[99] M. Bauer, G. Su, C. Casper, R. He, W. Rehrauer, and A. Friedl, "Heterogeneity of gene expression in stromal fibroblasts of human breast carcinomas and normal breast," *Oncogene*, vol. 29, no. 12, pp. 1732–1740, 2010.

[100] O. de Wever, Q.-D. Nguyen, L. van Hoorde et al., "Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac," *The FASEB Journal*, vol. 18, no. 9, pp. 1016–1018, 2004.

[101] O. de Wever and M. Mareel, "Role of tissue stroma in cancer cell invasion," *The Journal of Pathology*, vol. 200, no. 4, pp. 429–447, 2003.

[102] S. Semba, Y. Kodama, K. Ohnuma et al., "Direct cancer-stromal interaction increases fibroblast proliferation and enhances invasive properties of scirrhous-type gastric carcinoma cells," *British Journal of Cancer*, vol. 101, no. 8, pp. 1365–1373, 2009.

[103] D. Liao, Y. Luo, D. Markowitz, R. Xiang, and R. A. Reisfeld, "Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model," *PLoS ONE*, vol. 4, no. 11, Article ID e7965, 2009.

[104] A. Orimo, P. B. Gupta, D. C. Sgroi et al., "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion," *Cell*, vol. 121, no. 3, pp. 335–348, 2005.

[105] Y. Zhang, H. Tang, J. Cai et al., "Ovarian cancer-associated fibroblasts contribute to epithelial ovarian carcinoma metastasis

by promoting angiogenesis, lymphangiogenesis and tumor cell invasion," *Cancer Letters*, vol. 303, no. 1, pp. 47–55, 2011.

[106] B. Pula, A. Jethon, A. Piotrowska et al., "Podoplanin expression by cancer-associated fibroblasts predicts poor outcome in invasive ductal breast carcinoma," *Histopathology*, vol. 59, no. 6, pp. 1249–1260, 2011.

[107] E. L. Spaeth, J. L. Dembinski, A. K. Sasser et al., "Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression," *PLoS ONE*, vol. 4, no. 4, Article ID e4992, 2009.

[108] J. Guan and J. Chen, "Mesenchymal stem cells in the tumor microenvironment," *Biomedical Reports*, vol. 1, pp. 517–521, 2013.

[109] B. M. Beckermann, G. Kallifatidis, A. Groth et al., "VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma," *British Journal of Cancer*, vol. 99, no. 4, pp. 622–631, 2008.

[110] K. Buttler, M. Badar, V. Seiffart et al., "De novo hem- and lymphangiogenesis by endothelial progenitor and mesenchymal stem cells in immunocompetent mice," *Cellular and Molecular Life Sciences*, vol. 71, no. 8, pp. 1513–1527, 2014.

[111] K. Kajiya, S. Hirakawa, B. Ma, I. Drinnenberg, and M. Detmar, "Hepatocyte growth factor promotes lymphatic vessel formation and function," *The EMBO Journal*, vol. 24, no. 16, pp. 2885–2895, 2005.

[112] M. T. Dellinger and R. A. Brekken, "Phosphorylation of Akt and ERK1/2 is required for VEGF-A/VEGFR2-induced proliferation and migration of lymphatic endothelium," *PLoS ONE*, vol. 6, no. 12, Article ID e28947, 2011.

[113] S. Shinriki, H. Jono, M. Ueda et al., "Interleukin-6 signalling regulates vascular endothelial growth factor-C synthesis and lymphangiogenesis in human oral squamous cell carcinoma," *The Journal of Pathology*, vol. 225, no. 1, pp. 142–150, 2011.

[114] Y. H. Huang, H. Y. Yang, Y. F. Hsu, P. T. Chiu, G. Ou, and M. J. Hsu, "Src contributes to IL6-induced vascular endothelial growth factor-C expression in lymphatic endothelial cells," *Angiogenesis*, vol. 17, no. 2, pp. 407–418, 2014.

[115] C. Conrad, H. Niess, R. Huss et al., "Multipotent mesenchymal stem cells acquire a lymphendothelial phenotype and enhance lymphatic regeneration in vivo," *Circulation*, vol. 119, no. 2, pp. 281–289, 2009.

[116] A. E. Karnoub, A. B. Dash, A. P. Vo et al., "Mesenchymal stem cells within tumour stroma promote breast cancer metastasis," *Nature*, vol. 449, no. 7162, pp. 557–563, 2007.

[117] M. B. Fuertes, A. K. Kacha, J. Kline et al., "Host type I IFN signals are required for antitumor $CD8^+$ T cell responses through $CD8\alpha^+$ dendritic cells," *The Journal of Experimental Medicine*, vol. 208, no. 10, pp. 2005–2016, 2011.

[118] T. F. Gajewski, H. Schreiber, and Y. X. Fu, "Innate and adaptive immune cells in the tumor microenvironment," *Nature Immunology*, vol. 14, pp. 1014–1022, 2013.

[119] K. Steinbrink, M. Wölfl, H. Jonuleit, J. Knop, and A. H. Enk, "Induction of tolerance by IL-10-treated dendritic cells," *The Journal of Immunology*, vol. 159, no. 10, pp. 4772–4780, 1997.

[120] K. Palucka and J. Banchereau, "Cancer immunotherapy via dendritic cells," *Nature Reviews Cancer*, vol. 12, no. 4, pp. 265–277, 2012.

[121] N. J. Bahlis, A. M. King, D. Kolonias et al., "CD28-mediated regulation of multiple myeloma cell proliferation and survival," *Blood*, vol. 109, no. 11, pp. 5002–5010, 2007.

[122] S. Wei, I. Kryczek, L. Zou et al., "Plasmacytoid dendritic cells induce $CD8^+$ regulatory T cells in human ovarian carcinoma," *Cancer Research*, vol. 65, no. 12, pp. 5020–5026, 2005.

[123] T. J. Curiel, P. Cheng, P. Mottram et al., "Dendritic cell subsets differentially regulate angiogenesis in human ovarian cancer," *Cancer Research*, vol. 64, no. 16, pp. 5535–5538, 2004.

[124] A. Thiel, R. Kesselring, R. Pries, N. Wittkopf, A. Puzik, and B. Wollenberg, "Plasmacytoid dendritic cell subpopulations in head and neck squamous cell carcinoma," *Oncology Reports*, vol. 26, no. 3, pp. 615–620, 2011.

[125] I. Treilleux, J.-Y. Blay, N. Bendriss-Vermare et al., "Dendritic cell infiltration and prognosis of early stage breast cancer," *Clinical Cancer Research*, vol. 10, no. 22, pp. 7466–7474, 2004.

[126] R.-C. Ji, "Macrophages are important mediators of either tumor- or inflammation-induced lymphangiogenesis," *Cellular and Molecular Life Sciences*, vol. 69, no. 6, pp. 897–914, 2012.

[127] S. Sozzani, M. Rusnati, E. Riboldi, S. Mitola, and M. Presta, "Dendritic cell-endothelial cell cross-talk in angiogenesis," *Trends in Immunology*, vol. 28, no. 9, pp. 385–392, 2007.

[128] E. Gottfried, M. Kreutz, S. Haffner et al., "Differentiation of human tumour-associated dendritic cells into endothelial-like cells: an alternative pathway of tumour angiogenesis," *Scandinavian Journal of Immunology*, vol. 65, no. 4, pp. 329–335, 2007.

[129] J. R. Conejo-Garcia, F. Benencia, M.-C. Courreges et al., "Tumor-infiltrating dendritic cell precursors recruited by a β-defensin contribute to vasculogenesis under the influence of Vegf-A," *Nature Medicine*, vol. 10, no. 9, pp. 950–958, 2004.

[130] D. I. Gabrilovich, T. Ishida, S. Nadaf, J. E. Ohm, and D. P. Carbone, "Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function," *Clinical Cancer Research*, vol. 5, no. 10, pp. 2963–2970, 1999.

[131] L. Chen, P. Hamrah, C. Cursiefen et al., "Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity," *Nature Medicine*, vol. 10, no. 8, pp. 813–815, 2004.

[132] M. Darash-Yahana, E. Pikarsky, R. Abramovitch et al., "Role of high expression levels of CXCR4 in tumor growth, vascularization, and metastasis," *FASEB Journal*, vol. 18, no. 11, pp. 1240–1242, 2004.

[133] H. Dong, S. E. Strome, D. R. Salomao et al., "Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion," *Nature Medicine*, vol. 8, no. 8, pp. 793–800, 2002.

[134] J. A. Brown, D. M. Dorfman, F.-R. Ma et al., "Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production," *Journal of Immunology*, vol. 170, no. 3, pp. 1257–1266, 2003.

[135] L. Chen, "Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity," *Nature Reviews Immunology*, vol. 4, no. 5, pp. 336–347, 2004.

[136] W. Yang, H. Li, P. W. Chen et al., "PD-L1 expression on human ocular cells and its possible role in regulating immune-mediated ocular inflammation," *Investigative Ophthalmology and Visual Science*, vol. 50, no. 1, pp. 273–280, 2009.

[137] S. T. Haile, J. J. Bosch, N. I. Agu et al., "Tumor cell programmed death ligand 1-mediated T cell suppression is overcome by coexpression of CD80," *Journal of Immunology*, vol. 186, no. 12, pp. 6822–6829, 2011.

[138] A. S. Mansfield, P. Heikkila, K. von Smitten, J. Vakkila, and M. Leidenius, "Metastasis to sentinel lymph nodes in breast cancer is associated with maturation arrest of dendritic cells and poor co-localization of dendritic cells and $CD8^+$ T cells," *Virchows Archiv*, vol. 459, no. 4, pp. 391–398, 2011.

[139] M. E. Polak, P. Johnson, S. Di Palma et al., "Presence and maturity of dendritic cells in melanoma lymph node metastases," *The Journal of Pathology*, vol. 207, no. 1, pp. 83–90, 2005.

[140] A. Kusume, T. Sasahira, Y. Luo et al., "Suppression of dendritic cells by HMGB1 is associated with lymph node metastasis of human colon cancer," *Pathobiology*, vol. 76, no. 4, pp. 155–162, 2009.

[141] S. Ohashi, S. Okamura, F. Urano, and M. Maeda, "Clinicopathological variables associated with lymph node metastasis in submucosal invasive gastric cancer," *Gastric Cancer*, vol. 10, no. 4, pp. 241–250, 2007.

[142] W. Vermi, A. Micheletti, S. Lonardi et al., "slanDCs selectively accumulate in carcinoma-draining lymph nodes and marginate metastatic cells," *Nature Communications*, vol. 5, article 3029, 2014.

[143] F. G. Gomes, F. Nedel, A. M. Alves, J. E. Nör, and S. B. C. Tarquinio, "Tumor angiogenesis and lymphangiogenesis: tumor/endothelial crosstalk and cellular/microenvironmental signaling mechanisms," *Life Sciences*, vol. 92, no. 2, pp. 101–107, 2013.

[144] C. E. Lewis and J. W. Pollard, "Distinct role of macrophages in different tumor microenvironments," *Cancer Research*, vol. 66, no. 2, pp. 605–612, 2006.

[145] V. Riabov, A. Gudima, N. Wang, A. Mickley, A. Orekhov, and J. Kzhyshkowska, "Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis," *Frontiers in Physiology*, vol. 5, article 75, 2014.

[146] D. Kerjaschki, "The crucial role of macrophages in lymphangiogenesis," *The Journal of Clinical Investigation*, vol. 115, no. 9, pp. 2316–2319, 2005.

[147] K. Schledzewski, M. Falkowski, G. Moldenhauer et al., "Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macropahages in malignant tumours and wound healing tissue in vivo and in bone marrow cultures in vitro: implications for the assessment of lymphangiogenesis," *Journal of Pathology*, vol. 209, no. 1, pp. 67–77, 2006.

[148] A. Zumsteg, V. Baeriswyl, N. Imaizumi, R. Schwendener, C. Rüegg, and G. Christofori, "Myeloid cells contribute to tumor lymphangiogenesis," *PLoS ONE*, vol. 4, no. 9, Article ID e7067, 2009.

[149] I. H. G. Bronkhorst, L. V. Ly, E. S. Jordanova et al., "Detection of M2-macrophages in uveal melanoma and relation with survival," *Investigative Ophthalmology & Visual Science*, vol. 52, no. 2, pp. 643–650, 2011.

[150] R. D. Leek, C. E. Lewis, R. Whitehouse, M. Greenall, J. Clarke, and A. L. Harris, "Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma," *Cancer Research*, vol. 56, no. 20, pp. 4625–4629, 1996.

[151] A. Nishie, M. Ono, T. Shono et al., "Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas," *Clinical Cancer Research*, vol. 5, no. 5, pp. 1107–1113, 1999.

[152] H. Torisu, M. Ono, H. Kiryu et al., "Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFalpha and IL-1alpha," *International Journal of Cancer*, vol. 85, pp. 182–188, 2000.

[153] D. Moussai, H. Mitsui, J. S. Pettersen et al., "The human cutaneous squamous cell carcinoma microenvironment is characterized by increased lymphatic density and enhanced expression of macrophage-derived VEGF-C," *Journal of Investigative Dermatology*, vol. 131, no. 1, pp. 229–236, 2011.

[154] M. S. Kluger and O. R. Colegio, "Lymphangiogenesis linked to VEGF-C from tumor-associated macrophages: accomplices to metastasis by cutaneous squamous cell carcinoma," *Journal of Investigative Dermatology*, vol. 131, no. 1, pp. 17–19, 2011.

[155] L. V. Ly, A. Baghat, M. Versluis et al., "In aged mice, outgrowth of intraocular melanoma depends on proangiogenic M2-type macrophages," *Journal of Immunology*, vol. 185, no. 6, pp. 3481–3488, 2010.

[156] T. Mäkitie, P. Summanen, A. Tarkkanen, and T. Kivelä, "Tumor-infiltrating macrophages ($CD68^+$ cells) and prognosis in malignant uveal melanoma," *Investigative Ophthalmology and Visual Science*, vol. 42, no. 7, pp. 1414–1421, 2001.

[157] J. A. Nagy, E. Vasile, D. Feng et al., "Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis," *The Journal of Experimental Medicine*, vol. 196, no. 11, pp. 1497–1506, 2002.

[158] O. Fainaru, N. Almog, C. W. Yung et al., "Tumor growth and angiogenesis are dependent on the presence of immature dendritic cells," *The FASEB Journal*, vol. 24, no. 5, pp. 1411–1418, 2010.

[159] F. Pagès, A. Berger, M. Camus et al., "Effector memory T cells, early metastasis, and survival in colorectal cancer," *New England Journal of Medicine*, vol. 353, no. 25, pp. 2654–2666, 2005.

[160] J. Galon, A. Costes, F. Sanchez-Cabo et al., "Type, density, and location of immune cells within human colorectal tumors predict clinical outcome," *Science*, vol. 313, no. 5795, pp. 1960–1964, 2006.

[161] F. Azimi, R. A. Scolyer, P. Rumcheva et al., "Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma," *Journal of Clinical Oncology*, vol. 30, no. 21, pp. 2678–2683, 2012.

[162] B. Kreike, M. van Kouwenhove, H. Horlings et al., "Gene expression profiling and histopathological characterization of triple-negative/basal-like breast carcinomas," *Breast Cancer Research*, vol. 9, no. 5, article R65, 2007.

[163] S. M. A. Mahmoud, E. C. Paish, D. G. Powe et al., "Tumor-infiltrating $CD8^+$ lymphocytes predict clinical outcome in breast cancer," *Journal of Clinical Oncology*, vol. 29, no. 15, pp. 1949–1955, 2011.

[164] L. Zhang, J. R. Conejo-Garcia, D. Katsaros et al., "Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer," *New England Journal of Medicine*, vol. 348, no. 3, pp. 203–213, 2003.

[165] T. J. Curiel, G. Coukos, L. Zou et al., "Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival," *Nature Medicine*, vol. 10, no. 9, pp. 942–949, 2004.

[166] W. Zou, "Immunosuppressive networks in the tumour environment and their therapeutic relevance," *Nature Reviews Cancer*, vol. 5, no. 4, pp. 263–274, 2005.

[167] R. C. Taylor, A. Patel, K. S. Panageas, K. J. Busam, and M. S. Brady, "Tumor-infiltrating lymphocytes predict sentinel lymph node positivity in patients with cutaneous melanoma," *Journal of Clinical Oncology*, vol. 25, no. 7, pp. 869–875, 2007.

[168] J.-Y. Shin, I.-H. Yoon, J.-S. Kim, B. Kim, and C.-G. Park, "Vascular endothelial growth factor-induced chemotaxis and IL-10 from T cells," *Cellular Immunology*, vol. 256, no. 1-2, pp. 72–78, 2009.

[169] A. Ruddell, P. Mezquita, K. A. Brandvold, A. Farr, and B. M. Iritani, "B lymphocyte-specific c-Myc expression stimulates early and functional expansion of the vasculature and lymphatics

during lymphomagenesis," *the American Journal of Pathology*, vol. 163, no. 6, pp. 2233–2245, 2003.

[170] A. Ruddell, M. I. Harrell, M. Furuya, S. B. Kirschbaum, and B. M. Iritani, "B lymphocytes promote lymphogenous metastasis of lymphoma and melanoma," *Neoplasia*, vol. 13, no. 8, pp. 748–757, 2011.

[171] M. I. Harrell, B. M. Iritani, and A. Ruddell, "Tumor-induced sentinel lymph node lymphangiogenesis and increased lymph flow precede melanoma metastasis," *The American Journal of Pathology*, vol. 170, no. 2, pp. 774–786, 2007.

[172] D. B. Mendel, A. D. Laird, X. Xin et al., "In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship," *Clinical Cancer Research*, vol. 9, no. 1 I, pp. 327–337, 2003.

[173] E. P. Rock, V. Goodman, J. X. Jiang et al., "Food and Drug Administration drug approval summary: sunitinib malate for the treatment of gastrointestinal stromal tumor and advanced renal cell carcinoma," *Oncologist*, vol. 12, no. 1, pp. 107–113, 2007.

[174] S. M. Wilhelm, C. Carter, L. Tang et al., "BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis," *Cancer Research*, vol. 64, no. 19, pp. 7099–7109, 2004.

[175] R. C. Kane, A. T. Farrell, H. Saber et al., "Sorafenib for the treatment of advanced renal cell carcinoma," *Clinical Cancer Research*, vol. 12, no. 24, pp. 7271–7278, 2006.

[176] X.-W. He, X. Yu, T. Liu, S.-Y. Yu, and D.-J. Chen, "Vector-based RNA interference against vascular endothelial growth factor-C inhibits tumor lymphangiogenesis and growth of colorectal cancer in vivo in mice," *Chinese Medical Journal*, vol. 121, no. 5, pp. 439–444, 2008.

[177] Z. Lui, Q. Ma, X. Wang, and Y. Zhang, "Inhibiting tumor growth of colorectal cancer by blocking the expression of vascular endothelial growth factor receptor 3 using interference vector-based RNA interference," *International Journal of Molecular Medicine*, vol. 25, no. 1, pp. 59–64, 2010.

[178] R. V. Frolov and S. Singh, "Celecoxib and ion channels: a story of unexpected discoveries," *European Journal of Pharmacology*, vol. 730, pp. 61–71, 2014.

[179] L. Wang, W. Chen, X. Xie, Y. He, and X. Bai, "Celecoxib inhibits tumor growth and angiogenesis in an orthotopic implantation tumor model of human colon cancer," *Experimental Oncology*, vol. 30, no. 1, pp. 42–51, 2008.

[180] H. Liu, Y. Yang, J. Xiao et al., "Inhibition of cyclooxygenase-2 suppresses lymph node metastasis via VEGF-C," *Anatomical Record*, vol. 292, no. 10, pp. 1577–1583, 2009.

[181] S. Takahashi, "Vascular Endothelial Growth Factor (VEGF), VEGF receptors and their inhibitors for antiangiogenic tumor therapy," *Biological and Pharmaceutical Bulletin*, vol. 34, no. 12, pp. 1785–1788, 2011.

[182] S. Yao and L. Chen, "Reviving exhausted T lymphocytes during chronic virus infection by B7-H1 blockade," *Trends in Molecular Medicine*, vol. 12, no. 6, pp. 244–246, 2006.

[183] S. Yao, Y. Zhu, and L. Chen, "Advances in targeting cell surface signalling molecules for immune modulation," *Nature Reviews Drug Discovery*, vol. 12, no. 2, pp. 130–146, 2013.

[184] D. S. Chen and I. Mellman, "Oncology meets immunology: the cancer-immunity cycle," *Immunity*, vol. 39, no. 1, pp. 1–10, 2013.

[185] O. Hamid and R. D. Carvajal, "Anti-programmed death-1 and anti-programmed death-ligand 1 antibodies in cancer therapy," *Expert Opinion on Biological Therapy*, vol. 13, no. 6, pp. 847–861, 2013.

[186] L. H. Butterfield, "Dendritic cells in cancer immunotherapy clinical trials: are we making progress?" *Frontiers in Immunology*, vol. 4, article 454, 2013.

[187] S. A. Grupp, M. Kalos, D. Barrett et al., "Chimeric antigen receptor-modified T cells for acute lymphoid leukemia," *The New England Journal of Medicine*, vol. 368, no. 16, pp. 1509–1518, 2013.

[188] R. J. Brentjens, I. Rivière, J. H. Park et al., "Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias," *Blood*, vol. 118, no. 18, pp. 4817–4828, 2011.

[189] D. L. Porter, B. L. Levine, M. Kalos, A. Bagg, and C. H. June, "Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia," *New England Journal of Medicine*, vol. 365, no. 8, pp. 725–733, 2011.

[190] O. S. Qureshi, Y. Zheng, K. Nakamura et al., "Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4," *Science*, vol. 332, no. 6029, pp. 600–603, 2011.

[191] G. Q. Phan, J. C. Yang, R. M. Sherry et al., "Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 14, pp. 8372–8377, 2003.

[192] P. Waterhouse, J. M. Penninger, E. Timms et al., "Lymphoproliferative disorders with early lethality in mice deficient in *Ctla-4*," *Science*, vol. 270, no. 5238, pp. 985–988, 1995.

[193] U. Grohmann, C. Orabona, F. Fallarino et al., "CTLA-4-Ig regulates tryptophan catabolism *in vivo*," *Nature Immunology*, vol. 3, no. 11, pp. 1097–1101, 2002.

[194] M. Sasaki, H. Hasegawa, M. Kohno, A. Inoue, M. R. Ito, and S. Fujita, "Antagonist of secondary lymphoid-tissue chemokine (CCR ligand 21) prevents the development of chronic graft-versus-host disease in mice," *Journal of Immunology*, vol. 170, no. 1, pp. 588–596, 2003.

[195] S. Schlereth, H. S. Lee, P. Khandelwal, and D. R. Saban, "Blocking CCR7 at the ocular surface impairs the pathogenic contribution of dendritic cells in allergic conjunctivitis," *The American Journal of Pathology*, vol. 180, no. 6, pp. 2351–2360, 2012.

[196] Y. Shuyi, D. Juping, Z. Zhiqun et al., "A critical role of CCR7 in invasiveness and metastasis of SW620 colon cancer cell in vitro and in vivo," *Cancer Biology and Therapy*, vol. 7, no. 7, pp. 1037–1043, 2008.

[197] F. E. Gonzalez, C. Ortiz, M. Reyes et al., "Melanoma cell lysate induces CCR7 expression and in vivo migration to draining lymph nodes of therapeutic human dendritic cells," *Immunology*, vol. 142, no. 3, pp. 369–405, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 756347, 8 pages
http://dx.doi.org/10.1155/2014/756347

Research Article

MicroRNA Expression in Salivary Supernatant of Patients with Pancreatic Cancer and Its Relationship with ZHENG

Song Gao,[1,2] Lian-Yu Chen,[1,2] Peng Wang,[1,2] Lu-Ming Liu,[1,2] and Zhen Chen[1,2]

[1] *Department of Integrative Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China*
[2] *Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China*

Correspondence should be addressed to Zhen Chen; cz120@mail.sh.cn

Received 14 March 2014; Accepted 27 May 2014; Published 14 July 2014

Academic Editor: Zhiqiang Meng

In traditional Chinese medicine (TCM), diagnosis and prescriptions are based on the signs and symptoms which are recognized as ZHENG. The cornerstone of TCM is to differentially treat one ZHENG from others, which is also known as syndrome differentiation, and this relies on the gathering of clinical information through inspection, auscultation and olfaction, inquiry, and palpation. However, the biomolecular basis of the ZHENG remains unclear. In this study, the expressions of 384 cancer-related miRNAs in salivary supernatant of patients with pancreatic cancer were assessed by miRNA polymerase chain reaction (PCR) array, and the different expression patterns of miRNA in three different groups of ZHENG were studied with use of real-time quantitative PCR (qRT-PCR). Some miRNAs were found to be specifically expressed in some ZHENGs, for instance, miR-17, miR-21, and miR-181b in Shi-Re ZHENG and miR-196a in Pi-Xu ZHENG. This indicates that these miRNAs may play important roles in different ZHENG condition. Therefore, this study to some extent revealed the molecular basis of TCM ZHENG in pancreatic cancer.

1. Introduction

Traditional Chinese medicine (TCM), with a history of more than 3,000 years, emphasizes the comprehensive analysis of clinical information collected through four combined diagnostic methods: observation, auscultation and olfaction, inquiry, and palpation. The patients' disease condition will be classified into a specific ZHENG, which is the basis of prescription of Chinese herbs. ZHENG is the cornerstone in traditional Chinese medicine (TCM) theories. In cancer occurrence and its progression, a series of ZHENGs are involved. In recent years, many studies showed that TCM has certain advantages in the treatment of pancreatic cancer, while in most such studies it is difficult to interpret the advantages due to the lack of evidence-based medicine design. In TCM theory, the pathogenesis of pancreatic carcinoma may be the accumulation of dampness and heat, or the accumulation of dampness, heat, and toxicity. The related literature review indicated that Shi-Re, Pi-Xu, and Xue-Yu (dampness-heat, spleen-deficiency, and blood-stasis, resp.) are the top three types of TCM ZHENG in pancreatic cancer [1]. Accordingly, in this work, the expressions of some miRNAs related to pancreatic cancer (PC) under different ZHENG conditions were studied, and this work is expected to partially explore the molecular mechanism of TCM ZHENG in pancreatic cancer.

MicroRNA (miRNA), which is closely associated with the development of cancer, autoimmune disease, and other diseases, is single-stranded noncoding RNA in a variety of body fluids. During tumor development, aberrant expression of miRNAs can either inactivate tumor suppressor genes or activate oncogenes and thus promote tumor formation [2–8]. The expression of miRNAs is tissue-specific, detectable in blood [9], and correlated with clinical cancer development [10]. MicroRNA is highly stable in body fluids and can be easily detected. More and more scholars pay their attention to its expression in body fluids. Body fluids collection is convenient and trauma-free and thus can be easily accepted by patients, and there is no risk of hematogenous spread of diseases in sampling. Because detection of the expression levels of salivary miRNAs is convenient and noninvasive, it may be an option for early screening of pancreatic cancer. In conclusion, the role of salivary miRNAs is worthy of further

study. This study focused on the specific expression of miRNA in the salivary supernatant in patients with pancreatic cancer, and the correlation between ZHENG and microRNA expression in pancreatic cancer was also investigated. Based on our results, the molecular basis of TCM ZHENG in pancreatic cancer is partially revealed, which may help making clinical syndrome differentiation and curative effect assessment for the pancreatic cancer more objective and standardized.

2. Materials and Methods

2.1. Patients. Thirty patients with newly diagnosed pancreatic cancer in Shanghai Cancer Center were enrolled in this study. Of all the patients (patient group), 17 were males and 13 were females, with age ranged from 33 to 78 years, and all were not treated before this study, that is, not receiving surgery, radiological, and interventional treatments. The diagnosis of pancreatic ductal carcinoma was confirmed by pathological results after surgery. Saliva from 32 healthy volunteers was collected as control (control group), and all volunteers had no history of cancer and family history of cancer. The two groups were comparable regarding sex and age ($P > 0.05$). The including criteria for patients were as follows. (1) The diagnosis was confirmed by tissue pathology. (2) All cases were newly diagnosed pancreatic cancer. The excluding criteria for patients were as follows. (1) Patients received surgery, radiological, and chemotherapeutic treatments before the study. (2) Patients were complicated with organic or functional heart, liver, kidney, and brain diseases.

2.2. Saliva Supernatant Collection. Saliva was centrifugated at 2500 g for 10 min at 4°C, and the supernatant was collected and centrifuged at 10,000 g for 1 min to remove remaining cells. The resultant supernatant was transferred into a new tube and stored at −80°C within 30 min of collection prior to RNA extraction (Figure 1).

2.3. RNA Isolation and Processing. The samples were centrifuged at 2500 g for 10 min, and only the supernatant was used for RNA extraction. Soluble miRNAs in supernatant were isolated with use of the miRNeasy Serum/Plasma Kit (Qiagen) according to the manufacturer's protocol. RNA was eluted in 14 uL of RNase-free water. cDNA was generated by miScript RTII kit (Qiagen) according to the manufacturer's recommendations. The prepared cDNA was diluted 1 : 4 in RNase-free water, 5 uL of the diluted cDNA was preamplified with the Qiagen miScript preAMP PCR kit, and then the preamplified cDNA was diluted 5-fold before qPCR detection.

2.4. Measurement of miRNAs. miRNA profiling was performed with use of miScript miRNA PCR array human miRNome (384-well plate) from Qiagen. For subsequent evaluations of candidate miRNAs, custom Qiagen 384-well plates with specific primer probes were used. A SYBR Green-based qPCR was performed with 2 uL cDNA in a 10 uL reaction volume in the Applied Biosystems 7900HT Fast Real-Time PCR System instrument, and the reaction conditions

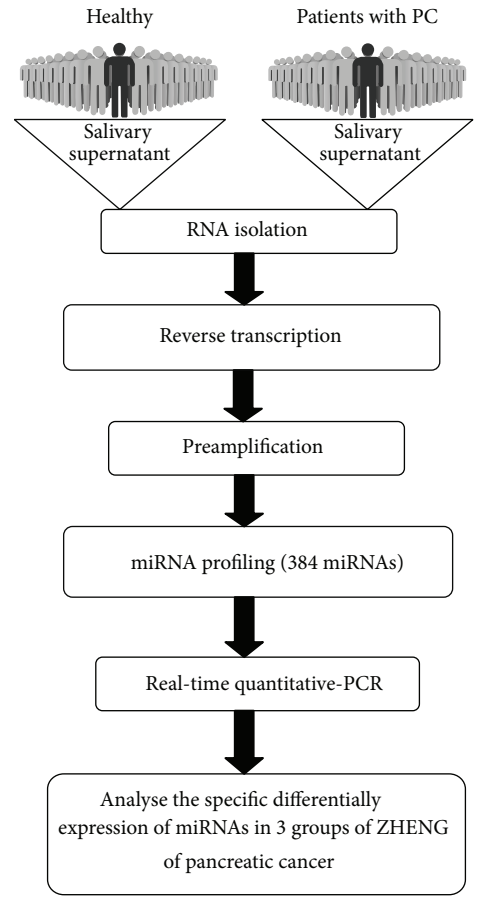


Figure 1: Work flow for processing salivary supernatant from 32 healthy volunteers and 30 PC patients and then screening 384 cancer-related miRNAs.

were as follows: 15 min at 95°C and 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. All qPCR experiments were performed in 384-well plates, with a set of technical controls included on each plate (*Caenorhabditis elegans* miR-39 (C.el-miR-39), which was used as the spike-in control, and miRTC, which is a Qiagen proprietary synthetic oligonucleotide). To assess recoveries after RNA isolation, C.el-miR-39 was spiked into the sample before the extraction process. The efficiency of reverse transcription with miRTC was assessed, which was present in the nucleic acid mixture that was reverse-transcribed, preamplified, and detected by real-time qPCR.

2.5. miRNA Expression by Real-Time Quantitative Polymerase Chain Reaction. Selected microRNAs were measured using individual Qiagen microRNA arrays, with TaqMan reagents (RT mix and Universal Master Mix II). For saliva samples, Megaplex RT and preamplification were conducted to increase the limit of miRNA detection. RQ Manager 1.2 Software (ABI) was applied to generate Ct values. Relative miRNA levels were determined by $^{\Delta\Delta}$Ct using endogenous controls (miR-16). Ct values ≥ 35 were considered as negative

Table 1: RT primers for amplification of the human mature miRNAs used in validation of microRNA microarray results.

Name	RT primer	Product length (bp)
miR-16	F primer 5′-CGCGCTAGCAGCACGTAAATA-3′ R primer 5′-GTGCAGGGTCCGAGGT-3′ Probe 5′-TTCGCACTGGATACGACCGCCAA-3′	**71**
miR-17	F primer 5′-CGGCGGCAAAGTGCTTACAG-3′ R primer 5′-GTGCAGGGTCCGAGGT-3′ Probe 5′-TTCGCACTGGATACGACCTACCTGCA-3′	**73**
miR-21	F primer 5′-GCGGCGGCTAGCTTATCAGAC-3′ R primer 5′-GTGCAGGGTCCGAGGT-3′ Probe 5′-TTCGCACTGGATACGACTCAACATCAG-3′	**74**
miR-181a	F primer 5′-GCCCGAACATTCAACGCTGT-3′ R primer 5′-GTGCAGGGTCCGAGGT-3′ Probe 5′-TTCGCACTGGATACGACACTCACCG-3′	**72**
miR-181b	F primer 5′-CGCGCAACATTCATTGCTGT-3′ R primer 5′-GTGCAGGGTCCGAGGT-3′ Probe 5′-TTCGCACTGGATACGACACCCAC-3′	**66**
miR-196a	F primer 5′-CGGCTTTGGCACTAGCACATT-3′ R primer 5′-GTGCAGGGTCCGAGGT-3′ Probe 5′-TTTGCGCACTGGATACGACAGCAA-3′	**71**

amplification. Real-time PCR was performed using the miScript SYBR green PCR kit (Qiagen) according to the manufacturer's instructions. Five selective miRNA-specific RT primers were designed by primer premier 5.0 (Table 1). miR-16 were used as the endogenous normalizer to calculate the respective relative concentrations for the candidate miRNAs (Qiagen). Relative levels of miRNAs were assessed using the $^{\Delta\Delta}$Ct method.

2.6. Analysis of miRNAs in Salivary Supernatant and Statistical Analysis. The results from the qPCR reaction with SDS 2.4 software (Applied Biosystems) were obtained as threshold cycle values with automatic threshold and baseline values. All data were presented as mean ± standard deviation (SD). All statistics were performed with use of SPSS version 16.0. The results were then compared with use of one-way ANOVA with LSD among multiple groups or student t-test between two groups. Values of $P < 0.05$ were considered statistically significant in all tests.

3. Results

3.1. Candidate miRNAs Differentially Expressed in Patients with Pancreatic Cancer and in Healthy Controls. An miRNA PCR array (miRBase version 18, containing 384 miRNAs; Qiagen) was used to detect the miRNAs fraction in salivary supernatant of 30 patients with pancreatic cancer and 32 healthy controls (Figure 1). Nineteen miRNAs were screened for the next phase of our study (Table 2). Custom qRT-PCR was performed to analyze the relative concentrations of the 19 candidate miRNAs in the saliva supernatant of the 30 patients and the 32 healthy volunteers (Figure 1). Of the 384 miRNAs investigated, 130 were upregulated more than 5-fold in patients with PC, among which 111 miRNAs were excluded since the difference was not significant ($P > 0.01$). Among the 19 eligible miRNAs (Figure 2), the top 5 miRNA candidates (miR-17, miR-21, miR-181a, miR-181b, and miR-196a) differentially expressed in the saliva samples of PC patients and healthy volunteers were chosen to be further confirmed in a larger cohort (based on miRWalk database https://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-53325/?query=saliva).

3.2. Relative Expression Levels of miR-17, miR-21, miR-181b, and miR-196a Were Different in Three ZHENGs of Pancreatic Cancer. The relative expression levels of miRNAs in 3 ZHENGs were studied, including Shi-Re ZHENG ($n = 58$), Pi-Xu ZHENG ($n = 50$), and Xue-Yu ZHENG ($n = 45$). Demographic data and clinical information of the participants were summarized in Table 3. The mean relative expression levels of miR-17, miR-21, and miR-181b in the saliva were significantly higher in patients with Shi-Re ZHENG than in patients with the other two ZHENGs ($P < 0.05$), whereas expression level of miR-196a was significantly higher in the Pi-Xu ZHENG group than in other two groups ($P < 0.05$) (Figure 3). The degree of upregulation of miR-196a was approximately 10-fold ($P < 0.05$) (Figure 3). The specific expressions of miR-17, miR-21, and miR-181b were able to distinguish Shi-Re ZHENG from the other two ZHENGs ($P < 0.05$), while Pi-Xu ZHENG was related to the higher expression of miR-196a. This may indicate the different molecular mechanisms of the syndromes in traditional Chinese medicine (ZHENG). The miRNA expression difference in the three different ZHENGs highlights the fact that these miRNAs play an important role, not only in the diseased human physiology but also in different ZHENG condition.

Table 2: The expression level of pancreatic cancer related miRNAs in salivary supernatant.

miRNA	Function	The relative quantitative expression	Fold change	Reference
let-7i-3p	Antioncogene	Down	67.96	[11]
miR-15b	Antioncogene	Down	77.73	[12]
miR-17	Oncogene	Up	425.26	[13]
miR-20a	Oncogene	Up	5.19	[13]
miR-21	Oncogene	Up	388.16	[14]
miR-27a	Oncogene	Up	5.65	[15]
miR-181a	Oncogene	Up	156.43	[16]
miR-181b	Oncogene	Up	362.49	[16]
miR-132	Oncogene	Up	9.415	[17]
miR-155	Oncogene	Up	9.56	[18]
miR-212	Oncogene	Up	11.87	[17]
miR-222	Oncogene	Up	6.38	[16]
miR-200a	Oncogene	Up	10.7	[19]
miR-200b	Oncogene	Up	5.39	[12]
miR-10a	Oncogene	Up	32.82	[20]
miR-190	Oncogene	Up	6.63	[12]
miR-192	Oncogene	Up	6.22	[21]
miR-194	Oncogene	Up	18.2	[22]
miR-196a	Oncogene	Up	6.14	[16]

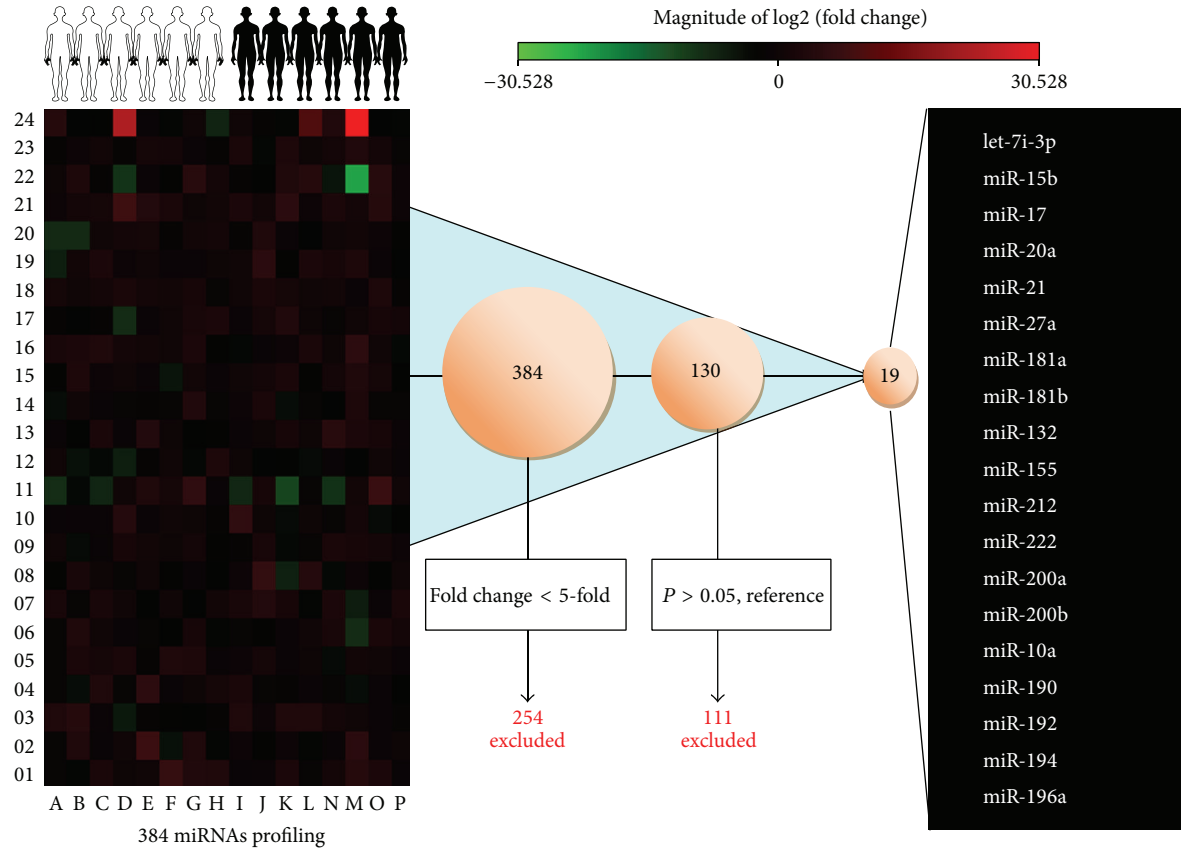


Figure 2: Heat map showing the fold changes in values of the 384 miRNAs in 32 healthy volunteers and 30 PC patients individually, compared with the mean of the healthy volunteers, a panel of the 19 selected miRNAs with proper melting curves, with miRNA values > 5-fold higher ($P < 0.05$) in PC patients than in healthy individuals.

Table 3: Baseline patient characteristics in the 3 groups of ZHENG associated with pancreatic cancer.

Clinical features	153 patients
Sex	
Male	76 (49.7%)
Female	77 (50.3%)
Median age (years)	58.8
Three TCM ZHENGs	
Shi-Re (dampness-heat)	58 (37.9%)
Pi-Xu (spleen-deficiency)	50 (32.7%)
Xue-Yu (blood-stasis)	45 (29.4%)
Treatment	
Cycle of TCM-based therapy	≥2
Median duration (day)	62 (54.1–69.9)
Response	
Disease progression	74 (49.7%)
Grade 3-4 toxicity	36 (24.6%)
Alternative treatment	43 (25.6%)

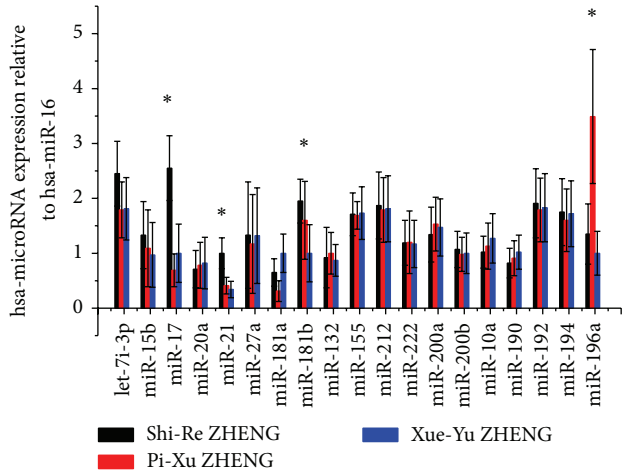


Figure 3: Comparison of differentially expression of 19 miRNAs among 3 ZHENGs of PC (Shi-Re ZHENG, Pi-Xu ZHENG, and Xue-Yu ZHENG) using the qRT-PCR; the bars indicate the fold change which was determined for the ratio of the expression of the PC salivary to the expression of the controls $^{*}P < 0.05$.

3.3. Inter- and Intra-Assay Imprecision. The entire experiment was performed with use of 384-well custom plates, each of which contained probes for the snoRNA/snRNA PCR Controls, C.el-miR-39 Primer, the miRTC oligonucleotide, and Positive PCR Control. All samples demonstrated good RNA recoveries (as measured by C.el-miR-39), with a mean intra-assay coefficient of variation (CV) of 7.2% and a mean interassay CV of 8.4% across all 153 samples and 2 plates. miRTC was used to measure the efficiency of reverse transcription. The mean intraplate CV (2.7%) and interplate CV (4.7%) indicated that the reverse-transcription reactions occurred at equivalent efficiencies across all samples. No template-control reactions and reactions without the reverse transcriptase were performed to determine whether the reverse transcription and PCR reactions worked properly. The melting temperatures of each miRNA were checked for all 153 samples to ensure the specificity of the probe and the mean (SD) melting temperature across all samples.

4. Discussion

In the clinical practice of traditional Chinese medicine (TCM), remedy prescription is made for different ZHENG (also known as syndrome), which is the objective classification of the patients' disease condition. ZHENG is kind of classification based on experience according to the patients conditions, and the prescription of herbs is made for different syndromes of TCM. ZHENG is also a kind of pathology of the disease development of a body in a certain stage. It reflects the nature of pathological change at a certain stage and reveals the intrinsic quality of disease more completely, profoundly, and accurately than symptoms [23]. However, the biomolecular basis of the ZHENG remains unclear. In recent years, TCM has shown certain advantages in treating pancreatic cancer, while the results from most of these studies are difficult to be widely applied due to the lack of enough evidence-based medicine arguments as well as few studies on TCM ZHENG of pancreatic cancer. Compared with biomolecular science and Western medicine, ZHENG in TCM is short of objectivity, accuracy, and reproducibility. Since their discovery in human plasma, extracellular miRNAs have been explored as biomarkers of cancer. Method for miRNA detection in peripheral blood has been well developed, and it is accurate and stable. The value of miRNA as a diagnostic marker has been confirmed due to its highly stable expression in peripheral blood and close association with tumor pathological process, tumor source, and particular functions [10–12, 14].

Several studies have found stable miRNA profiles in bodily fluids, such as saliva, which is a complex liquid that comprises secretions from the major and minor salivary glands. There is also an extensive blood supply to these glands. Molecules present in plasma are also present in saliva, such as DNA, RNA, and proteins [24]. Therefore, tumor-related miRNAs may similarly exist in saliva. There are emerging studies which have demonstrated that cancer-derived exosome-like microvesicles are capable of activating transcription in salivary gland cells and altering the salivary gland cell-derived exosome-like microvesicles [25]. The finding indicated that tumor-derived exosomes could function as the shuttle between the distal tumor and the oral cavity leading to the development of discriminatory salivary biomarkers. In general, the salivary tumor-related miRNAs may come from the blood supplying to salivary glands or the cancer-derived exosome-like microvesicles, which need further studies to be confirmed.

Saliva is a mixture of proteins, DNA, RNA, fatty acids, and a variety of microbes, and it can be the feasible sample for the diagnosis of a variety of diseases. Researchers from Scripps Research Institute of University of Rochester, UCLA, and UCSF analyzed the protein components from parotid

gland, submaxillary gland, and sublingual gland secretions and finally identified a total of 1116 different proteins [26]. They compared these proteins with proteins in blood and tears and finally found some proteins in saliva, matched with proteins in blood, which play a role in Alzheimer's disease, breast cancer, and diabetes. The proteins studied in this work can be used to construct a proteomic map of saliva and also can be used as the biomarkers for PC diagnosis.

In recent years, there are tremendous works of body fluids for the diagnosis of disease. MicroRNA in saliva, as a new diagnostic marker, is also a hot topic. Park et al. [27] reported 50 kinds of miRNAs in the supernatants of saliva from patients with oral squamous cell carcinoma. Further study showed that levels of miR-125a and miR-200a were significantly lower in saliva of patients than in that of healthy subjects. Liu et al. [28] found that expression of miR-31 in supernatant of body fluids from OSCC patients was significantly higher than in that of healthy subjects, and the expression level decreased after tumor resection, which indicates that miRNAs could be used in the diagnosis and prognosis of OSCC. At the same time, researchers have isolated miRNAs from saliva of healthy people and patients with Sjogren's syndrome [29]. Similarly, there could be some miRNAs in saliva, which can reflect the tumorigenesis, development, and metastasis, as well as prognosis of pancreatic cancer. Our study aims to find the new pancreatic cancer biomarkers in saliva by screening differentially expressed miRNAs in pancreatic cancer patients and healthy population and further to identify the specific miRNAs particularly expressed in different ZHENGs of pancreatic cancer. To our knowledge, there were no such studies described the salivary miRNome in the clinical setting of ZHENG before this study.

In this work, we isolated and analyzed 384 miRNAs in the salivary supernatant from patients with PC and healthy controls; 19 miRNAs were identified to be differentially expressed between patients with PC ($n = 30$) and healthy controls ($n = 32$). This is in agreement with previously published microRNA profiling studies, most of which have shown that microRNA expression in serum or tumor samples seems globally higher than in normal samples [11–13, 15–22]. Interestingly, the differentially expressed miRNAs described here (including let-7, miR-21, and miR-181a) are similar to those previously published microRNA profiling studies. However, some of the differentially expressed microRNAs described in this study are not consistent with the reported study [30]. This discrepancy could be due to the patient individual differences, RNA degradation due to salivary amylase, and the influence of salivary collection method. Our results indicate that differentially expressed miRNAs may play an important role in development and progression of PC by deregulating specific target oncogenes or tumor suppressors. Furthermore, the same miRNA may play opposite roles in tumor pathogenesis, that is, as an oncogene in certain cancers and as a tumor suppressor in others [31]. Therefore, targets of most of the miRNAs including their pathways and functions still remain to be studied urgently. The sample size for our microarray analysis is relatively small. However, this is not like those studies of mRNA gene marker screening, which may need large sample size to obtain reliable profile. In this work, a limited number of microRNAs were studied.

Nineteen miRNAs were chosen for the further analysis in another 153 patients. There were significant differences in the expression of 4 microRNAs (miR-17, miR-21, miR-181b, and miR-196a) in three patient groups with different ZHENG. These results suggest that microRNAs may play an important role in the molecular pathogenesis of PC and could be included as one of the indicators for distinguishing the syndromes of traditional Chinese medicine (ZHENG). No such data was reported concerning the miR-17 miR-21, miR-181b, and miR-196a and their relevance to clinicopathologic behavior and syndromes of traditional Chinese medicine in patients with PC previously.

However, some studies have demonstrated a link between these 4 miRNAs and the prognosis in pancreatic cancer. MiR-17-5p is reported to be overexpressed in pancreatic cancer, and it plays an important role in carcinogenesis and cancer progression [32]. Serum exosomal miR-17 was higher in PC patients than in non-PC patients and healthy participants. High levels of miR-17 were significantly correlated with metastasis and advanced stage of PC [33]. The former studies have identified miR-21 as overexpressed in early pancreatic cancer lesions, pancreatic tumors, and pancreatic cancer-derived cell lines [34]. miR-21 is one of the most cited miRNAs and has emerged as the miRNA most frequently associated with poor outcome in cancer [35]. MiR-21 and miR-196a were overexpressed in pancreatic carcinomas compared to benign aspirates [36]. Some study suggested that the expression of miR-181b was higher in cell lines of pancreatic cancer, such as BxPC3, Panc1, and PSN1 cells [37]. In brief, these 4 miRNAs may play a role of oncogene in pancreatic cancer and it may become a new early diagnosis mark and therapy target of pancreatic cancer. Unlike miR-17 and miR-21, which are all deregulated in the same direction in a variety of cancers [13, 14], members of the miR-181 family are upregulated in some cancers and downregulated in others [38–41]. It has been reported that miR-181 downregulates the homeobox protein Hox-A11, which acts as a suppressor in differentiation process, and thus a functional link between miR-181 family and the complicated process of differentiation was established. Based on this, the discrepancies could also be due to microRNA target differences in different tumor differentiation stage and microenvironment. However, the precise targets of miRNA in patients with various ZHENGs remain unclear at present, and how miRNA is involved in tumor formation and affects the prognosis also remains unclear.

This study mainly focuses on the investigation of searching for the correlation between the expression of miRNAs in saliva and the syndromes. At the same time, we hoped to find the discriminatory salivary biomarkers, which could be readily detected upon the development of pancreatic cancer. The main purpose of this paper is exploring the relationship between miRNAs in salivary supernatant and the syndromes, making clinical syndrome differentiation and curative effect assessment for the pancreatic cancer more objective and standardized.

5. Conclusions

In summary, miRNAs in salivary supernatant of PC patients were studied and 4 miRNAs were identified, that is, miR-17, miR-21, miR-181b, and miR-196a, as sensitive indicators for treatment based on syndrome differentiation. However, there are some limitations in this work. Firstly, the sample size of this study is relatively small, and therefore, to confirm the results, further study with larger sample size is required. Secondly, although salivary supernatant from healthy people is used as control in gene profiling analysis, oral foreign body, such as food debris, may contaminate the results by changing the ingredient and percentage of miRNA. This may contribute to controversial reports on miRNA expression in cancer. Thirdly, the precise roles of these 4 miRNAs (miR-17, miR-21, miR-181b, and miR-196a) and their target mRNAs regulation in PC progression remain unclear. Further in vitro and in vivo studies should be performed to enrich the knowledge of this nascent field. Nevertheless, our results indicate that these 4 miRNAs may be good candidate molecular markers in ZHENG differentiation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study was supported by Grants of Chinese Integrative Medicine Science Fund of Shanghai (no. ZXYK-1214), Shanghai TCM Development Action Plan for the Research Projects (ZYSNXD-CC-ZDYJ037), and National Natural Science Foundation of China (no. 81273953).

References

[1] Z. Chen and P. Wang, "Clinical distribution and molecular basis of traditional Chinese medicine ZHENG in cancer," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 783923, 8 pages, 2012.

[2] W. Zhang, J. E. Dahlberg, and W. Tam, "MicroRNAs in tumorigenesis: a primer," *The American Journal of Pathology*, vol. 171, no. 3, pp. 728–738, 2007.

[3] A. Y. Nikitin and D. C. Corney, "MicroRNA and ovarian cancer," *Histology and Histopathology*, vol. 23, no. 9, pp. 1161–1169, 2008.

[4] L. Zhang, S. Volinia, M. J. Birrer et al., "Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 19, pp. 7004–7009, 2008.

[5] A. Giannakakis, G. Coukos, A. Hatzigeorgiou, R. Sandaltzopoulos, and L. Zhang, "miRNA genetic alterations in human cancers," *Expert Opinion on Biological Therapy*, vol. 7, no. 9, pp. 1375–1386, 2007.

[6] N. Yang, G. Coukos, and L. Zhang, "MicroRNA epigenetic alterations in human cancer: one step forward in diagnosis and treatment," *International Journal of Cancer*, vol. 122, no. 5, pp. 963–968, 2008.

[7] F. J. Slack and J. B. Weidhaas, "MicroRNA in cancer prognosis," *The New England Journal of Medicine*, vol. 359, no. 25, pp. 2720–2722, 2008.

[8] S. Deng, G. A. Calin, C. M. Croce, G. Coukos, and L. Zhang, "Mechanisms of microRNA deregulation in human cancer," *Cell Cycle*, vol. 7, no. 17, pp. 2643–2646, 2008.

[9] K. E. Resnick, H. Alder, J. P. Hagan, D. L. Richardson, C. M. Croce, and D. E. Cohn, "The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform," *Gynecologic Oncology*, vol. 112, no. 1, pp. 55–59, 2009.

[10] S.-L. Yu, H.-Y. Chen, G.-C. Chang et al., "MicroRNA signature predicts survival and relapse in lung cancer," *Cancer Cell*, vol. 13, no. 1, pp. 48–57, 2008.

[11] J. Torrisani, B. Bournet, M. C. Du Rieu et al., "Let-7 microRNA transfer in pancreatic cancer-derived cells inhibits in vitro cell proliferation but fails to alter tumor progression," *Human Gene Therapy*, vol. 20, no. 8, pp. 831–844, 2009.

[12] Y. Zhang, M. Li, H. Wang et al., "Profiling of 95 MicroRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis," *World Journal of Surgery*, vol. 33, no. 4, pp. 698–709, 2009.

[13] S. Volinia, G. A. Calin, C. G. Liu et al., "A microRNA expression signature of human solid tumors defines cancer gene targets," *Proceeding of the National Academy of Sciences of the USA*, vol. 103, no. 7, pp. 2257–2261, 2006.

[14] T. Moriyama, K. Ohuchida, K. Mizumoto et al., "MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance," *Molecular Cancer Therapeutics*, vol. 8, no. 5, pp. 1067–1074, 2009.

[15] Y. Ma, S. Yu, W. Zhao, Z. Lu, and J. Chen, "MiR-27 a regulates the growth, colony formation and migration of pancreatic cancer cells by targeting Sprouty2," *Cancer Letters*, vol. 298, no. 2, pp. 150–158, 2010.

[16] X. Kong, Y. Du, G. Wang et al., "Detection of differentially expressed microRNAs in serum of pancreatic ductal adenocarcinoma patients: miR-196a could be a potential marker for poor prognosis," *Digestive Diseases and Sciences*, vol. 56, no. 2, pp. 602–609, 2011.

[17] J. Park, J. C. Henry, J. Jiang et al., "MiR-132 and miR-212 are increased in pancreatic cancer and target the retinoblastoma tumor suppressor," *Biochemical and Biophysical Research Communications*, vol. 406, no. 4, pp. 518–523, 2011.

[18] M. Gironella, M. Seux, M. Xie et al., "Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 41, pp. 16170–16175, 2007.

[19] A. Li, N. Omura, S. M. Hong et al., "Pancreatic cancers epigenetically silence SIP1 and hypomethylate and overexpress miR-200a/200b in association with elevated circulating miR-200a and miR-200b levels," *Cancer Research*, vol. 70, no. 13, pp. 5226–5237, 2010.

[20] F. U. Weiss, I. J. Marques, J. M. Woltering et al., "Retinoic acid receptor antagonists inhibit miR-10a expression and block metastatic behavior of pancreatic cancer," *Gastroenterology*, vol. 137, no. 6, pp. 2136–2145, 2009.

[21] M. H. Zhu, C. Y. Zhao, M. Shi et al., "Pancreatic cancer specific microRNA as serological markers, Chinese Medical Association branch pathology," in *Academic Conference Proceedings*, pp. 128–213, 2010.

[22] S. T. Mees, W. A. Mardin, C. Wendel et al., "EP300—a miRNA-regulated metastasis suppressor gene in ductal adenocarcinomas of the pancreas," *International Journal of Cancer*, vol. 126, no. 1, pp. 114–124, 2010.

[23] Z. Chen, L. Chen, P. Wang, H. Dai, S. Gao, and K. Wang, "Tumor microenvironment varies under different TCM ZHENG models and correlates with treatment response to herbal medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 635702, 10 pages, 2012.

[24] Y. Lee and D. T. Wong, "Saliva: an emerging biofluid for early detection of diseases," *The American Journal of Dentistry*, vol. 22, no. 4, pp. 241–248, 2009.

[25] C. S. Lau and D. T. W. Wong, "Breast cancer exosome-like microvesicles and salivary gland cells interplay alters salivary gland cell-derived exosome-like microvesicles in vitro," *PLoS ONE*, vol. 7, no. 3, Article ID e33037, 9 pages, 2012.

[26] P. Denny, F. K. Hagen, M. Hardt et al., "The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions," *Journal of Proteome Research*, vol. 7, no. 5, pp. 1994–2006, 2008.

[27] N. J. Park, H. Zhou, D. Elashoff et al., "Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection," *Clinical Cancer Research*, vol. 15, no. 17, pp. 5473–5477, 2009.

[28] C.-J. Liu, S.-Y. Kao, H.-F. Tu, M.-M. Tsai, K.-W. Chang, and S.-C. Lin, "Increase of microRNA miR-31 level in plasma could be a potential marker of oral cancer," *Oral Diseases*, vol. 16, no. 4, pp. 360–364, 2010.

[29] A. Michael, S. D. Bajracharya, P. S. T. Yuen et al., "Exosomes from human saliva as a source of microRNA biomarkers," *Oral Diseases*, vol. 16, no. 1, pp. 34–38, 2010.

[30] N. Yanaihara, N. Caplen, E. Bowman et al., "Unique microRNA molecular profiles in lung cancer diagnosis and prognosis," *Cancer Cell*, vol. 9, no. 3, pp. 189–198, 2006.

[31] W. Gao, Y. Yu, H. Cao et al., "Deregulated expression of miR-21, miR-143 and miR-181a in non small cell lung cancer is related to clinicopathologic characteristics or patient prognosis," *Biomedicine and Pharmacotherapy*, vol. 64, no. 6, pp. 399–408, 2010.

[32] H. J. Yan, W. S. Liu, W. H. Sun et al., "MiR-17-5p inhibitor enhances chemosensitivity to gemcitabine via upregulating Bim expression in pancreatic cancer cells," *Digestive Diseases and Sciences*, vol. 57, no. 12, pp. 3160–3167, 2012.

[33] R. Que, G. Ding, J. Chen, and L. Cao, "Analysis of serum exosomal microRNAs and clinicopathologic features of patients with pancreatic adenocarcinoma," *World Journal of Surgical Oncology*, vol. 11, article 219, 2013.

[34] M. C. Du Rieu, J. Torrisani, J. Selves et al., "MicroRNA-21 is induced early in pancreatic ductal adenocarcinoma precursor lesions," *Clinical Chemistry*, vol. 56, no. 4, pp. 603–612, 2010.

[35] V. S. Nair, L. S. Maeda, and J. P. A. Ioannidis, "Clinical outcome prediction by microRNAs in human cancer: a systematic review," *Journal of the National Cancer Institute*, vol. 104, no. 7, pp. 528–540, 2012.

[36] N. C. Panarelli, Y. T. Chen, X. K. Zhou, N. Kitabayashi, and R. K. Yantiss, "MicroRNA expression aids the preoperative diagnosis of pancreatic ductal adenocarcinoma," *Pancreas*, vol. 41, no. 5, pp. 685–690, 2012.

[37] D. Takiuchi, H. Eguchi, H. Nagano et al., "Involvement of microRNA-181b in the gemcitabine resistance of pancreatic cancer cells," *Pancreatology*, vol. 13, no. 5, pp. 517–523, 2013.

[38] L. Shi, Z. Cheng, J. Zhang et al., "hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells," *Brain Research*, vol. 1236, pp. 185–193, 2008.

[39] A. Bottoni, M. C. Zatelli, M. Ferrcin et al., "Identification of differentially expressed microRNAs by microarray: a possible role for microRNA genes in pituitary adenomas," *Journal of Cellular Physiology*, vol. 210, no. 2, pp. 370–377, 2007.

[40] R. Visone, L. Z. Rassenti, A. Veronese et al., "Karyotype-specific microRNA signature in chronic lymphocytic leukemia," *Blood*, vol. 114, no. 18, pp. 3872–3879, 2009.

[41] I. Naguibneva, M. Ameyar-Zazoua, A. Polesskaya et al., "The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation," *Nature Cell Biology*, vol. 8, no. 3, pp. 278–284, 2006.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 751392, 33 pages
http://dx.doi.org/10.1155/2014/751392

Review Article

The Role of Chemoattractant Receptors in Shaping the Tumor Microenvironment

Jiamin Zhou,[1,2] Yi Xiang,[1,3] Teizo Yoshimura,[1] Keqiang Chen,[1] Wanghua Gong,[4] Jian Huang,[5] Ye Zhou,[6] Xiaohong Yao,[5] Xiuwu Bian,[5] and Ji Ming Wang[1]

[1] *Laboratory of Molecular Immunoregulation, Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702, USA*
[2] *Endoscopic Center, Zhongshan Hospital, Fudan University, Shanghai 200032, China*
[3] *Department of Pulmonary Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China*
[4] *Basic Research Program, Leidos Biomedical Research, Inc., Frederick, MD 21702, USA*
[5] *Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing 400038, China*
[6] *Department of Gastric Cancer and Soft Tissue Surgery, Fudan University Cancer Center, Shanghai 200032, China*

Correspondence should be addressed to Ji Ming Wang; wangji@mail.nih.gov

Received 30 April 2014; Accepted 17 June 2014; Published 10 July 2014

Academic Editor: Zhen Chen

Chemoattractant receptors are a family of seven transmembrane G protein coupled receptors (GPCRs) initially found to mediate the chemotaxis and activation of immune cells. During the past decades, the functions of these GPCRs have been discovered to not only regulate leukocyte trafficking and promote immune responses, but also play important roles in homeostasis, development, angiogenesis, and tumor progression. Accumulating evidence indicates that chemoattractant GPCRs and their ligands promote the progression of malignant tumors based on their capacity to orchestrate the infiltration of the tumor microenvironment by immune cells, endothelial cells, fibroblasts, and mesenchymal cells. This facilitates the interaction of tumor cells with host cells, tumor cells with tumor cells, and host cells with host cells to provide a basis for the expansion of established tumors and development of distant metastasis. In addition, many malignant tumors of the nonhematopoietic origin express multiple chemoattractant GPCRs that increase the invasiveness and metastasis of tumor cells. Therefore, GPCRs and their ligands constitute targets for the development of novel antitumor therapeutics.

1. Introduction

Chemoattractant receptors are a family of G protein coupled seven transmembrane cell surface receptors (GPCRs). According to their source of ligands and expression patterns, the family members are categorized into classical GPCRs and chemokine GPCRs. The former include formyl peptide receptor and its variants (FPR1, FPR2, and FPR3), platelet activating factor receptor (PAFR), activated complement component 5a receptor (C5aR), and leukotriene B4 receptor and its variants (BLT1 and BLT2). Chemokine GPCRs are composed of four subfamilies based on the conserved N-terminal cysteine residues in the mature proteins of the ligands, CC-, CXC-, CX3C-, and C-, and thus are termed CCR, CXCR, CX3CR, and XCR, respectively. So far, approximately 50 chemokines and at least 18 chemokine GPCRs have been identified [1] (Table 1). Promiscuity is a characteristic of GPCRs and their ligands. Some chemoattractants bind to more than one GPCR. Conversely, some GPCRs display overlapping ligand specificities with variable affinity and functions [2]. Although chemoattractant GPCRs are mainly expressed by leukocytes and their major function has been considered as mediators of leukocyte trafficking and homing, over the past two decades, the role of GPCRs and their ligands in tumor progression began to be increasingly recognized. The expression of some GPCRs or ligands in tumor tissues has been shown to be correlated with the therapeutic outcome of tumor patients [3–10]. It is undeniable that tumor cells are one of the major sources of chemoattractants in tumor tissues and many tumor cells express one or more chemoattractant GPCRs to their advantage [11]. In addition, tumor-derived

Table 1: Chemoattractant GPCRs and ligands.

	Expression	Ligands	Functions	References
"Classical"				
FPR				
FPR1	Myeloid cells, lymphocytes Tumor cells	Bacteria and host derived peptides	Chemotaxis and activation Tumor growth, invasion, angiogenesis	[12, 13]
FPR2	Myeloid cells Tumor cells	Bacteria and host derived peptides	Chemotaxis and activation Antitumor defense, tumor invasion	[13]
FPR3	Monocytes, dendritic cells Tumor cells	Synthetic and host derived peptides	Chemotaxis and activation Tumor invasion	[13]
PAFR	Macrophages, polymorphonuclear leucocytes, and various tissue cells Tumor cells	PAF	Chemotaxis and activation Tumor growth and metastasis; inhibiting tumor angiogenesis	[14, 15]
C5aR	Neutrophils, monocytes, eosinophils, basophils, dendritic cells, mast cells, and various nonimmune cells Tumor cells	C5a	Chemotaxis and activation Tumor metastasis; opposite function in angiogenesis	[16–18]
LTB4R				
BLT1	Neutrophils, macrophages, T lymphocytes Tumor cells	LTB4	Chemotaxis and activation Tumor growth	[19]
BLT2	Most human tissues cells and leukocytes Tumor cells	LTB4	Chemotaxis and activation Tumor growth, metastasis	[19]
"Chemokine"				
CCR				
CCR1	Monocytes, neutrophils, T lymphocytes, dendritic cells Tumor cells	CCL3/4/6/7/8/9/10/14/15/16/23	Chemotaxis and activation Tumor growth, metastasis, angiogenesis	[20]
CCR2	Monocytes, basophils, T lymphocytes, dendritic cells, NK cells, endothelial cells Tumor cells	CCL2/7/8/11/13/16	Chemotaxis and activation Tumor growth, metastasis, angiogenesis	[21, 22]
CCR3	Eosinophils, basophils, Th2 lymphocytes, mast cells Tumor cells	CCL7/11/13/15/24/26/28	Chemotaxis and activation Tumor growth, metastasis	[23]
CCR4	Macrophages, monocytes, basophils, T and B lymphocytes, dendritic cells, NK cells, mast cells, platelets Tumor cells	CCL2/4/5/17/22	Chemotaxis and activation Tumor growth, metastasis, angiogenesis	[24]
CCR5	Macrophages, T lymphocytes, dendritic cells, NK cells Tumor cells	CCL3/4/5/7/11/13/16	Chemotaxis and activation Tumor growth, metastasis, angiogenesis	[25, 26]
CCR6	Neutrophils, T and B lymphocytes, dendritic cells, epithelial cells of some tissues Tumor cells	CCL20	Chemotaxis and activation Tumor growth, metastasis	[27, 28]
CCR7	T and B lymphocytes, dendritic cells Tumor cells	CCL19/21	Lymphoid tissue chemotaxis and activation Tumor growth, metastasis	[29, 30]
CCR8	Macrophages, Th2 lymphocytes, endothelial cells Tumor cells	CCL1/16	Chemotaxis and activation Tumor metastasis	[31, 32]
CCR9	T lymphocytes Tumor cells	CCL25	Small intestinal specific chemotaxis and activation Tumor growth, metastasis; inhibiting tumor metastasis in some tumors	[33, 34]

Table 1: Continued.

	Expression	Ligands	Functions	References
CCR10	T lymphocytes Tumor cells	CCL27/28	Skin-specific chemotaxis and activation Tumor growth, metastasis, angiogenesis	[35, 36]
CXCR				
CXCR1	Neutrophils, polymorphonuclear leukocytes, endothelial cells Tumor cells	CXCL6/8	Chemotaxis and activation Tumor growth, metastasis, angiogenesis	[37–39]
CXCR2	Neutrophils, basophils, T lymphocytes, oligodendrocytes, endothelial cells Tumor cells	CXCL1/2/3/5/6/8	Chemotaxis and activation Tumor growth, metastasis, angiogenesis	[40, 41]
CXCR3	Macrophages, T lymphocytes, NK cells, NKT cells, endothelial cells Tumor cells	CXCL4/9/10/11	Chemotaxis and activation Two variants CXCR3-A and CXCR3-B have opposite function in tumor progression	[42, 43]
CXCR4	Numerous cell types: hematopoietic cells and stem cells Tumor cells	CXCL12	Chemotaxis and activation Maintenance of stem phenotype Tumor growth, metastasis, angiogenesis	[1, 44]
CXCR5	T and B lymphocytes Tumor cells	CXCL13	Chemotaxis and activation Tumor growth, metastasis; inhibiting tumor metastasis in some tumors	[45, 46]
CXCR6	T and B lymphocytes, NK cells, NKT cells, plasma cells Tumor cells	CXCL16	Chemotaxis and activation Tumor growth, metastasis, angiogenesis; inhibiting tumor migration in some tumors	[47]
CXCR7	T and B lymphocytes, dendritic cells, endothelial cells, fetal hepatocytes Tumor cells	CXCL11/12	Chemotaxis and activation Tumor growth, metastasis, angiogenesis; assisting with CXCR4 to regulate tumor progression	[48–50]
CX3CR				
CX3CR1	Monocytes, T and B lymphocytes, mast cells, dendritic cells, NK cells Tumor cells	CX3CL1	Chemotaxis and activation Tumor growth, metastasis; inhibiting tumor invasion in some tumors	[51, 52]
XCR				
XCR1	Neutrophils, T lymphocytes, dendritic cells Tumor cells	XCL1/2	Chemotaxis and activation Tumor cell growth, metastasis	[53]

chemoattractants are mediators of leukocyte, in particular macrophage (tumor-associated macrophages, TAMs), infiltration that may result in the persistence of chronic inflammation in the tumor microenvironment together with a vigorous angiogenesis. Therefore, chemoattractant GPCRs are believed to play a crucial role in tumor progression via signaling based on dissociation of trimeric G proteins in response to ligands binding culminating in cell chemotaxis, invasion, production of mediators promoting angiogenesis, transactivation of growth factor receptors, such as epidermal growth factor receptor (EGFR), and tumor cell metastasis. (Figure 1 shows the signaling.)

A tumor has been recognized as a complicated "organ," other than a simple collection of relatively homogeneous cancer cells, whose entire biology could be understood by elucidating the autonomous properties of these cells. In contrast, various types of host cells are known to contribute in important ways to the biology of tumors, including endothelial cells (ECs), pericytes, immune cells, cancer-associated fibroblasts (CAFs), and stem and progenitor cells of the tumor stroma [54]. The interaction between these cells and their secreting factors results in an environment which markedly affects tumor progression. (Figure 2 shows the tumor.) Therefore, understanding the contribution of GPCRs and their ligands to the complexity of the tumor microenvironment is critical for the identification of novel therapeutic targets.

2. GPCRs in Recruiting Tumor-Associated Immune Cells

The infiltration of immune cells is a characteristic of the tumor microenvironment, which is the basis for the presence of chronic inflammation. Chemoattractants are characterized

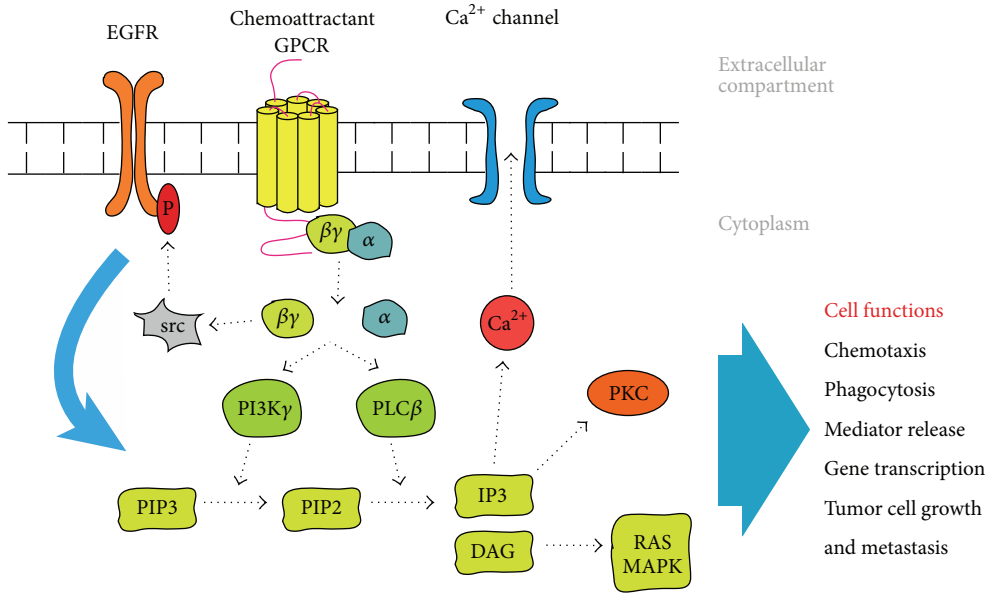


Figure 1: The signaling pathway of chemoattractant GPCRs. Chemoattractant GPCRs activated by ligands elicit a cascade of signal transduction pathways involving G proteins, phospholipase C (PLC), phosphoinositide (PI) 3 kinases, protein kinase C (PKC), Ca^{2+}, RAS, and MAPKs to mediate leukocyte migration and activation. Chemoattractant GPCRs also play a crucial role in tumor progression upon activation by their ligands culminating in cell chemotaxis, invasion, production of mediators promoting angiogenesis, and transactivation of EGFR.

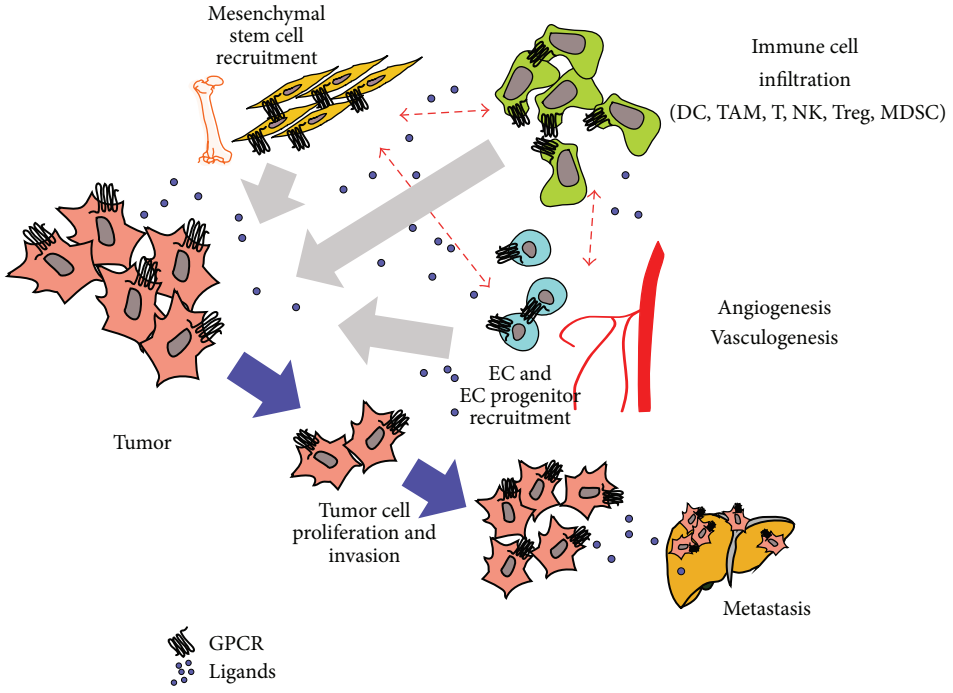


Figure 2: Chemoattractant GPCRs in tumor microenvironment. A tumor has been recognized as a complicated "organ." Various types of tumor and host cells, including immune cells, fibroblasts, endothelial cells, and progenitor cells of the tumor stroma, contribute to the tumor development, growth, metastasis, immune escape, and neovascularization.

by their ability to induce directional migration and activation of leukocytes by stimulating specific GPCRs [2]. (Figure 1 shows the signaling.) The infiltrating immune cells play an important role in shaping a tumor-promoting or tumor-suppressive microenvironment [55, 56].

2.1. Tumor Infiltrating Tumor Suppressive Immune Cells. In general, infiltration of antigen presenting dendritic cells (DCs) into the tumor represents an early tumor-triggered host immune response. In hepatocellular carcinoma (HCC), tumor infiltrating DCs express the chemokine GPCRs, CCR1

and CCR5. Tumor cell apoptosis induced by suicide genes increases the number of DCs migrating into the draining lymph nodes to generate a specific cytotoxic cell population against HCC cells [57], although apoptotic tumor cells are also believed to generate tolerogenic DCs. In addition to CCR1 and CCR5, CCR6 is also commonly expressed by circulating immature DCs (iDCs). In melanoma, the profiling of GPCRs expressed by plasmacytoid DCs (pDCs) showed that the only significantly elevated GPCR is CCR6, which mediates the recruitment of pDCs from blood by the chemokine ligand CCL20 produced by melanoma cells [58]. Other immune cells in tumor microenvironment may also promote DC recruitment, such as Th9 cells, which increase DC infiltration of the tumor mediated by CCR6/CCL20 interaction that generates CD8(+) cytotoxic T lymphocyte (CTLs) responses and inhibit tumor growth [59]. After capturing antigens, DCs undergo maturation and express high levels of the chemokine GPCR CCR7 that enables DCs to migrate to T cell zones in the draining lymph nodes that produce the CCR7 ligands, CCL19 and CCL21. However, the results of interaction between DCs and tumor cells could be multifaceted based on CCR7/CCL19 or CCR7/CCL21 interaction [60, 61]. These chemokines may decide the distribution of immature or mature DCs within tumor tissues and generate opposing immunological consequences. For example, in renal cell carcinoma, tumor cells secrete CCL20 to recruit CCR6(+) immature DCs that mostly elicit tolerance, while, in the tumor invasion margin, only CCL19 and CCL21 are detected and they recruit CCR7(+) mature DCs as well as CCR7(+) T cells to form clusters that provide local foci of antitumor immune responses [62].

In addition to T cells, DCs may also cooperate with other immune competent cells, such as nature killer (NK) cells, to enhance antitumor effect. TLR9-activated pDCs could induce CTLs cross primed against multiple B16 tumor antigens, which is completely dependent on early recruitment and activation of NK cells. CCR5 expressing NK cells are recruited by CCL3, CCL4, and CCL5 secreted by pDCs, while IFN-γ was produced by NK cells stimulated by OX40L expressed on pDCs [63]. Conversely, IL-18-primed NK cells produce high levels of the iDC-attracting chemokines CCL3 and CCL4 to recruit iDCs in a CCR5-dependent manner and induce the production of CXCR3 and CCR5 ligands, CXCL9, CXCL10, and CCL5, by iDCs to facilitate the subsequent recruitment of CD8(+) T cells [64]. In breast cancer, NK cells take advantage of their own production of IFN-γ to enhance the secretion of chemokines CXCL9, CXCL10, and CXCL11 by tumor cells, which in turn accelerate the infiltration of CXCR3 expressing NK cells into the tumor site [65]. Hence, a positive feedback of DCs, NK cells, and tumor cells may result in the enhancement of antitumor immune responses. In addition, CCR5 and CXCR3 expressing CD8(+) T cells recruited by DCs are predominantly of the Th1 type that executes antitumor effect and colocalizes with macrophages and neutrophils to amplify the cell-mediated immune responses [56].

2.2. Tumor Infiltrating Immune Suppressive Cells. Immune suppressive cells recruited into tumor microenvironment subvert the host defense and create a microenvironment favoring tumor escape. These cells include myeloid-derived suppressor cells (MDSCs), TAMs, and regulatory CD4(+) T cells (Tregs). For example, in a melanoma model, when CTLs are injected intravenously into tumor-bearing mice, the cells are detected in the tumor as early as on day 1, peaking on day 3, and inhibit tumor growth. However, the antitumor effect is soon diminished with accumulation of MDSCs in the tumor, which outnumber CTLs by day 5. MDSCs produce nitric oxide, arginase I, and reactive oxygen species that inhibit the proliferation of antigen-specific CD8(+) T cells and reduce tumor cell killing. In CCR2−/− mice, the accumulation of MDSCs is significantly reduced, indicating that MDSC infiltration in the tumor is dependent on the chemokine GPCR CCR2 and its ligands, mainly CCL2 produced in the tumor [66].

Different T cell types appear in tumors at different stages of progression. In human ovarian cancer, recruitment of high numbers of Th1 cells was observed in stage II tumors, whereas activated Tregs along with high numbers of monocytes/macrophages and myeloid DCs (mDCs) were observed in disseminated tumors (stages III-IV). All tumor cells, monocytes/macrophages, and mDCs produce CCL22 to recruit Tregs via the GPCR CCR4. The specific recruitment of Tregs results in immune suppression in the advanced stages of ovarian cancer [67]. The paradox that early stage tumors are inhibited by infiltrating antitumor immune cells which is reversed by suppressive Tregs through CCR4/CCL22 interaction is also observed in myeloma [68]. Thus, chemokines and GPCRs play a crucial role in regulating pro- and antitumor responses by recruiting different types of immune cells (Table 2).

2.2.1. Tregs. Treg is a CD4(+)CD25(+)FoxP3(+) T cell subtype. Treg expresses chemokine GPCR CCR4 and responds to the ligands CCL1 and CCL22 to accumulate in tumors. The degree of Treg infiltration is correlated with the prognosis of tumor patients [108, 110, 135]. A similar prognostic value was also obtained by the ratio of CD8(+) T cell/CCR4(+) Treg [136]. In melanoma, deletion of CD45RA(−)FoxP3(hi)CD4(+) Tregs (effector Tregs) using anti-CCR4 antibody significantly augmented CD8(+) T cell infiltration in the tumor and unmasked a nascent antitumor host response [137]. The recruitment of Tregs into the tumor microenvironment depended on the presence of CD8(+) T cells that produce ligands for CCR4 [138]. Therefore, the balance of infiltrating CCR4(+) Tregs and CD8(+) T cells in tumor tends to be a seesaw. Tregs can also interact with other cells in the tumor microenvironment. For instance, in a highly metastatic breast cancer model, only a proportion of CCR4(+) tumor cells in the primary tumor establish lung metastasis. Implanted orthotopic primary tumors "remotely" stimulate the expression of CCL17 and CCL22 in the lungs, which attract both CCR4(+) Tregs and tumor cells. CCR4(+) Tregs protect CCR4(+) tumor cells from being attacked by antitumor host immune cells. In fact, in the absence of CCR4(+) Tregs, CCR4(+) tumor cells disseminated into the lung are efficiently eliminated by NK cells, because CCR4(+) Tregs directly kill NK cells using beta-galactoside-binding protein [139]. Interestingly, in return, NK cells themselves

Table 2: Chemoattractant GPCRs associated with stromal cell infiltration.

	GPCRs	Tumor types
Immune cells		
Dendritic cells	CCR1	Hepatocellular carcinoma [57]
	CCR5	Hepatocellular carcinoma [57], ovarian cancer [69]
	CCR6	Breast cancer [70, 71], colorectal cancer and lung cancer [72], lymphoma [73], melanoma [58, 72], lymphocyte-rich gastric cancer [74], renal cell carcinoma [62], thyroid cancer [75]
	CCR7	Breast cancer [76], renal cell carcinoma [62]
	CXCR1/2	Colorectal cancer [77–79], gastric cancer [79], hepatocellular carcinoma [78], pancreatic cancer [78]
Myeloid-derived suppressor cells	CCR2	Basal cell carcinomas [80], melanoma [66]
	CXCR2	Colitis-associated cancer [81]
	CXCR4	Gastric cancer [82], ovarian cancer [83]
Tumor-associated macrophages	PAFR	Melanoma [84]
	CCR2	Breast cancer [85], cervical cancer [86], colitis-associated cancer [87], lymphoma [88], nasopharyngeal carcinoma [89], oral cancer [90], prostate cancer [91], pancreatic cancer [92], thyroid cancer [93]
	CCR4	Lung cancer [94]
	CCR5	Hepatocellular carcinoma [95], nasopharyngeal carcinoma [89]
	CXCR3	Breast cancer [42]
	CX3CR1	Breast cancer [96], glioma [97]
Regulatory T cells	CCR4	Breast cancer [98], cervical cancer [99], Hodgkin lymphoma [100], gastric cancer [101], glioma [102], melanoma [103]
	CCR5	Colorectal cancer [104], lymphoma [105], pancreatic cancer [106], renal cell carcinoma [107]
	CCR6	Breast cancer [108], colorectal cancer [109], hepatocellular carcinoma [110], Hodgkin lymphoma [111], renal cell carcinoma [107]
	CCR7	Melanoma [112], ovarian cancer [113]
	CCR10	Ovarian cancer [35]
	CXCR1	Lung cancer, mesothelioma, melanoma [114]
	CXCR3	Renal cell carcinoma [107]
	CXCR4	Breast cancer [115], B cell lymphoma [116], hepatocellular carcinoma [117], lung cancer [118], glioma [119], ovarian cancer [120, 121]
	CXCR6	Nasopharyngeal carcinoma [122], renal cell carcinoma [107]
Stromal cells		
Mesenchymal stem cells	FPR2	Ovarian tumor [123]
	CCR2	Breast cancer [124], glioma [125], lymphoma [88]
	CXCR1	Glioma [126, 127]
	CXCR2	Kidney cancer [128], glioma [127]
	CXCR4	Breast cancer [129], gastric cancer [130], glioma [125, 131, 132]
	CXCR6	Glioma [132], prostate cancer [133]
	CX3CR1	Colorectal cancer [134]

also may attract Tregs through the CCR4/CCL22 interaction. In a Lewis lung cancer (LLC) implantation model, mouse lungs bearing LLC secrete CCL22 to recruit Tregs to suppress the proliferation of endogenous CD4(+)CD25(−) cells and the only cell type in the lung to produce CCL22 is NK cells [140]. CCR4/CCL22 even induces Tregs to selectively infiltrate into a particular site in the tumor, such as the area of lymphoid aggregates where Tregs are activated and proliferate in response to tumor-associated antigens presented by DCs. However, this process does not occur in the tumor bed [98, 141]. In addition, there are other GPCRs and ligands that may recruit Tregs, such as CCR5/CCL5 in colorectal cancer (CRC) and pancreatic cancer [104, 106], CCR6/CCL20 in HCC and breast cancer [108, 110], and CCR10/CCL28 in ovarian cancer [35], while CXCR3 and CXCR6 are expressed by Tregs infiltrating renal cell carcinoma [107]. Since Tregs are believed to be one of the major suppressive host cells that interfere with antitumor immune response, targeting

GPCRs should be one of the effective measures to diminish Treg infiltration of the tumor environment thereby restoring tumor immunity.

2.2.2. TAMs. In addition to the complicated interaction between Tregs and other tumor suppressing immune cells in the microenvironment, there are also other tumor supporting immune cells as important constituents. In a mouse CRC model, CCR6(+) Tregs are recruited into the tumor by responding to CCL20 secreted not only by tumor cells but also by TAMs. After targeted deletion of TAMs, Treg recruitment was abrogated with reduced tumor growth [109].

Macrophages are a major tumor infiltrating immune cell type that may affect tumor growth by either anti- or protumor effects [142]. Blood-derived monocytes infiltrate tumor tissues and differentiate into macrophages followed by further polarization into M1 or M2 phenotype, which differs in their patterns of cytokine secretion and biological function [143]. M1 macrophages mediate tumor suppression through type I cytokine production and tumor antigen acquisition and presentation [142, 144], whereas M2 macrophages promote tumor progression by producing type II cytokines [145]. Unfortunately, TAMs largely are of the M2 phenotype and promote the progression of almost all known solid tumors. Tumors produce many cytokines and other mediators that propel TAMs into the M2 phenotype [146]. Chemoattractant GPCRs are critical for TAM infiltration in the tumor, including chemokine GPCRs and the classical GPCR PAFR [84]. In certain tumor models, phagocytosis of apoptotic tumor cells by macrophages may induce M2 polarization, with the production of anti-inflammatory mediators [84, 147]. The main GPCR and ligand favoring TAM accumulation are CCR2/CCL2, which occurs in numerous tumors, such as pancreatic cancer, cervical cancer, papillary thyroid cancer, and prostate cancer [86, 91–93]. Some tumors also secrete other CCR2 ligands to recruit TAMs, such as HBD-3 in oral cancer [90]. In breast cancer, CCR2/CCL2 interaction recruits macrophages into the lung, where the cells "create" an appropriate microenvironment to facilitate tumor cell lodging and the development of metastatic foci [148]. FPR2 is also a GPCR expressed mainly on macrophages and neutrophils with the capacity to respond to bacterial chemotactic peptides [12]. In the mouse LLC model, tumors implanted subcutaneously grow more rapidly in mice deficient in Fpr2, the orthologue of human FPR2, and show significantly increased infiltration of TAMs with M2 polarization. Macrophages derived from Fpr2 deficient mice express higher levels of the chemokine GPCR, CCR4, which in cooperation with CCR2 mediate a marked increase in macrophage chemotaxis in response to CCL2. In addition, macrophages from Fpr2 deficient mice are more prone to M2 polarization after stimulation with LLC-derived supernatant. In contrast, in the presence of Fpr2, some macrophages develop an M1 phenotype after conditioning with LLC supernatant. Therefore, Fpr2 appears to sustain M1 differentiation of macrophages which participate in anti-LLC host responses [94]. Similarly, mice deficient in the chemokine GPCR CXCR3 exhibit polarization of TAMs into M2 phenotype in breast cancer [42]. Another chemokine GPCR, CX3CR1, and its ligand, CX3CL1, recruit TAMs and sustain the survival of TAMs to promote tumor metastasis [96, 149]. Therefore, chemoattractant GPCRs, in addition to mediating TAM recruitment, also favor TAM polarization to the M2 phenotype in response to tumor microenvironmental factors that promote tumor growth.

2.2.3. MDSCs. Another type of immunosuppressive cells that shape the protumor microenvironment is MDSCs, which consist of subsets of immature myeloid cells with either monocytic or granulocytic morphology [150]. MDSCs are recruited into tumors via the chemokine GPCRs CCR2, CXCR2, or CXCR4 and are believed to promote tumor progression, such as facilitating metastasis in CRC [151, 152]. MDSCs exert their protumor activity by suppressing antitumor effectors, as by inhibiting T cell function via iNOS and arginase [80, 153, 154]. Deletion of CCR2(+) MDSCs using a toxin-mediated ablation strategy increased recruitment of activated CD8(+) T cells into the tumor and thus restored antitumor defense [150]. MDSCs are also capable of sustaining a protumor microenvironment by recruiting Tregs via chemoattractant GPCRs and ligands. For instance, MDSCs release CCL3, CCL4, and CCL5, which activate CCR5 expressed by Tregs and result in their recruitment in both in vitro and in vivo experimental models [105]. In addition to recruiting Tregs, a group of CD11b(+)CCR8(+) myeloid cells similar to MDSCs recruited by CCR8/CCL1 interaction in urothelial and renal carcinomas also "educate" tumor infiltrating T cells to express FoxP3, a marker for Tregs [31]. Thus, MDSCs have been recognized as an important component in the tumor microenvironment that are regulated by chemoattractant GPCRs and ligands. MDSCs also utilize the GPCR/ligand interactions to amplify protumor host response.

2.2.4. Other Tumor Infiltrating Cells. In addition to immune cells, stromal cells in the tumor microenvironment also take part in the regulation of tumor growth. Mesenchymal stem cells (MSCs) are one of the major components in the tumor stroma and are believed to be the precursors of CAFs [155, 156]. MSCs may be recruited into the tumor through FPR2, CCR2, CXCR1, CXCR2, CXCR4, CXCR6, and CX3CR1 depending on the types and locations [125, 126, 128, 133]. Tumor-resident MSCs are often constantly exposed to immune cells and inflammatory cytokines in the microenvironment. They may have acquired functions distinct from normal tissue MSCs that alter the balance of host tumor interaction [88]. For example, compared with bone marrow MSCs, MSCs isolated from spontaneous mouse lymphomas (L-MSCs) promote tumor growth in association with recruitment of large numbers of CD11b(+) Ly6C(+) monocytes, F4/80(+) macrophages, and CD11b(+) Ly6G(+) neutrophils into the tumor. Depletion of monocytes/macrophages, but not neutrophils, completely abolishes the tumor promoting activity of L-MSCs. Such tumor infiltrating monocytes/macrophages are recruited by CCL2 produced by L-MSCs and CCR2 expressed on TAMs [88]. Similarly, CAFs are associated with immune suppressive microenvironment. In Hodgkin lymphoma and cutaneous T cell lymphoma, CAFs secrete the chemokines CCL11 and

CCL26 that recruit CCR3(+) T lymphocytes into the tumor and produce high levels of IL-4, a signature of a Th2-dominant microenvironment [157].

In conclusion, GPCRs and ligands are critical for the recruitment of a variety of immune and nonimmune cells into the tumor microenvironment where these cells interact to establish host responses, which, unfortunately, mostly tip the balance to protumor elements.

3. The Role of Chemoattractant GPCRs Expressed by Tumor Cells

While chemoattractant GPCRs contribute to tumor growth by promoting the recruitment of protumor stromal cells and angiogenesis, many tumor cells also express a variety of GPCRs, which, by responding to autocrine and/or paracrine agonists produced in the microenvironment, directly stimulate tumor cell proliferation and tumor spread and expansion (Table 3).

In anaplastic large cell lymphomas, the CCR3/CCL11 interaction promotes tumor cell proliferation and inhibits apoptosis through ERK1/2, Bcl-xL and the production of survivin [192]. Similarly, through an AKT signaling pathway, CCR7 and its ligands CCL19 and CCL21 induce squamous cell carcinoma of the head and neck growth in vitro and in vivo [230]. In addition, CCR6/CCL20 interaction in endometrial adenocarcinoma, CXCR1/2/CXCL7 interaction in clear cell renal cell carcinoma, CXCR2/CXCL8 interaction in nasopharyngeal carcinoma, and CXCR6/CXCR16 interaction in HCC are reported to promote tumor cell growth [3, 27, 37, 265]. Hypoxia, which occurs during tumor expansion, induces the production of GPCR ligands that promote tumor cell proliferation in an autocrine manner. In cervical carcinoma, hypoxia stimulates tumor cells to express high levels of CXCR1/2 and CXCL8 that respond to ligands in the microenvironment by proliferating [246]. Actually, numerous chemoattractant GPCRs, such as CCR1, CCR5, CXCR5, CXCR7, and PAFR, are expressed by various types of tumor cells and are implicated in tumor growth [1]. In the case of the same GPCR, CXCR3, its two variants have opposite functions. CXCR3-A promotes cells growth but CXCR3-B mediates growth-inhibitory signals and induces apoptosis in various tumors [270].

In addition to tumor cells, stromal cells in the microenvironment also secrete GPCR ligands that stimulate the receptors on tumor cells in a paracrine manner which may represent a more important yet complicated stimulating loop. This is exemplified by observations in human glioma in which CXCR4/CXCL12 interaction favors an autocrine or paracrine loop for tumor cell proliferation [314, 315]. CXCR4/CXCL12 growth stimulating effects were also detected in glioma stem cells via an AKT-mediated prosurvival and self-renewal pathway. Highly malignant human glioblastoma cells (GBM) express a classical chemoattractant GPCR, FPR1, which recognizes a ligand, Annexin A1, released by necrotic GBM cells that mediates the proliferation of live GBM cells to increase their invasiveness and the production of angiogenic factors vascular endothelial growth factor (VEGF) and CXCL8 (IL-8), which stimulate VEGF receptor (VEGFR) and CXCR1/CXCR2 on vascular ECs to promote their migration and formation of new vasculature [316, 317]. It is interesting to note that FPR1 in GBM cells does not act alone; instead, the GPCR transactivates EGFR which accounts for part of the GBM growth stimulating activity of FPR1. GBM cells are able to maximally exploit the supportive mediators in the microenvironment to their advantage [1, 318]. By stimulating GPCR, tumor cells may even change the phenotype of neighboring stromal cells. Breast tumor cells secrete CCL20 to activate the ERK1/2/MAPK pathway in surrounding "normal" breast epithelial cells via CCR6 and promote their malignant transformation [319].

CAFs have been recognized as important regulators of tumor initiation by secreting CXCL12 to activate CXCR4 on breast cancer cells and stimulate tumor growth [320]. Studies have also shown that, after activation by CXCL12, breast cancer cells secrete another chemokine CCL20 that activates CCR6 expressed by tumor cells and facilitates their proliferation [321], while, in Hodgkin lymphoma, CAFs from tumor-involved lymph nodes cocultured with Reed-Sternberg cells produce CCL5, which activates CCR5 on tumor cells to stimulate tumor growth [205]. Multiple myeloma (MM) cells and osteoclasts (OCs) form yet another example of tumor promoting activity of GPCR/ligand interactions. MM growth in the bone marrow niche depends on bone resorption and interaction with active OCs [322, 323]. MM cells secrete CCL3 to activate OCs through its receptor CCR1 [324]. CCR1/CCL3 interaction inhibits the function of osteoblasts (OBs), resulting in the loss of OB/OC balance, which could facilitate MM growth [325]. Also, OCs in the tumor microenvironment sustain MM cell proliferation through production of chemokine that activate CCR2 on tumor cells [187]. These pathways culminate in MM outgrowth.

Based on these observations, it is now clear that chemokine GPCRs expressed by tumor cells and autocrine or paracrine ligands form a formidable interaction in the microenvironment that orchestrates the crisscross interaction between tumor cells and stromal cells stimulating further growth of the tumors.

4. The Role of Chemoattractant GPCRs in Tumor Metastasis

Metastasis is the major cause of cancer death. In order for cancer cells to metastasize, the cells should acquire a motile phenotype and be able to detach from the primary tumor mass to degrade basement membrane and intravasate into the blood or lymph vessels. After trafficking in the blood or lymphatic vessels, tumor cells tend to form emboli extravasating into distant organs or lymph nodes [1, 326]. Nearly each step of metastasis is heavily dependent on the tumor microenvironment and chemoattractant GPCRs are active participants in the processes.

A historical discovery of the role of chemoattractant GPCR/ligand interactions in promoting cancer metastasis was reported in 1998, in which the chemokine CCL2 (MCP-1) was shown to mediate kidney specific metastasis of a subpopulation of a murine experimental lymphoma [327]. This was followed by a more detailed study of several

Table 3: The functions of chemoattractant GPCRs expressed by tumor cells.

GPCRs	Tumor	Function	References
FPR			
FPR1	Colorectal cancer	Invasion	[158]
	Gastric cancer	Invasion	[159]
	Glioblastoma	Growth, invasion, vasculogenesis, angiogenesis	[160–163]
FPR2	Gastric cancer	Invasion	[159]
	Ovarian cancer	Invasion	[164]
FPR3	Gastric cancer	Invasion	[159]
PAFR	Breast cancer	Migration, proliferation, angiogenesis	[165]
	Melanoma	Metastasis	[166]
	Ovarian cancer	Proliferation, invasion	[167]
C5aR	Bile duct cancer, colorectal cancer	Invasion	[168]
	Non-small-cell lung cancer	Metastasis	[169]
LTB4R			
BLT1	Colorectal cancer	Proliferation	[170]
BLT2	Bladder cancer	Metastasis, antiapoptosis	[171, 172]
	Breast cancer	Metastasis	[173]
	Pancreatic cancer	Growth, migration	[174, 175]
	Prostate cancer	Antianoikis, antiapoptosis	[176, 177]
	Ovarian cancer	Metastasis	[178]
CCR			
CCR1	Breast cancer	Invasion	[179]
	Glioma	Proliferation, tumorigenesis	[180]
	Hepatocellular carcinoma	Migration, invasion	[181, 182]
	Oral squamous cell carcinoma	Migration	[183]
	Ovarian cancer	Invasion	[184]
	Bladder cancer	Migration, invasion	[185]
CCR2	Breast cancer	Migration, proliferation, antiapoptosis	[186]
	Hepatocellular carcinoma	Migration, invasion	[181]
	Multiple myeloma	Growth	[187]
	Ovarian cancer	Invasion, adhesion, proliferation	[188, 189]
	Prostate cancer	Proliferation, migration, invasion	[190, 191]
	Lymphoma	Growth	[192]
CCR3	Glioma	Proliferation, tumorigenesis	[180]
	Oral squamous cell carcinoma	Migration, invasion	[183]
	Ovarian cancer	Invasion, proliferation	[184, 189]
	Renal cell carcinoma	Growth, dissemination	[193]
	Breast cancer	Growth, metastasis, angiogenesis	[139, 194]
CCR4	Colorectal cancer	Migration	[195]
	Gastric cancer	Migration	[196]
	Melanoma	Metastasis	[197]
	Squamous cell carcinoma of the head and neck	Metastasis	[198]
CCR5	Breast cancer	Proliferation, metastasis	[25, 199–202]
	Colorectal cancer	Growth	[203]
	Gastric cancer	Metastasis	[204]
	Glioma	Proliferation, tumorigenesis	[180]
	Hodgkin lymphoma	Growth, metastasis	[205]
	Oral cancer	Migration	[206]
	Ovarian cancer	Invasion, proliferation	[189]

Table 3: Continued.

GPCRs	Tumor	Function	References
	Colorectal cancer	Proliferation, metastasis	[207, 208]
	Endometrial adenocarcinoma	Proliferation	[27]
CCR6	Hepatocellular carcinoma	Metastasis	[209, 210]
	Non-small-cell lung cancer	Metastasis	[211]
	Pancreatic cancer	Invasion	[212–214]
	Squamous cell carcinoma of the head and neck	Metastasis	[215, 216]
	Breast cancer	Metastasis, antianoikis	[217, 218]
	Colorectal cancer	Metastasis	[219, 220]
	Melanoma	Growth, metastasis, tumorigenesis	[221, 222]
	Non-small-cell lung cancer	Proliferation, antiapoptosis, metastasis	[29, 223–226]
CCR7	Oral squamous cell carcinoma	Metastasis	[227]
	Pancreatic ductal adenocarcinoma	Metastasis	[228]
	Prostate cancer	Metastasis	[229]
	Squamous cell carcinoma of the head and neck	Proliferation, antiapoptosis, metastasis, adhesion	[230–236]
	T cell lymphoma	Dissemination	[237]
CCR8	Melanoma, breast cancer, leukemia	Metastasis	[32]
	Breast cancer	Migration, invasion	[238]
	Colorectal cancer	Inhibiting metastasis	[239]
CCR9	Ovarian cancer	Migration, invasion	[240]
	Pancreatic cancer	Proliferation, invasion	[34, 241]
	Prostate cancer	Antiapoptosis	[242]
CCR10	Melanoma	Growth, metastasis	[243, 244]
CXCR			
	Breast cancer	Stem cell self-renewal	[245]
	Cervical carcinoma	Proliferation	[246]
	Colorectal cancer	Metastasis, antiapoptosis, angiogenesis	[247]
	Gastric cancer	Invasion	[248]
CXCR1	Glioblastoma	Growth, migration, invasion	[249, 250]
	Melanoma	Growth, migration, invasion, angiogenesis, tumorigenesis	[251–253]
	Prostate cancer	Growth, angiogenesis	[254]
	Renal cell carcinoma	Growth, angiogenesis	[37]
	Thyroid carcinoma	Metastasis	[255]
	Breast cancer	Migration, invasion, stem cell self-renewal	[245, 256, 257]
	Cervical carcinoma	Proliferation	[246]
	Colorectal cancer	Proliferation, migration, invasion, angiogenesis	[258–261]
	Gastric cancer	Metastasis	[262, 263]
	Glioblastoma	Growth, migration	[249, 264]
	Melanoma	Growth, migration, invasion, angiogenesis, tumorigenesis	[251–253]
CXCR2	Nasopharyngeal carcinoma	Growth	[265]
	Non-small-cell lung cancer	Growth, metastasis, angiogenesis	[266, 267]
	Ovarian cancer	Growth, angiogenesis	[268]
	Pancreatic cancer	Invasion, angiogenesis	[269]
	Prostate cancer	Growth, angiogenesis	[254]
	Renal cell carcinoma	Growth, angiogenesis	[37]
	Thyroid carcinoma	Metastasis	[255]
	Breast cancer	Metastasis; inhibiting growth	[270–273]
	Colorectal cancer	Metastasis	[274]
CXCR3	Glioma	Growth	[275, 276]
	Lung adenocarcinoma	Metastasis	[226]
	Melanoma	Migration	[277]
	Myeloma	Inhibiting/promoting proliferation and apoptosis	[43]

Table 3: Continued.

GPCRs	Tumor	Function	References
	Ovarian cancer	Growth, metastasis	[278]
	Prostate cancer	Metastasis	[279]
	Renal cell carcinoma	Growth, metastasis	[280, 281]
CXCR4	At least 23 haematopoietic and solid cancers	Growth, metastasis, angiogenesis	[1, 44]
	Breast cancer	Metastasis	[282]
CXCR5	Colorectal cancer	Growth, migration	[283]
	Neuroblastoma	Inhibiting/promoting metastasis	[45, 284]
	Prostate cancer	Proliferation, invasion, migration, adhesion	[285–288]
	Colorectal cancer	Growth, migration, invasion	[289]
	Hepatocellular carcinoma	Growth, metastases, angiogenesis	[3]
	Melanoma	Stem cell self-renewal	[290]
CXCR6	Nasopharyngeal carcinoma	Metastasis	[291]
	Pancreatic ductal adenocarcinoma	Invasion	[292]
	Prostate cancer	Proliferation, metastasis	[293–295]
	Renal cell carcinoma	Inhibiting migration	[296]
	Breast cancer	Inhibiting invasion; growth, angiogenesis	[297]
	Cervical carcinoma	Growth, adhesion	[298]
	Glioma	Growth, migration, sphere and tube formation	[49, 299]
CXCR7	Hepatocellular carcinoma	Growth, metastasis, angiogenesis	[300, 301]
	Lymphoma	Growth, adhesion	[298]
	Nasopharyngeal carcinoma	Metastasis	[291]
	Neuroblastoma	Inhibiting growth; metastasis	[50, 302]
CX3CR			
	Epithelial ovarian carcinoma	Proliferation, migration, adhesion	[303]
	Glioma	Inhibiting invasion	[304]
CX3CR1	Neuroblastoma	Migration	[305]
	Pancreatic ductal adenocarcinoma	Migration	[306, 307]
	Prostate cancer	Metastasis	[308–310]
	Renal cell carcinoma	Metastasis	[311]
XCR			
XCR1	Epithelial ovarian carcinoma	Proliferation, metastasis	[312]
	Oral squamous cell carcinoma	Proliferation, migration, invasion	[313]

human cancer cell lines including breast and lung cancer cells which metastasized into distant organs in nude mice by using several chemokine GPCRs. These findings enriched the "seed" and "soil" paradigm of cancer metastasis by including chemoattractant GPCRs as the requisite for tumor cells as qualified "seeds" and a ligand producing distant organ or draining lymph nodes as suitable "soil" [328]. Since then, studies of the role of chemoattractant GPCRs and ligands in cancer metastasis have become a burgeoning research field and many malignant tumors have been shown to utilize a variety of GPCR/ligand interactions for metastasis. For example, in lung cancer, hypoxia induces the expression of CCR7 by tumor cells that increases cell invasiveness and eventual lymph node metastasis [29]. Hypoxia also promotes lymph node metastasis of breast cancer by increasing the expression of CCR5 on tumor cells and the ligand CCL5 in lymph nodes via the transcription factor hypoxia-inducible factor- (HIF-) 1α [25]. In prostate cancer and pancreatic ductal adenocarcinoma, cancer metastasis is associated with CX3CR1 on tumor cells and the ligand CX3CL1 at metastasis site [306, 308]. The sources of chemoattractants in tumor microenvironment are from both tumor and stromal cells. In prostate cancer, hypoxia-preconditioned MSCs produce CCL21 to attract tumor cells expressing CCR7 which is associated with enhanced lymph node metastasis of the tumor [229]. Similarly, under hypoxia, MSCs promote breast cancer metastasis through CXCR3/CXCL10 interaction [271].

Chemoattractant GPCRs and their ligands reportedly involved in enhanced tumor metastasis are listed in Table 4. Recently, cancer stem cells (CSCs) have been shown to account for most of the cancer metastasis. Interestingly, chemoattractant GPCRs participate in the maintenance of the metastatic property of CSCs by forming an autocrine loop. In ovarian cancer, the invasiveness of CD133(+) CSCs is enhanced by the chemokine CCL5, which activates CCR3 and CCR5 expressed by the cells to increase matrix metalloproteinase (MMP) 9 secretion [184]. A number of studies that use exogenous chemokines to induce cell invasion are

Table 4: Chemoattractant GPCRs associated with tumor metastasis.

Tumor type	GPCRs	Ligands	Metastatic sites
Bladder cancer	BLT2	LTB4	Lung [171]
	CCR2	CCL2	Lung [329]
	CXCR6	CXCL16	Perineural and lymphovascular invasion [330]
Breast cancer	BLT2	LTB4	Lung [173]
	CCR2	CCL2	Lung [85, 148], bone [148]
	CCR4	CCL17/22	Lung [139, 194, 331]
	CCR5	CCL5	Lung [200, 201], lymph node [25, 332]
	CCR6		Pleura [333]
	CCR7	CCL19/21	Lymph node [218, 334–337]
			Skin [333]
	CCR8	CCL1	Lymph node [32]
	CCR9	CCL25	Lymph nodes and gastrointestinal tract [238]
	CXCR1	CXCL8	Bone [338, 339]
	CXCR2		Lung [340], bone [341]
	CXCR3	CXCL9	Lung [342]
		CXCL10	Bone [343], lung [344]
	CXCR4	CXCL12	Lymph node [328, 336, 337, 345], bone [346–348], lung [328, 346, 349], liver [333]
	CXCR5	CXCL13	Lymph node [282]
	CXCR6	CXCL16	Lymph node [350]
	CXCR7	CXCL12	Lung, greater omentum, and lymph nodes [351]
	CX3CR1		Brain [333]
Cervical cancer	CXCR4	CXCL12	Lymph node [352]
	CXCR4/7	CXCL12	Lymph node [353, 354]
Colorectal cancer	CCR1	CCL7/9/15	Liver [355–357]
	CCR2	CCL2	Liver [151, 358], lung [359]
		CCL7	Liver [356]
	CCR3	CCL7	Liver [356]
	CCR5	CCL5	Liver and lung [203]
	CCR6	CCL20	Liver [207, 360]
	CCR7	CCL21	Lymph node [219, 220, 361]
	CXCR1/2		Liver [247]
	CXCR2	CXCL1	Lymph node [261], liver [362]
		CXCL8	Skin [363]
	CXCR3	CXCL9	Lymph node [364]
		CXCL10	Lymph node [364], lung [365]
		CXCL11	Lung [365]
	CXCR6	CXCL16	Liver [289, 366]
	CXCR4	CXCL12	Liver [367–370], lymph node [371, 372], brain [373]
Esophageal cancer	CCR7	CCL21	Lymph node [374–376]
	CXCR2		Lymph node [377]
	CXCR4	CXCL12	Lung [378, 379], liver [378, 379], lymph node [378, 380], peritoneum [379], retroperitoneum [379]
Gastric cancer	FPR1/2/3	Annexin A1	Peritoneum [159]
	CCR2	CCL2	Lymph node [381]
	CCR4	CCL17	Lymph node, lung, and bone [194]
	CCR5	CCL5	Lymph node [204]
	CCR7		Lymph node [30, 382, 383]
	CXCR2	CXCL1	Lymph node [262]
	CXCR4	CXCL12	Lymph node [382, 384–387], peritoneum [388–390], liver [387]

Table 4: Continued.

Tumor type	GPCRs	Ligands	Metastatic sites
Glioma	CXCR4/7	CXCL12	Bone marrow [299] Lymph node, distant organs [391]
Head and neck squamous cell carcinoma	CCR4	CCL22	Lymph node [198]
	CCR6	CCL20	Lymph node [216, 392]
	CCR7	CCL19/21	Lymph node [227, 230–232, 393]
	CXCR2	CXCL1/8	Lymph node [394, 395]
	CXCR4	CXCL12	Lymph node [393, 396], lung [397, 398]
	CXCR5	CXCL13	Bone [399]
	XCR1	XCL1	Lymph node [313]
Hepatocellular carcinoma	CCR7		Intrahepatic metastasis, lymph node [400]
	CXCR4	CXCR12	Lung [401], bone [402, 403], lymph node [404]
	CXCR6	CXCL16	Lung [3]
	CXCR7	CXCL12	Lung [300, 405]
Lymphoma	CCR7	CCL21	Lymph node [237]
Leukemia	CCR8	CCL1	Lymph node [32]
	CXCR4	CXCL12	Extramedullary sites (liver, kidney, spleens, and peripheral blood) [406]
Melanoma	FPR1/2/3	Annexin A1	Lung [407]
	PAFR	PAF	Lung [166, 408, 409]
	CCR2	CCL2	Lung [410]
	CCR3		Brain [411]
	CCR4	CCL22	Brain [197, 411]
	CCR5	CCL4	Lung [412, 413]
	CCR7	CCL21	Lymph node [221, 222, 244, 414], liver [415]
	CCR8	CCL1	Lymph node [32]
	CCR9	CCL25	Small intestinal [416, 417]
	CCR10	CCL27	Skin [243, 244]
	CXCR2	CXCL8	Lung [418]
	CXCR3	CXCL10	Lymph node [419, 420], bone [421]
	CXCR4	CXCL2	Lung [244, 422–424]
Neuroblastoma	CXCR3	CXCL10	Bone marrow [425]
	CXCR4	CXCL12	Bone [426–428], liver [429, 430], kidney [430], bone marrow [428, 430]
	CXCR5	CXCL13	Bone marrow [284]
	CXCR4/7	CXCL12	Bone marrow [302]
	CX3CR1	CX3L1	Bone marrow [305]
Non-small-cell lung cancer	C5aR		Lymph node [169]
	CCR4	CCL22	Bone [431]
	CCR6	CCL20	Adrenal specific metastasis [211]
	CCR7	CCL19/21	Lymph node [29, 226, 432]
	CXCR2	CXCL5	Hilar and mediastinal lymph nodes, chest wall, and contralateral lung; extrathoracic distant metastases (para-aortic lymph nodes, liver, adrenal glands, kidneys, spleen, and diaphragm) [266]
	CXCR4	CXCL12	Lungs, liver, bone marrow, adrenal glands [433], pleural [434], brain [433, 435]
	CX3CR1		Brain and liver [436]
Osteosarcoma	CCR7	CCL21	Lymph node [334]
	CXCR3	CCL9/10/11	Lung [437]
	CXCR4	CXCL12	Lung [438]
	CXCR7	CXCL12	Lung [439]

Table 4: Continued.

Tumor type	GPCRs	Ligands	Metastatic sites
Ovarian carcinoma	BLT2		Diaphragm, intestine, and mesentery (intraperitoneal dissemination) [178]
	CCR3	CCL5	Liver, bowel, and spleen [184]
	CCR9	CCL25	Small intestinal [440]
	CXCR4	CXCL12	Pelvic [441], lymph node [442, 443], peritoneum [444]
	CXCR6	CXCL16	Lymph node [443]
	XCR1	XCL1/2	Diaphragm, peritoneal wall, colon, spleen, and liver [312], peritoneum [312]
Pancreatic cancer	CCR2	CCL2	Liver [92, 445], peritoneal [445]
	CCR7	CCL21	Lymph node [228, 446]
	CXCR4/7	CXCL12	Liver [447, 448], lung [448], lymph node [449]
Prostate cancer	CCR2	CCL2	Bone [450]
	CCR7	CCL21	Lymph node [229]
	CXCR1/2	CXCL8	Lymph node [451]
	CXCR3	CXCL4/10	Lymph node, liver, lung, adrenal [279]
	CXCR4	CXCL12	Bone [133, 452, 453]
	CXCR5	CXCL13	Bone [288]
	CXCR6	CXCL16	Bone [133, 294, 453], liver [294]
	CX3CR1	CX3CL1	Bone [310]
Renal cell carcinoma	CCR1/3	CCL15	Bone [454]
	CCR5	CCL3	Lung [326]
Thyroid papillary cancer	CCR7	CCL21	Lymph node [455, 456]
	CXCR1/2	CXCL8	Lymph node [255]
	CXCR4		Lymph node [455, 457, 458]
	CXCR7		Lymph node [459]

in the literature. However, there are also a small number of chemokine and GPCR interactions that may inhibit tumor cells invasion, such as CX3CR1/CX3CL1 interaction in glioma [304].

While the aberrant expression of chemoattractant GPCRs is an important feature for a motile phenotype of tumor cells, the next step of tumor cell metastasis from the primary mass is detachment. These cells must survive the loss of interactions with extracellular matrix (ECM) that causes anoikis for further invasion of blood or lymph vessels [217]. In breast cancer, the activation of both CXCR4/CXCL12 and CCR7/CCL21 may reduce the sensitivity of metastatic cancer cells to anoikis by upregulating antiapoptotic proteins. Consequently, blocking the chemokine and GPCR interactions attenuates breast cancer metastasis in vivo [217]. Recently, another classical chemoattractant GPCR, BLT2, has also been shown to establish resistance to anoikis in prostate cancer cells through a BLT2-NOX-ROS-NF-κB cascade [176].

Thus, accumulating evidence indicates an essential role of chemoattractant GPCRs and ligands in every step of cancer metastasis, including the acquisition of increased motility, detachment from the primary tumor mass by breaking down matrix proteins, intra- and extravasation, and lodgment in distant organs and lymph nodes. In addition, chemoattractant GPCRs and ligands also orchestrate the interaction of metastatic tumor cells with stromal cells, such as TAMs, ECs, and fibroblasts, which act either as "driving forces" for tumor cell dissemination or as "conditioners" of the "soil" that facilitates the settlement of metastatic tumor cells to develop secondary foci. Therefore, chemoattractant GPCRs and ligands provide promising molecular targets for prevention of tumor metastasis.

5. The Role of Chemoattractant GPCRs in Tumor Neovascularization

Neovascularization is critical for consolidation of the tumor microenvironment for tumor progression. Chemoattractant GPCRs provide pro- and antiangiogenic factors and receptors and are able to regulate two phases of neovascularization: vasculogenesis and angiogenesis (Table 5).

5.1. Vasculogenesis. Vasculogenesis is the formation of new blood vessels from circulating bone marrow-derived endothelial progenitor cells (EPCs). Coordinated events are required for the recruitment and incorporation of EPCs into the tumor tissue, including migration, invasion, differentiation, proliferation, and formation of vessels [461]. Although VEGF is a well-known angiogenic factor taking part in the vasculogenesis, other paracrine factors, such as chemoattractants produced by tumor cells, are also involved. EPCs expressing CXCR4 are mobilized by the ligand CXCL12 in an autocrine or paracrine manner [503]. Another chemokine CCL2 also mobilizes EPCs from the bone marrow [504]. These chemokines then promote EPC proliferation and guide the cells into tumor stroma to form functional neovasculature [505]. EPCs participating in neovascularization have also been reported in HCC, in which myeloid-derived EPCs

Table 5: Chemoattractant GPCRs associated with tumor neovascularization.

	Receptors	Tumors
Vasculogenesis	FPR1	Glioma [160]
	FPR2	Ovarian cancer [123]
	CCR2	Hepatocellular carcinoma [460]
	CCR5	Hepatocellular carcinoma [460]
	CCR6	Hepatocellular carcinoma [461]
	CXCR2	Pancreatic cancer [462]
	CXCR4	Breast cancer [320], melanoma [463]
Angiogenesis	FPR1	Glioma [161, 162, 316]
	C5aR	Epithelial ovarian cancer [17]
	CCR1	Hepatocellular carcinoma [464], lymphoma [465], multiple myeloma [466]
	CCR2	Breast cancer [22, 467, 468], esophageal cancer [469], gastric cancer [381], melanoma [470]
	CCR4	Breast cancer [194]
	CCR5	Multiple myeloma [466], renal cell carcinoma [326]
	CCR10	Ovarian cancer [35]
	CXCR1	Prostate cancer [471], renal cell carcinoma [37]
	CXCR2	Cervical cancer [472], colorectal cancer [258, 259], glioblastoma [473], lung adenocarcinoma [267, 474, 475], melanoma [418, 476], ovarian cancer [268], pancreatic cancer [269, 477–479], prostate cancer [480], renal cell carcinoma [37, 481]
	CXCR1/2	Glioblastoma [482], melanoma [251, 253], multiple myeloma [483], ovarian cancer [484], pancreatic cancer [485], prostate cancer [254, 451, 486], renal cell carcinoma [37]
	CXCR4	Breast cancer [487], colorectal cancer [488, 489], gastric cancer [490], glioblastoma [491–493], hepatocellular carcinoma [494], ovarian cancer [495], pancreatic cancer [269, 496], prostate cancer [497], squamous cell carcinoma [398]
	CXCR6	Hepatocellular carcinoma [3], prostate cancer [295]
	CXCR7	Bladder cancer [498], breast cancer [297], breast and lung cancer [499], colorectal cancer [488], hepatocellular carcinoma [301], prostate cancer [500], renal cell carcinoma [501]
	CX3CR1	Breast cancer [96], colorectal cancer [149], melanoma [502]

(colony forming unit-endothelial cells) as early EPCs highly express CCR6 and are mobilized by the ligand CCL20 produced by HCC cells for migration and invasion of tumor stroma to form vasculature. CCR6/CCL20 in tumor microenvironments in addition plays a crucial role in driving phenotypic switch of hematopoietic cells with increased potential for angiogenic EC differentiation and attenuated proinflammatory activity [461]. A classical chemoattractant receptor, FPR1, may also participate in vasculogenesis in human GBM. This was shown in a xenograft model in which the number of EPCs incorporated into intracranial GBM lesion was significantly reduced in tumors formed by GBM cells in which FPR1 was depleted by RNA interference. The EPC chemotactic and tubule-stimulating activities were also attenuated in the supernatant of GBM cells deficient in FPR1 [160]. Another classical chemoattractant GPCR, the FPR1 variant FPR2, has also been reported to participate in recruiting MSCs into tumor tissues to promote the formation of neovasculature in response to tumor-derived ligand LL-37 [123].

5.2. Angiogenesis. Angiogenesis is a process in which new blood vessels sprout from existing vasculature. In tumor microenvironment, various cells regulate this process through GPCRs, which are expressed on vascular ECs and mediate cell recruitment and proliferation thereby extending the new vasculature in response to the ligands produced by tumor and other stromal cells. Tumor cells, tumor stem cells, and infiltrating TAMs in particular also express GPCRs capable of promoting the release of proangiogenic factors recruiting and activating vascular ECs [1].

FPR1 selectively expressed by GBM cells when activated by exogenous and tumor derived agonists promotes tumor cells to produce proangiogenic factors VEGF and the angiogenic chemokine CXCL8 [161, 316, 506]. CXCR1/2 expressed by vascular ECs and CXCL8, the ligand produced by tumor and stromal cells, are known to promote angiogenesis through inducing EC migration and formation of tubules [484, 507]. GBM stem cells may also utilize chemoattractant GPCRs FPR1 and CXCR4 to participate in angiogenesis by releasing VEGF [162, 249].

In addition to the direct interaction between chemoattractant GPCRs expressed by ECs and ligands in the tumor microenvironment, tumors take the advantage of infiltrating stromal cells, such as CAFs, TAMs, and Tregs, to benefit angiogenesis through GPCRs. In lung cancer, CAFs express CCR5 and are activated by CCL3 to secrete hepatocyte growth factor (HGF) to accelerate angiogenesis [326]. CAFs also cooperate with tumor cells to promote angiogenesis through CXCR4 expressed by both cell types. In pancreatic cancer, tumor cells secrete CXCL8 and CAFs secrete CXCL12 to enhance the recruitment and proliferation of ECs. However, CXCL12 promotes EC infiltration and CXCL8 enhances

tubule formation by ECs revealing distinct functions of the CXCR2/CXCL8 and CXCR4/CXCL12 interactions in the process [269].

In addition, TAMs are an important source of angiogenic factors in tumor. For example, CCR2 and CD40 on TAMs are activated by CCL2 and CD40L produced in gastric cancer tissues and synergistically promote VEGF production to increase microvessel density [381, 508]. Moreover, Tregs expressing CCR10 are capable of accelerating angiogenesis through secreting VEGF in response to CCL28 produced by hypoxic tumor cells for EC infiltration and participation in angiogenesis [35].

It is interesting to note that alcohol consumption contributes to increased breast cancer angiogenesis, thus promoting the growth and metastasis of tumor cells in an animal model. This involves upregulated expression of CCR2 and CCL2 by tumor cells that increase the interaction between tumor and vascular ECs [467]. Another physical and chemical factor, radiation, exerts a similar effect through CXCR4/CXCL12 interaction on tumor angiogenesis [509].

Conversely, some chemoattractant GPCRs, such as CXCR3, are reported to mediate angiostatic activity through non-ELR CXC chemokines CXCR4/9/10/11 in various tumors [1]. The controversial results of angiogenesis are also found in C5aR [16, 17]. Therefore, angiogenesis may be regulated by a complex balancing process between opposing pro- and antiangiogenic GPCR and ligand interactions.

6. Perspectives

Accumulating evidence indicates crucial roles of chemoattractant GPCRs and their ligands in tumor progression by shaping tumor microenvironment. Almost all cell types including tumor cells per se are able to take the advantage of GPCRs and ligands to affect tumor progression. Chemoattractant GPCRs and ligands are involved in almost every step of tumor development and progression such as increasing tumor cell motility, invasiveness, intra- and extravasation, dissemination, leukocyte infiltration, and angiogenesis. These render the GPCRs and ligands promising drug targets for disruption of the tumor progression cascade. Recently, new agents targeting chemoattractant GPCRs have been developed and are being tested in the clinic, such as a humanized anti-CCR4 monoclonal antibody, mogamulizumab (KW-0761), aiming at curtailing cutaneous T cell lymphoma [510]. Therefore, gaining a better understanding of the GPCRs and their ligands in tumor microenvironment is vital and will provide novel therapeutic opportunities.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jiamin Zhou and Yi Xiang contributed equally to this paper.

Acknowledgments

This project has been funded in part by Federal funds from the National Cancer Institute, National Institutes of Health, under Contract no. HHSN261200800001E. The research was also supported in part by the Intramural Research Program of the NCI, NIH. Jiamin Zhou is funded in part by China Scholarship Council, National Natural Science Foundation of China (no. 81101566), and Scientific Funds of Shanghai Government (11DZ2280400, 12QA1400600, XYQ2011017, and 11411950500). Yi Xiang is funded in part by the National Natural Science Foundation of China (no. 81101771) and Shanghai Municipal Commission "Exchange Scholars." The authors thank Dr. Joost J Oppenheim for critical review of the paper and Ms. Cheryl Lamb and Ms. Sharon Livingstone for secretarial assistance.

References

[1] J. Huang, K. Chen, W. Gong, N. M. Dunlop, and J. M. Wang, "G-protein coupled chemoattractant receptors and cancer," *Frontiers in Bioscience*, vol. 13, no. 9, pp. 3352–3363, 2008.

[2] Y. Le, Y. Cui, P. Iribarren, G. Ying, and J. M. Wang, "Manipulating chemoattractant and receptor genes," *In Vivo*, vol. 16, no. 1, pp. 1–23, 2002.

[3] Q. Gao, Y. Zhao, X. Wang et al., "CXCR6 upregulation contributes to a proinflammatory tumor microenvironment that drives metastasis and poor patient outcomes in hepatocellular carcinoma," *Cancer Research*, vol. 72, no. 14, pp. 3546–3556, 2012.

[4] N. Chakravarti, V. G. R. Peddareddigari, C. L. Warneke et al., "Differential expression of the G-protein-coupled formyl peptide receptor in melanoma associates with aggressive phenotype," *The American Journal of Dermatopathology*, vol. 35, no. 2, pp. 184–190, 2013.

[5] D. Kitagawa, A. Taketomi, H. Kayashima et al., "Expression of platelet-activating factor receptor: a novel prognosticator in patients with hepatocellular carcinoma following hepatectomy," *Oncology*, vol. 72, no. 5-6, pp. 381–387, 2008.

[6] M. Han, S. Lv, Y. Zhang et al., "The prognosis and clinicopathology of CXCR4 in gastric cancer patients: a meta-analysis," *Tumour Biology*, vol. 35, no. 5, pp. 4589–4597, 2014.

[7] J. Wu, X. Wu, W. Liang, C. Chen, L. Zheng, and H. An, "Clinicopathological and prognostic significance of chemokine receptor CXCR4 overexpression in patients with esophageal cancer: a meta-analysis," *Tumor Biology*, vol. 35, no. 4, pp. 3709–3715, 2014.

[8] R. J. Byers, E. Sakhinia, P. Joseph et al., "Clinical quantitation of immune signature in follicular lymphoma by RT-PCR-based gene expression profiling," *Blood*, vol. 111, no. 9, pp. 4764–4770, 2008.

[9] H. Sugasawa, T. Ichikura, H. Tsujimoto et al., "Prognostic significance of expression of CCL5/RANTES receptors in patients with gastric cancer," *Journal of Surgical Oncology*, vol. 97, no. 5, pp. 445–450, 2008.

[10] P. A. Cassier, I. Treilleux, T. Bachelot et al., "Prognostic value of the expression of C-Chemokine Receptor 6 and 7 and their ligands in non-metastatic breast cancer," *BMC Cancer*, vol. 11, article 213, 2011.

[11] J. M. Wang, X. Deng, W. Gong, and S. Su, "Chemokines and their role in tumor growth and metastasis," *Journal of Immunological Methods*, vol. 220, no. 1-2, pp. 1–17, 1998.

[12] Y. Le, P. M. Murphy, and J. M. Wang, "Formyl-peptide receptors revisited," *Trends in Immunology*, vol. 23, no. 11, pp. 541–548, 2002.

[13] J. Huang, K. Chen, W. Gong et al., "Receptor "hijacking" by malignant glioma cells: a tactic for tumor progression," *Cancer Letters*, vol. 267, no. 2, pp. 254–261, 2008.

[14] M. A. N. D. Ferreira, L. S. Barcelos, M. M. Teixeira, Y. S. Bakhle, and S. P. Andrade, "Tumor growth, angiogenesis and inflammation in mice lacking receptors for platelet activating factor (PAF)," *Life Sciences*, vol. 81, no. 3, pp. 210–217, 2007.

[15] Z. Honda, S. Ishii, and T. Shimizu, "Platelet-activating factor receptor," *Journal of Biochemistry*, vol. 131, no. 6, pp. 773–779, 2002.

[16] H. F. Langer, K. Chung, V. V. Orlova et al., "Complement-mediated inhibition of neovascularization reveals a point of convergence between innate immunity and angiogenesis," *Blood*, vol. 116, no. 22, pp. 4395–4403, 2010.

[17] S. Nunez-Cruz, P. A. Gimotty, M. W. Guerra et al., "Genetic and pharmacologic inhibition of complement impairs endothelial cell function and ablates ovarian cancer neovascularization," *Neoplasia*, vol. 14, no. 11, pp. 994–1004, 2012.

[18] H. Lee, P. L. Whitfeld, and C. R. Mackay, "Receptors for complement C5a. The importance of C5aR and the enigmatic role of C5L2," *Immunology and Cell Biology*, vol. 86, no. 2, pp. 153–160, 2008.

[19] A. M. Tager and A. D. Luster, "BLT1 and BLT2: the leukotriene B4 receptors," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 69, no. 2-3, pp. 123–134, 2003.

[20] J. F. Cheng and R. Jack, "CCR1 antagonists," *Molecular Diversity*, vol. 12, no. 1, pp. 17–23, 2008.

[21] M. Struthers and A. Pasternak, "CCR2 antagonists," *Current Topics in Medicinal Chemistry*, vol. 10, no. 13, pp. 1278–1298, 2010.

[22] R. Salcedo, M. L. Ponce, H. A. Young et al., "Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression," *Blood*, vol. 96, no. 1, pp. 34–40, 2000.

[23] L. I. Willems and A. P. IJzerman, "Small molecule antagonists for chemokine CCR3 receptors," *Medicinal Research Reviews*, vol. 30, no. 5, pp. 778–817, 2010.

[24] A. V. Purandare and J. E. Somerville, "Antagonists of CCR4 as immunomodulatory agents," *Current Topics in Medicinal Chemistry*, vol. 6, no. 13, pp. 1335–1344, 2006.

[25] S. Lin, S. Wan, L. Sun et al., "Chemokine C-C motif receptor 5 and C-C motif ligand 5 promote cancer cell migration under hypoxia," *Cancer Science*, vol. 103, no. 5, pp. 904–912, 2012.

[26] C. E. de Oliveira, J. M. Oda, R. Losi Guembarovski et al., "CC chemokine receptor 5: the interface of host immunity and cancer," *Disease Markers*, vol. 2014, Article ID 126954, 8 pages, 2014.

[27] A. E. Wallace, R. D. Catalano, R. A. Anderson, and H. N. Jabbour, "Chemokine (C-C) motif ligand 20 is regulated by PGF2α-F-prostanoid receptor signalling in endometrial adenocarcinoma and promotes cell proliferation," *Molecular and Cellular Endocrinology*, vol. 331, no. 1, pp. 129–135, 2011.

[28] W. Wan and P. M. Murphy, "Regulation of atherogenesis by chemokine receptor CCR6," *Trends in Cardiovascular Medicine*, vol. 21, no. 5, pp. 140–144, 2011.

[29] Y. Li, X. Qiu, S. Zhang, Q. Zhang, and E. Wang, "Hypoxia induced CCR7 expression via HIF-1α and HIF-2α correlates with migration and invasion in lung cancer cells," *Cancer Biology & Therapy*, vol. 8, no. 4, pp. 322–330, 2009.

[30] S. Zhou, S. Xu, H. Tao et al., "CCR7 expression and intratumoral FOXP3$^+$ regulatory T cells are correlated with overall survival and lymph node metastasis in gastric cancer," *PLoS ONE*, vol. 8, no. 9, Article ID e74430, 2013.

[31] E. Eruslanov, T. Stoffs, W. Kim et al., "Expansion of CCR8+ inflammatory myeloid cells in cancer patients with urothelial and renal carcinomas," *Clinical Cancer Research*, vol. 19, no. 7, pp. 1670–1680, 2013.

[32] S. Das, E. Sarrou, S. Podgrabinska et al., "Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses," *The Journal of Experimental Medicine*, vol. 210, no. 8, pp. 1509–1528, 2013.

[33] M. Svensson and W. W. Agace, "Role of CCL25/CCR9 in immune homeostasis and disease," *Expert Review of Clinical Immunology*, vol. 2, no. 5, pp. 759–773, 2006.

[34] E. L. Heinrich, A. K. Arrington, M. E. Ko et al., "Paracrine activation of chemokine receptor CCR9 enhances the invasiveness of pancreatic cancer cells," *Cancer Microenvironment*, vol. 6, no. 3, pp. 241–245, 2013.

[35] A. Facciabene, X. Peng, I. S. Hagemann et al., "Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and Treg cells," *Nature*, vol. 475, no. 7355, pp. 226–230, 2011.

[36] H. Kai, T. Kadono, T. Kakinuma et al., "CCR10 and CCL27 are overexpressed in cutaneous squamous cell carcinoma," *Pathology Research and Practice*, vol. 207, no. 1, pp. 43–48, 2011.

[37] R. Grepin, M. Guyot, S. Giuliano et al., "The CXCL7/CXCR1/2 axis is a key driver in the growth of clear cell renal cell carcinoma," *Cancer Research*, vol. 74, no. 3, pp. 873–883, 2014.

[38] J. Busch-Petersen, "Small molecule antagonists of the CXCR2 and CXCR1 chemokine receptors as therapeutic agents for the treatment of inflammatory diseases," *Current Topics in Medicinal Chemistry*, vol. 6, no. 13, pp. 1345–1352, 2006.

[39] S. Segerer and P. J. Nelson, "Chemokines in renal diseases.," *TheScientificWorldJournal*, vol. 5, pp. 835–844, 2005.

[40] A. Stofas, G. Levidou, C. Piperi et al., "The role of CXC-chemokine receptor CXCR2 and suppressor of cytokine signaling-3 (SOCS-3) in renal cell carcinoma," *BMC Cancer*, vol. 14, article 149, 2014.

[41] M. Veenstra and R. M. Ransohoff, "Chemokine receptor CXCR2: physiology regulator and neuroinflammation controller?" *Journal of Neuroimmunology*, vol. 246, no. 1-2, pp. 1–9, 2012.

[42] S. Oghumu, S. Varikuti, C. Terrazas et al., "CXCR3 deficiency enhances tumor progression by promoting macrophage M2 polarization in a murine breast cancer model," *Immunology*, 2014.

[43] N. Giuliani, S. Bonomini, P. Romagnani et al., "CXCR3 and its binding chemokines in myeloma cells: expression of isoforms and potential relationships with myeloma cell proliferation and survival," *Haematologica*, vol. 91, no. 11, pp. 1489–1497, 2006.

[44] M. Cojoc, C. Peitzsch, F. Trautmann, L. Polishchuk, G. D. Telegeev, and A. Dubrovska, "Emerging targets in cancer management: role of the CXCL12/CXCR4 axis," *OncoTargets and Therapy*, vol. 6, pp. 1347–1361, 2013.

[45] F. del Grosso, S. Coco, P. Scaruffi et al., "Role of CXCL13-CXCR5 crosstalk between malignant neuroblastoma cells and Schwannian stromal cells in neuroblastic tumors," *Molecular Cancer Research*, vol. 9, no. 7, pp. 815–823, 2011.

[46] M. Krumbholz, D. Theil, S. Cepok et al., "Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment," *Brain*, vol. 129, no. 1, pp. 200–211, 2006.

[47] C. A. M. La Porta, "CXCR6: the role of environment in tumor progression. Challenges for therapy," *Stem Cell Reviews and Reports*, vol. 8, no. 4, pp. 1282–1285, 2012.

[48] X. Sun, G. Cheng, M. Hao et al., "CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression," *Cancer and Metastasis Reviews*, vol. 29, no. 4, pp. 709–722, 2010.

[49] M. Esencay, Y. Sarfraz, and D. Zagzag, "CXCR7 is induced by hypoxia and mediates glioma cell migration towards SDF-1alpha," *BMC Cancer*, vol. 13, article 347, 2013.

[50] J. Liberman, H. Sartelet, M. Flahaut et al., "Involvement of the CXCR7/CXCR4/CXCL12 axis in the malignant progression of human neuroblastoma," *PLoS ONE*, vol. 7, no. 8, Article ID e43665, 2012.

[51] E. Ferretti, M. Bertolotto, S. Deaglio et al., "A novel role of the CX_3CR1/CX_3CL1 system in the cross-talk between chronic lymphocytic leukemia cells and tumor microenvironment," *Leukemia*, vol. 25, no. 8, pp. 1268–1277, 2011.

[52] J. Zhang and J. M. Patel, "Role of the CX3CL1-CX3CR1 axis in chronic inflammatory lung diseases," *International Journal of Clinical and Experimental Medicine*, vol. 3, no. 3, pp. 233–244, 2010.

[53] Y. Lei and Y. Takahama, "XCL1 and XCR1 in the immune system," *Microbes and Infection*, vol. 14, no. 3, pp. 262–267, 2012.

[54] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell*, vol. 144, no. 5, pp. 646–674, 2011.

[55] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, no. 6917, pp. 860–867, 2002.

[56] K. Franciszkiewicz, A. Boissonnas, M. Boutet, C. Combadière, and F. Mami-Chouaib, "Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response," *Cancer Research*, vol. 72, no. 24, pp. 6325–6332, 2012.

[57] N. Iida, Y. Nakamoto, T. Baba et al., "Tumor cell apoptosis induces tumor-specific immunity in a CC chemokine receptor 1- and 5-dependent manner in mice," *Journal of Leukocyte Biology*, vol. 84, no. 4, pp. 1001–1010, 2008.

[58] J. Charles, J. Di Domizio, D. Salameire et al., "Characterization of circulating dendritic cells in melanoma: Role of CCR6 in plasmacytoid dendritic cell recruitment to the tumor," *Journal of Investigative Dermatology*, vol. 130, no. 6, pp. 1646–1656, 2010.

[59] Y. Lu, S. Hong, H. Li et al., "Th9 cells promote antitumor immune responses in vivo," *The Journal of Clinical Investigation*, vol. 122, no. 11, pp. 4160–4171, 2012.

[60] J. Pahne-Zeppenfeld, N. Schroer, B. Walch-Ruckheim et al., "Cervical cancer cell-derived interleukin-6 impairs CCR7-dependent migration of MMP-9 expressing dendritic cells," *International Journal of Cancer*, vol. 134, no. 9, pp. 2061–2073, 2014.

[61] L. V. Ramanathapuram, D. Hopkin, and Z. B. Kurago, "Dendritic cells (DC) facilitate detachment of squamous carcinoma cells (SCC), while SCC promote an immature CD16+ DC phenotype and control DC migration," *Cancer Microenvironment*, vol. 6, no. 1, pp. 41–55, 2013.

[62] P. Middel, S. Brauneck, W. Meyer, and H. Radzun, "Chemokine-mediated distribution of dendritic cell subsets in renal cell carcinoma," *BMC Cancer*, vol. 10, article 578, 2010.

[63] C. Liu, Y. Lou, G. Lizée et al., "Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice," *Journal of Clinical Investigation*, vol. 118, no. 3, pp. 1165–1175, 2008.

[64] J. L. Wong, E. Berk, R. P. Edwards, and P. Kalinski, "IL-18-primed helper NK cells collaborate with dendritic cells to promote recruitment of effector $CD8^+$ T cells to the tumor microenvironment," *Cancer Research*, vol. 73, no. 15, pp. 4653–4662, 2013.

[65] K. Kajitani, Y. Tanaka, K. Arihiro, T. Kataoka, and H. Ohdan, "Mechanistic analysis of the antitumor efficacy of human natural killer cells against breast cancer cells," *Breast Cancer Research and Treatment*, vol. 134, no. 1, pp. 139–155, 2012.

[66] A. Hosoi, H. Matsushita, K. Shimizu et al., "Adoptive cytotoxic T lymphocyte therapy triggers a counter-regulatory immunosuppressive mechanism via recruitment of myeloid-derived suppressor cells," *International Journal of Cancer*, vol. 134, no. 8, pp. 1810–1822, 2014.

[67] A. Fialová, S. Partlová, L. Sojka et al., "Dynamics of T-cell infiltration during the course of ovarian cancer: the gradual shift from a Th17 effector cell response to a predominant infiltration by regulatory T-cells," *International Journal of Cancer*, vol. 132, no. 5, pp. 1070–1079, 2013.

[68] S. Cho, K. Koizumi, N. Takeno et al., "Anti-tumor effect of combining CC chemokine 22 and an anti-CD25 antibody on myeloma cells implanted subcutaneously into mice," *Molecular Medicine Reports*, vol. 2, no. 5, pp. 773–777, 2009.

[69] Y. C. Nesbeth, D. G. Martinez, S. Toraya et al., "$CD4^+$ T cells elicit host immune responses to MHC class II^- ovarian cancer through CCL5 secretion and CD40-mediated licensing of dendritic cells," *The Journal of Immunology*, vol. 184, no. 10, pp. 5654–5662, 2010.

[70] D. Bell, P. Chomarat, D. Broyles et al., "In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas," *The Journal of Experimental Medicine*, vol. 190, no. 10, pp. 1417–1425, 1999.

[71] M. C. Thomachot, N. Bendriss-Vermare, C. Massacrier et al., "Breast carcinoma cells promote the differentiation of $CD34^+$ progenitors towards 2 different subpopulations of dendritic cells with $CD1a^{high}CD86^-$Langerin- and $CD1a^+CD86^+$Langerin$^+$ phenotypes," *International Journal of Cancer*, vol. 110, no. 5, pp. 710–720, 2004.

[72] T. Fushimi, A. Kojima, M. A. S. Moore, and R. G. Crystal, "Macrophage inflammatory protein 3α transgene attracts dendritic cells to established murine tumors and suppresses tumor growth," *Journal of Clinical Investigation*, vol. 105, no. 10, pp. 1383–1393, 2000.

[73] A. Biragyn, M. Surenhu, D. Yang et al., "Mediators of innate immunity that target immature, but not mature, dendritic cells induce antitumor immunity when genetically fused with nonimmunogenic tumor antigens," *Journal of Immunology*, vol. 167, no. 11, pp. 6644–6653, 2001.

[74] H. Ohtani, T. Nakayama, and O. Yoshie, "*In situ* expression of the CCL20-CCR6 axis in lymphocyte-rich gastric cancer and its potential role in the formation of lymphoid stroma," *Pathology International*, vol. 61, no. 11, pp. 645–651, 2011.

[75] K. Tsuge, H. Takeda, S. Kawada, K. Maeda, and M. Yamakawa, "Characterization of dendritic cells in differentiated thyroid cancer," *Journal of Pathology*, vol. 205, no. 5, pp. 565–576, 2005.

[76] S. Wu, W. Xing, J. Peng et al., "Tumor transfected with CCL21 enhanced reactivity and apoptosis resistance of human

monocyte-derived dendritic cells," *Immunobiology*, vol. 213, no. 5, pp. 417–426, 2008.

[77] C. Alfaro, N. Suárez, I. Martínez-Forero et al., "Carcinoma-derived Interleukin-8 disorients dendritic cell migration without impairing T-cell stimulation," *PLoS ONE*, vol. 6, no. 3, Article ID e17922, 2011.

[78] E. Feijoó, C. Alfaro, G. Mazzolini et al., "Dendritic cells delivered inside human carcinomas are sequestered by interleukin-8," *International Journal of Cancer*, vol. 116, no. 2, pp. 275–281, 2005.

[79] S. Asfaha, A. N. Dubeykovskiy, H. Tomita et al., "Mice that express human interleukin-8 have increased mobilization of immature myeloid cells, which exacerbates inflammation and accelerates colon carcinogenesis," *Gastroenterology*, vol. 144, no. 1, pp. 155–166, 2013.

[80] Q. Fan, D. Gu, H. Liu et al., "Defective TGF-beta signaling in bone marrow-derived cells prevents hedgehog-induced skin tumors," *Cancer Research*, vol. 74, no. 2, pp. 471–483, 2014.

[81] H. Katoh, D. Wang, T. Daikoku, H. Sun, S. K. Dey, and R. N. Dubois, "CXCR2-expressing myeloid-derived suppressor cells are essential to promote colitis-associated tumorigenesis," *Cancer Cell*, vol. 24, no. 5, pp. 631–644, 2013.

[82] Y. Zhuang, L. S. Peng, Y. L. Zhao et al., "$CD8^+$ T cells that produce interleukin-17 regulate myeloid-derived suppressor cells and are associated with survival time of patients with gastric cancer," *Gastroenterology*, vol. 143, no. 4, pp. 951.e8–962.e8, 2012.

[83] N. Obermajer, R. Muthuswamy, K. Odunsi, R. P. Edwards, and P. Kalinski, "PGE 2-induced CXCL 12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment," *Cancer Research*, vol. 71, no. 24, pp. 7463–7470, 2011.

[84] A. L. L. Bachi, L. C. dos Santos, S. Nonogaki, S. Jancar, and M. G. Jasiulionis, "Apoptotic cells contribute to melanoma progression and this effect is partially mediated by the platelet-activating factor receptor," *Mediators of Inflammation*, vol. 2012, Article ID 610371, 6 pages, 2012.

[85] B. Qian, J. Li, H. Zhang et al., "CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis," *Nature*, vol. 475, no. 7355, pp. 222–225, 2011.

[86] J. C. Pahler, S. Tazzyman, N. Erez et al., "Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response," *Neoplasia*, vol. 10, no. 4, pp. 329–339, 2008.

[87] B. K. Popivanova, F. I. Kostadinova, K. Furuichi et al., "Blockade of a chemokine, CCL2, reduces chronic colitis-associated carcinogenesis in mice," *Cancer Research*, vol. 69, no. 19, pp. 7884–7892, 2009.

[88] G. Ren, X. Zhao, Y. Wang et al., "CCR2-dependent recruitment of macrophages by tumor-educated mesenchymal stromal cells promotes tumor development and is mimicked by TNFα," *Cell Stem Cell*, vol. 11, no. 6, pp. 812–824, 2012.

[89] K. F. Tang, S. Y. Tan, S. H. Chan et al., "A distinct expression of CC chemokines by macrophages in nasopharyngeal carcinoma: implication for the intense tumor infiltration by T lymphocytes and macrophages," *Human Pathology*, vol. 32, no. 1, pp. 42–49, 2001.

[90] G. Jin, H. I. Kawsar, S. A. Hirsch et al., "An antimicrobial peptide regulates tumor-associated macrophage trafficking via the chemokine receptor CCR2, a model for tumorigenesis," *PLoS ONE*, vol. 5, no. 6, Article ID e10993, 2010.

[91] K. Izumi, L.-Y. Fang, A. Mizokami et al., "Targeting the androgen receptor with siRNA promotes prostate cancer metastasis through enhanced macrophage recruitment via CCL2/CCR2-induced STAT3 activation," *EMBO Molecular Medicine*, vol. 5, no. 9, pp. 1383–1401, 2013.

[92] D. E. Sanford, B. A. Belt, R. Z. Panni et al., "Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 axis," *Clinical Cancer Research*, vol. 19, no. 13, pp. 3404–3415, 2013.

[93] M. Ryder, M. Gild, T. M. Hohl et al., "Genetic and pharmacological targeting of CSF-1/CSF-1R inhibits tumor-associated macrophages and impairs BRAF-induced thyroid cancer progression," *PLoS ONE*, vol. 8, no. 1, Article ID e54302, 2013.

[94] Y. Liu, K. Chen, C. Wang et al., "Cell surface receptor FPR2 promotes antitumor host defense by limiting M2 polarization of macrophages," *Cancer Research*, vol. 73, no. 2, pp. 550–560, 2013.

[95] N. Barashi, I. D. Weiss, O. Wald et al., "Inflammation-induced hepatocellular carcinoma is dependent on CCR5 in mice," *Hepatology*, vol. 58, no. 3, pp. 1021–1030, 2013.

[96] J. R. Reed, M. D. Stone, T. C. Beadnell, Y. Ryu, T. J. Griffin, and K. L. Schwertfeger, "Fibroblast growth factor receptor 1 activation in mammary tumor cells promotes macrophage recruitment in a CX3CL1-dependent manner," *PLoS ONE*, vol. 7, no. 9, Article ID e45877, 2012.

[97] J. Held-Feindt, K. Hattermann, S. S. Müerköster et al., "CX3CR1 promotes recruitment of human glioma-infiltrating microglia/macrophages (GIMs)," *Experimental Cell Research*, vol. 316, no. 9, pp. 1553–1566, 2010.

[98] M. Gobert, I. Treilleux, N. Bendriss-Vermare et al., "Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical utcome," *Cancer Research*, vol. 69, no. 5, pp. 2000–2009, 2009.

[99] A. Battaglia, A. Buzzonetti, E. Martinelli et al., "Selective changes in the immune profile of tumor-draining lymph nodes after different neoadjuvant chemoradiation regimens for locally advanced cervical cancer," *International Journal of Radiation Oncology Biology Physics*, vol. 76, no. 5, pp. 1546–1553, 2010.

[100] T. Ishida, T. Ishii, A. Inagaki et al., "Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege," *Cancer Research*, vol. 66, no. 11, pp. 5716–5722, 2006.

[101] K. Enarsson, A. Lundgren, B. Kindlund et al., "Function and recruitment of mucosal regulatory T cells in human chronic *Helicobacter pylori* infection and gastric adenocarcinoma," *Clinical Immunology*, vol. 121, no. 3, pp. 358–368, 2006.

[102] J. T. Jordan, W. Sun, S. F. Hussain, G. DeAngulo, S. S. Prabhu, and A. B. Heimberger, "Preferential migration of regulatory T cells mediated by glioma-secreted chemokines can be blocked with chemotherapy," *Cancer Immunology, Immunotherapy*, vol. 57, no. 1, pp. 123–131, 2008.

[103] S. Kimpfler, A. Sevko, S. Ring et al., "Skin melanoma development in ret transgenic mice despite the depletion of CD25+Foxp3+ regulatory T cells in lymphoid organs," *Journal of Immunology*, vol. 183, no. 10, pp. 6330–6337, 2009.

[104] L. Y. Chang, Y. C. Lin, J. Mahalingam et al., "Tumor-derived chemokine CCL5 enhances TGF-β-mediated killing of CD8+ T cells in colon cancer by T-regulatory cells," *Cancer Research*, vol. 72, no. 5, pp. 1092–1102, 2012.

[105] E. Schlecker, A. Stojanovic, C. Eisen et al., "Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth," *Journal of Immunology*, vol. 189, no. 12, pp. 5602–5611, 2012.

[106] M. C. B. Tan, P. S. Goedegebuure, B. A. Belt et al., "Disruption of CCR5-dependent homing of regulatory T cells inhibits tumor growth in a murine model of pancreatic cancer," *Journal of Immunology*, vol. 182, no. 3, pp. 1746–1755, 2009.

[107] K. A. Oldham, G. Parsonage, R. I. Bhatt et al., "T lymphocyte recruitment into renal cell carcinoma tissue: a role for chemokine receptors CXCR3, CXCR6, CCR5, and CCR6," *European Urology*, vol. 61, no. 2, pp. 385–394, 2012.

[108] L. Xu, W. Xu, S. Qiu, and S. Xiong, "Enrichment of $CCR6^{+}Foxp3^{+}$ regulatory T cells in the tumor mass correlates with impaired $CD8^{+}$ T cell function and poor prognosis of breast cancer," *Clinical Immunology*, vol. 135, no. 3, pp. 466–475, 2010.

[109] J. Liu, N. Zhang, Q. Li et al., "Tumor-associated macrophages recruit CCR6+ regulatory T cells and promote the development of colorectal cancer via enhancing CCL20 production in mice," *PLoS ONE*, vol. 6, no. 4, Article ID e19495, 2011.

[110] K. Chen, S. Lin, L. Zhou et al., "Selective recruitment of regulatory T cell through CCR6-CCL20 in hepatocellular carcinoma fosters tumor progression and predicts poor prognosis," *PLoS ONE*, vol. 6, no. 9, Article ID e24671, 2011.

[111] B. Lamprecht, S. Kreher, I. Anagnostopoulos et al., "Aberrant expression of the Th2 cytokine IL-21 in Hodgkin lymphoma cells regulates STAT3 signaling and attracts T_{reg} cells via regulation of MIP-3α," *Blood*, vol. 112, no. 8, pp. 3339–3347, 2008.

[112] J. D. Shields, I. C. Kourtis, A. A. Tomei, J. M. Roberts, and M. A. Swartz, "Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL_{21}," *Science*, vol. 328, no. 5979, pp. 749–752, 2010.

[113] S. Wei, I. Kryczek, L. Zou et al., "Plasmacytoid dendritic cells induce CD8+ regulatory T cells in human ovarian carcinoma," *Cancer Research*, vol. 65, no. 12, pp. 5020–5026, 2005.

[114] S. Eikawa, Y. Ohue, K. Kitaoka et al., "Enrichment of $Foxp3^{+}$ CD4 regulatory T cells in migrated T cells to IL-6-and IL-8-expressing tumors through predominant induction of CXCR1 by IL-6," *Journal of Immunology*, vol. 185, no. 11, pp. 6734–6740, 2010.

[115] M. Yan, N. Jene, D. Byrne et al., "Recruitment of regulatory T cells is correlated with hypoxia-induced CXCR4 expression, and is associated with poor prognosis in basal-like breast cancers," *Breast Cancer Research*, vol. 13, article R47, no. 2, 2011.

[116] C. Dürr, D. Pfeifer, R. Claus et al., "CXCL12 mediates immunosuppression in the lymphoma microenvironment after allogeneic transplantation of hematopoietic cells," *Cancer Research*, vol. 70, no. 24, pp. 10170–10181, 2010.

[117] Q. Li, J. M. Bao, X. L. Li, T. Zhang, and X. H. Shen, "Inhibiting effect of astragalus polysaccharides on the functions of CD4+CD25 hightreg cells in the tumor microenvironment of human hepatocellular carcinoma," *Chinese Medical Journal*, vol. 125, no. 5, pp. 786–793, 2012.

[118] O. Wald, U. Izhar, G. Amir et al., "CD4+CXCR4highCD69+ T cells accumulate in lung adenocarcinoma," *Journal of Immunology*, vol. 177, no. 10, pp. 6983–6990, 2006.

[119] O. M. Grauer, S. Nierkens, E. Bennink et al., "CD4+FoxP3+ regulatory T cells gradually accumulate in gliomas during tumor growth and efficiently suppress antiglionia immune responses in vivo," *International Journal of Cancer*, vol. 121, no. 1, pp. 95–105, 2007.

[120] S. Wei, I. Kryczek, R. P. Edwards et al., "Interleukin-2 administration alters the CD4+FOXP3+ T-cell pool and tumor trafficking in patients with ovarian carcinoma," *Cancer Research*, vol. 67, no. 15, pp. 7487–7494, 2007.

[121] E. Righi, S. Kashiwagi, J. Yuan et al., "CXCL12/CXCR4 blockade induces multimodal antitumor effects that prolong survival in an immunocompetent mouse model of ovarian cancer," *Cancer Research*, vol. 71, no. 16, pp. 5522–5534, 2011.

[122] G. Parsonage, L. R. MacHado, J. W. Hui et al., "CXCR6 and CCR5 localize T lymphocyte subsets in nasopharyngeal carcinoma," *The American Journal of Pathology*, vol. 180, no. 3, pp. 1215–1222, 2012.

[123] S. B. Coffelt, F. C. Marini, K. Watson et al., "The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through recruitment of multipotent mesenchymal stromal cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 10, pp. 3806–3811, 2009.

[124] A. H. Klopp, E. L. Spaeth, J. L. Dembinski et al., "Tumor irradiation increases the recruitment of circulating mesenchymal stem cells into the tumor microenvironment," *Cancer Research*, vol. 67, no. 24, pp. 11687–11695, 2007.

[125] F. Xu, J. Shi, B. Yu, W. Ni, X. Wu, and Z. Gu, "Chemokines mediate mesenchymal stem cell migration toward gliomas in vitro," *Oncology Reports*, vol. 23, no. 6, pp. 1561–1567, 2010.

[126] S. M. Kim, D. S. Kim, C. H. Jeong et al., "CXC chemokine receptor 1 enhances the ability of human umbilical cord blood-derived mesenchymal stem cells to migrate toward gliomas," *Biochemical and Biophysical Research Communications*, vol. 407, no. 4, pp. 741–746, 2011.

[127] D. Kim, J. H. Kim, J. Kwon Lee et al., "Overexpression of CXC chemokine receptors is required for the superior glioma-tracking property of umbilical cord blood-derived mesenchymal stem cells," *Stem Cells and Development*, vol. 18, no. 3, pp. 511–519, 2009.

[128] B. Liang-Kuan, Z. Nan, L. Cheng et al., "Kidney cancer cells secrete IL-8 to activate Akt and promote migration of mesenchymal stem cells," *Urologic Oncology*, vol. 32, no. 5, pp. 607–612, 2014.

[129] H. Gao, W. Priebe, J. Glod, and D. Banerjee, "Activation of signal transducers and activators of transcription 3 and focal adhesion kinase by stromal cell-derived factor 1 is required for migration of human mesenchymal stem cells in response to tumor cell-conditioned medium," *Stem Cells*, vol. 27, no. 4, pp. 857–865, 2009.

[130] S. Fakhari, E. Kalantar, M. Nikzaban et al., "Effect of Helicobacter pylori infection on stromal-derived factor-1/CXCR4 axis in bone marrow-derived mesenchymal stem cells," *Advanced Biomedical Research*, vol. 3, p. 19, 2014.

[131] S. A. Park, C. H. Ryu, S. M. Kim et al., "CXCR4-transfected human umbilical cord blood-derived mesenchymal stem cells exhibit enhanced migratory capacity toward gliomas," *International Journal of Oncology*, vol. 38, no. 1, pp. 97–103, 2011.

[132] J. Behnan, P. Isakson, M. Joel et al., "Recruited brain tumor-derived mesenchymal stem cells contribute to brain tumor progression," *Stem Cells*, vol. 32, no. 5, pp. 1110–1123, 2014.

[133] Y. Jung, J. K. Kim, Y. Shiozawa et al., "Recruitment of mesenchymal stem cells into prostate tumours promotes metastasis," *Nature Communications*, vol. 4, article 1795, 2013.

[134] Y. F. Huang, M. J. Chen, M. H. Wu, and S. C. Hung, "The use of hypoxic cultured mesenchymal stem cell for oncolytic virus therapy," *Cancer Gene Therapy*, vol. 20, no. 5, pp. 308–316, 2013.

[135] J. F. M. Jacobs, A. J. Idema, K. F. Bol et al., "Prognostic significance and mechanism of Treg infiltration in human brain tumors," *Journal of Neuroimmunology*, vol. 225, no. 1-2, pp. 195–199, 2010.

[136] Y. Watanabe, F. Katou, H. Ohtani, T. Nakayama, O. Yoshie, and K. Hashimoto, "Tumor-infiltrating lymphocytes, particularly the balance between $CD8^+$ T cells and $CCR4^+$ regulatory T cells, affect the survival of patients with oral squamous cell carcinoma," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*, vol. 109, no. 5, pp. 744–752, 2010.

[137] D. Sugiyama, H. Nishikawa, Y. Maeda et al., "Anti-CCR4 mAb selectively depletes effector-type $FoxP3^+CD4^+$ regulatory T cells, evoking antitumor immune responses in humans," *Proceedings of the National Academy of Sciences of the USA*, vol. 110, no. 44, pp. 17945–17950, 2013.

[138] S. Spranger, R. M. Spaapen, Y. Zha et al., "Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells," *Science Translational Medicine*, vol. 5, no. 200, p. 200ra116, 2013.

[139] P. B. Olkhanud, D. Baatar, M. Bodogai et al., "Breast cancer lung metastasis requires expression of chemokine receptor CCR4 and regulatory T cells," *Cancer Research*, vol. 69, no. 14, pp. 5996–6004, 2009.

[140] A. W. Mailloux and M. R. I. Young, "NK-dependent increases in CCL22 secretion selectively recruits regulatory T cells to the tumor microenvironment," *The Journal of Immunology*, vol. 182, no. 5, pp. 2753–2765, 2009.

[141] C. Ménétrier-Caux, M. Gobert, and C. Caux, "Differences in tumor regulatory T-cell localization and activation status impact patient outcome," *Cancer Research*, vol. 69, no. 20, pp. 7895–7898, 2009.

[142] J. A. Joyce and J. W. Pollard, "Microenvironmental regulation of metastasis," *Nature Reviews Cancer*, vol. 9, no. 4, pp. 239–252, 2009.

[143] J. Vakkila and M. T. Lotze, "Inflammation and necrosis promote tumour growth," *Nature Reviews Immunology*, vol. 4, no. 8, pp. 641–648, 2004.

[144] J. E. Talmadge, M. Donkor, and E. Scholar, "Inflammatory cell infiltration of tumors: Jekyll or Hyde," *Cancer and Metastasis Reviews*, vol. 26, no. 3-4, pp. 373–400, 2007.

[145] D. M. Mosser and J. P. Edwards, "Exploring the full spectrum of macrophage activation," *Nature Reviews Immunology*, vol. 8, no. 12, pp. 958–969, 2008.

[146] J. W. Pollard, "Tumour-educated macrophages promote tumour progression and metastasis," *Nature Reviews Cancer*, vol. 4, no. 1, pp. 71–78, 2004.

[147] V. A. Fadok, D. L. Bratton, and P. M. Henson, "Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences," *Journal of Clinical Investigation*, vol. 108, no. 7, pp. 957–962, 2001.

[148] X. Lu and Y. Kang, "Chemokine (C-C motif) ligand 2 engages $CCR2^+$ stromal cells of monocytic origin to promote breast cancer metastasis to lung and bone," *The Journal of Biological Chemistry*, vol. 284, no. 42, pp. 29087–29096, 2009.

[149] J. Zheng, M. Yang, J. Shao, Y. Miao, J. Han, and J. Du, "Chemokine receptor CX3CR1 contributes to macrophage survival in tumor metastasis," *Molecular Cancer*, vol. 12, no. 1, article 141, 2013.

[150] A. M. Lesokhin, T. M. Hohl, S. Kitano et al., "Monocytic $CCR2^+$ myeloid-derived suppressor cells promote immune escape by limiting activated CD8 T-cell infiltration into the tumor microenvironment," *Cancer Research*, vol. 72, no. 4, pp. 876–886, 2012.

[151] L. Zhao, S. Y. Lim, A. N. Gordon-Weeks et al., "Recruitment of a myeloid cell subset (CD11b/Gr1mid) via CCL2/CCR2 promotes the development of colorectal cancer liver metastasis," *Hepatology*, vol. 57, no. 2, pp. 829–839, 2013.

[152] Y. Sawanobori, S. Ueha, M. Kurachi et al., "Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice," *Blood*, vol. 111, no. 12, pp. 5457–5466, 2008.

[153] S. Nagaraj and D. I. Gabrilovich, "Myeloid-derived suppressor cells," *Advances in Experimental Medicine and Biology*, vol. 601, pp. 213–223, 2007.

[154] A. Sica and V. Bronte, "Altered macrophage differentiation and immune dysfunction in tumor development," *The Journal of Clinical Investigation*, vol. 117, no. 5, pp. 1155–1166, 2007.

[155] M. Quante, S. P. Tu, H. Tomita et al., "Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth," *Cancer Cell*, vol. 19, no. 2, pp. 257–272, 2011.

[156] E. L. Spaeth, J. L. Dembinski, A. K. Sasser et al., "Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression," *PLoS ONE*, vol. 4, no. 4, Article ID e4992, 2009.

[157] T. Miyagaki, M. Sugaya, H. Fujita et al., "Eotaxins and CCR3 interaction regulates the Th2 environment of cutaneous T-Cell lymphoma," *Journal of Investigative Dermatology*, vol. 130, no. 9, pp. 2304–2311, 2010.

[158] B. A. Babbin, W. Y. Lee, C. A. Parkos et al., "Annexin I regulates SKCO-15 cell invasion by signaling through formyl peptide receptors," *Journal of Biological Chemistry*, vol. 281, no. 28, pp. 19588–19599, 2006.

[159] T. Y. Cheng, M. S. Wu, J. T. Lin et al., "Annexin A1 is associated with gastric cancer survival and promotes gastric cancer cell invasiveness through the formyl peptide receptor/extracellular signal-regulated kinase/integrin beta-1-binding protein 1 pathway," *Cancer*, vol. 118, no. 23, pp. 5757–5767, 2012.

[160] C. P. Xu, H. R. Zhang, F. L. Chen et al., "Human malignant glioma cells expressing functional formylpeptide receptor recruit endothelial progenitor cells for neovascularization," *International Immunopharmacology*, vol. 10, no. 12, pp. 1602–1607, 2010.

[161] X. H. Yao, Y. F. Ping, J. H. Chen et al., "Production of angiogenic factors by human glioblastoma cells following activation of the G-protein coupled formylpeptide receptor FPR," *Journal of Neuro-Oncology*, vol. 86, no. 1, pp. 47–53, 2008.

[162] X. H. Yao, Y. F. Ping, J. H. Chen et al., "Glioblastoma stem cells produce vascular endothelial growth factor by activation of a G-protein coupled formylpeptide receptor FPR," *Journal of Pathology*, vol. 215, no. 4, pp. 369–376, 2008.

[163] D. Chen, Y. Ping, S. Yu et al., "Downregulating FPR restrains xenograft tumors by impairing the angiogenic potential and invasive capability of malignant glioma cells," *Biochemical and Biophysical Research Communications*, vol. 381, no. 3, pp. 448–452, 2009.

[164] S. B. Coffelt, S. L. Tomchuck, K. J. Zwezdaryk, E. S. Danka, and A. B. Scandurro, "Leucine leucine-37 uses formyl peptide receptor-like 1 to activate signal transduction pathways, stimulate oncogenic gene expression, and enhance the invasiveness

of ovarian cancer cells," *Molecular Cancer Research*, vol. 7, no. 6, pp. 907–915, 2009.

[165] B. Bussolati, L. Biancone, P. Cassoni et al., "PAF produced by human breast cancer cells promotes migration and proliferation of tumor cells and neo-angiogenesis," *American Journal of Pathology*, vol. 157, no. 5, pp. 1713–1725, 2000.

[166] V. O. Melnikova, K. Balasubramanian, G. J. Villares et al., "Crosstalk between protease-activated receptor 1 and platelet-activating factor receptor regulates melanoma cell adhesion molecule (MCAM/MUC18) expression and melanoma metastasis," *The Journal of Biological Chemistry*, vol. 284, no. 42, pp. 28845–28855, 2009.

[167] M. Aponte, W. Jiang, M. Lakkis et al., "Activation of platelet-activating factor receptor and pleiotropic effects on tyrosine phospho-EGFR/Src/FAK/paxillin in ovarian cancer," *Cancer Research*, vol. 68, no. 14, pp. 5839–5848, 2008.

[168] H. Nitta, Y. Wada, Y. Kawano et al., "Enhancement of human cancer cell motility and invasiveness by anaphylatoxin C5a via aberrantly expressed C5a receptor (CD88)," *Clinical Cancer Research*, vol. 19, no. 8, pp. 2004–2013, 2013.

[169] J. Gu, J. Y. Ding, C. L. Lu et al., "Overexpression of CD88 predicts poor prognosis in non-small-cell lung cancer," *Lung Cancer*, vol. 81, no. 2, pp. 259–265, 2013.

[170] A. Ihara, K. Wada, M. Yoneda, N. Fujisawa, H. Takahashi, and A. Nakajima, "Blockade of leukotriene B4 signaling pathway induces apoptosis and suppresses cell proliferation in colon cancer," *Journal of Pharmacological Sciences*, vol. 103, no. 1, pp. 24–32, 2007.

[171] E. Kim, J. Seo, C. Kim, J. Lee, K. Lee, and J. Kim, "BLT2 promotes the invasion and metastasis of aggressive bladder cancer cells through a reactive oxygen species-linked pathway," *Free Radical Biology and Medicine*, vol. 49, no. 6, pp. 1072–1081, 2010.

[172] J. M. Seo, K. J. Cho, E. Y. Kim, M. H. Choi, B. C. Chung, and J. H. Kim, "Up-regulation of BLT2 is critical for the survival of bladder cancer cells," *Experimental and Molecular Medicine*, vol. 43, no. 3, pp. 129–137, 2011.

[173] H. Kim, J. Choi, G. Park, and J. Kim, "BLT2 up-regulates interleukin-8 production and promotes the invasiveness of breast cancer cells," *PLoS ONE*, vol. 7, no. 11, Article ID e49186, 2012.

[174] R. Hennig, T. Osman, I. Esposito et al., "BLT2 is expressed in PanINs, IPMNs, pancreatic cancer and stimulates tumour cell proliferation," *British Journal of Cancer*, vol. 99, no. 7, pp. 1064–1073, 2008.

[175] M. K. Park, Y. Park, J. Shim, H. J. Lee, S. Kim, and C. H. Lee, "Novel involvement of leukotriene B4 receptor 2 through ERK activation by PP2A down-regulation in leukotriene B4-induced keratin phosphorylation and reorganization of pancreatic cancer cells," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1823, no. 12, pp. 2120–2129, 2012.

[176] J. W. Lee and J. H. Kim, "Activation of the leukotriene B_4 receptor 2-reactive oxygen species (BLT2-ROS) cascade following detachment confers anoikis resistance in prostate cancer cells," *The Journal of Biological Chemistry*, vol. 288, no. 42, pp. 30054–30063, 2013.

[177] J. Lee, G. Kim, and J. Kim, "Androgen receptor is up-regulated by a BLT2-linked pathway to contribute to prostate cancer progression," *Biochemical and Biophysical Research Communications*, vol. 420, no. 2, pp. 428–433, 2012.

[178] J. M. Seo, S. Park, and J. H. Kim, "Leukotriene B_4 receptor-2 promotes invasiveness and metastasis of ovarian cancer cells through signal transducer and activator of transcription 3 (STAT3)-dependent up-regulation of matrix metalloproteinase 2," *The Journal of Biological Chemistry*, vol. 287, no. 17, pp. 13840–13849, 2012.

[179] M. Swamydas, K. Ricci, S. L. Rego, and D. Dréau, "Mesenchymal stem cell-derived CCL-9 and CCL-5 promote mammary tumor cell invasion and the activation of matrix metalloproteinases," *Cell Adhesion and Migration*, vol. 7, no. 3, pp. 315–324, 2013.

[180] J. Kouno, H. Nagai, T. Nagahata et al., "Up-regulation of CC chemokine, CCL3L1, and receptors, CCR3, CCR5 in human glioblastoma that promotes cell growth," *Journal of Neuro-Oncology*, vol. 70, no. 3, pp. 301–307, 2004.

[181] M. Dagouassat, N. Suffee, H. Hlawaty et al., "Monocyte chemoattractant protein-1 (MCP-1)/CCL2 secreted by hepatic myofibroblasts promotes migration and invasion of human hepatoma cells," *International Journal of Cancer*, vol. 126, no. 5, pp. 1095–1108, 2010.

[182] X. Wu, J. Fan, X. Wang et al., "Downregulation of CCR1 inhibits human hepatocellular carcinoma cell invasion," *Biochemical and Biophysical Research Communications*, vol. 355, no. 4, pp. 866–871, 2007.

[183] D. W. Jung, Z. M. Che, J. Kim, K. Y. Kim, D. Williams, and J. Kim, "Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7," *International Journal of Cancer*, vol. 127, no. 2, pp. 332–344, 2010.

[184] H. Long, R. Xie, T. Xiang et al., "Autocrine CCL5 signaling promotes invasion and migration of $CD133^+$ ovarian cancer stem-like cells via NF-κB-mediated MMP-9 upregulation," *Stem Cells*, vol. 30, no. 10, pp. 2309–2319, 2012.

[185] H. Y. Chiu, K. H. Sun, S. Y. Chen et al., "Autocrine CCL2 promotes cell migration and invasion via PKC activation and tyrosine phosphorylation of paxillin in bladder cancer cells," *Cytokine*, vol. 59, no. 2, pp. 423–432, 2012.

[186] W. B. Fang, I. Jokar, A. Zou, D. Lambert, P. Dendukuri, and N. Cheng, "CCL2/CCR2 chemokine signaling coordinates survival and motility of breast cancer cells through Smad3 protein- and p42/44 mitogen-activated protein kinase (MAPK)-dependent mechanisms," *The Journal of Biological Chemistry*, vol. 287, no. 43, pp. 36593–36608, 2012.

[187] J. Moreaux, D. Hose, A. Kassambara et al., "Osteoclast-gene expression profiling reveals osteoclast-derived CCR2 chemokines promoting myeloma cell migration," *Blood*, vol. 117, no. 4, pp. 1280–1290, 2011.

[188] S. Furukawa, S. Soeda, Y. Kiko et al., "MCP-1 promotes invasion and adhesion of human ovarian cancer cells," *Anticancer Research*, vol. 33, no. 11, pp. 4785–4790, 2013.

[189] V. Levina, B. M. Nolen, A. M. Marrangoni et al., "Role of eotaxin-1 signaling in ovarian cancer," *Clinical Cancer Research*, vol. 15, no. 8, pp. 2647–2656, 2009.

[190] J. Gao, A. Wang, M. Zhang et al., "RNAi targeting of CCR2 gene expression induces apoptosis and inhibits the proliferation, migration, and invasion of PC-3M cells," *Oncology Research*, vol. 21, no. 2, pp. 73–82, 2014.

[191] T. H. Lin, H. H. Liu, T. H. Tsai et al., "CCL2 increases $\alpha v\alpha 3$ integrin expression and subsequently promotes prostate cancer migration," *Biochimica et Biophysica Acta*, vol. 1830, no. 10, pp. 4917–4927, 2013.

[192] T. Miyagaki, M. Sugaya, T. Murakami et al., "CCL11-CCR3 interactions promote survival of anaplastic large cell lymphoma cells via ERK1/2 activation," *Cancer Research*, vol. 71, no. 6, pp. 2056–2065, 2011.

[193] K. Jöhrer, C. Zelle-Rieser, A. Perathoner et al., "Up-regulation of functional chemokine receptor CCR3 in human renal cell carcinoma," *Clinical Cancer Research*, vol. 11, no. 7, pp. 2459–2465, 2005.

[194] J. Y. Li, Z. L. Ou, S. J. Yu et al., "The chemokine receptor CCR4 promotes tumor growth and lung metastasis in breast cancer," *Breast Cancer Research and Treatment*, vol. 131, no. 3, pp. 837–848, 2012.

[195] A. A. Al-haidari, I. Syk, K. Jirström, and H. Thorlacius, "CCR4 mediates CCL17 (TARC)-induced migration of human colon cancer cells via RhoA/Rho-kinase signaling," *International Journal of Colorectal Disease*, vol. 28, no. 11, pp. 1479–1487, 2013.

[196] J. H. Lee, Y. Cho, J. Y. Lee et al., "The chemokine receptor CCR4 is expressed and associated with a poor prognosis in patients with gastric cancer," *Annals of Surgery*, vol. 249, no. 6, pp. 933–941, 2009.

[197] S. Izraely, A. Klein, O. Sagi-Assif et al., "Chemokine-chemokine receptor axes in melanoma brain metastasis," *Immunology Letters*, vol. 130, no. 1-2, pp. 107–114, 2010.

[198] T. Tsujikawa, T. Yaguchi, G. Ohmura et al., "Autocrine and paracrine loops between cancer cells and macrophages promote lymph node metastasis via CCR4/CCL22 in head and neck squamous cell carcinoma," *International Journal of Cancer*, vol. 132, no. 12, pp. 2755–2766, 2013.

[199] Y. Zhang, F. Yao, X. Yao et al., "Role of CCL5 in invasion, proliferation and proportion of CD44+/CD24- phenotype of MCF-7 cells and correlation of CCL5 and CCR5 expression with breast cancer progression," *Oncology Reports*, vol. 21, no. 4, pp. 1113–1121, 2009.

[200] M. Velasco-Velázquez, X. Jiao, M. de La Fuente et al., "CCR5 antagonist blocks metastasis of basal breast cancer cells," *Cancer Research*, vol. 72, no. 15, pp. 3839–3850, 2012.

[201] M. Velasco-Velazquez and R. G. Pestell, "The CCL5/CCR5 axis promotes metastasis in basal breast cancer," *Oncoimmunology*, vol. 2, no. 4, Article ID e23660, 2013.

[202] T. T. Murooka, R. Rahbar, and E. N. Fish, "CCL5 promotes proliferation of MCF-7 cells through mTOR-dependent mRNA translation," *Biochemical and Biophysical Research Communications*, vol. 387, no. 2, pp. 381–386, 2009.

[203] B. Cambien, P. Richard-Fiardo, B. F. Karimdjee et al., "CCL5 neutralization restricts cancer growth and potentiates the targeting of PDGFRβ in colorectal carcinoma," *PLoS ONE*, vol. 6, no. 12, Article ID e28842, 2011.

[204] Z. Cao, X. Xu, X. Luo et al., "Role of RANTES and its receptor in gastric cancer metastasis," *Journal of Huazhong University of Science and Technology—Medical Science*, vol. 31, no. 3, pp. 342–347, 2011.

[205] D. Aldinucci, D. Lorenzon, L. Cattaruzza et al., "Expression of CCR5 receptors on Reed-Sternberg cells and Hodgkin lymphoma cell lines: involvement of CCL5/Rantes in tumor cell growth and microenvironmental interactions," *International Journal of Cancer*, vol. 122, no. 4, pp. 769–776, 2008.

[206] J. Chuang, W. Yang, H. Chen et al., "CCL5/CCR5 axis promotes the motility of human oral cancer cells," *Journal of Cellular Physiology*, vol. 220, no. 2, pp. 418–426, 2009.

[207] C. Rubie, V. Oliveira, K. Kempf et al., "Involvement of chemokine receptor CCR6 in colorectal cancer metastasis," *Tumor Biology*, vol. 27, no. 3, pp. 166–174, 2006.

[208] S. Brand, T. Olszak, F. Beigel et al., "Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK, and Akt signaling resulting in proliferation and migration of colorectal cancer cells," *Journal of Cellular Biochemistry*, vol. 97, no. 4, pp. 709–723, 2006.

[209] C. Rubie, V. O. Frick, M. Wagner et al., "Chemokine expression in hepatocellular carcinoma versus colorectal liver metastases," *World Journal of Gastroenterology*, vol. 12, no. 41, pp. 6627–6633, 2006.

[210] H. Uchida, Y. Iwashita, A. Sasaki et al., "Chemokine receptor CCR6 as a prognostic factor after hepatic resection for hepatocellular carcinoma," *Journal of Gastroenterology and Hepatology*, vol. 21, no. 1, pp. 161–168, 2006.

[211] C. M. Raynaud, O. Mercier, P. Dartevelle et al., "Expression of chemokine receptor CCR6 as a molecular determinant of adrenal metastatic relapse in patients with primary lung cancer," *Clinical Lung Cancer*, vol. 11, no. 3, pp. 187–191, 2010.

[212] J. Kleeff, T. Kusama, D. L. Rossi et al., "Detection and localization of Mip-3alpha/LARC/Exodus, a macrophage proinflammatory chemokine, and its CCR6 receptor in human pancreatic cancer," *International Journal of Cancer*, vol. 81, no. 4, pp. 650–657, 1999.

[213] T. F. Kimsey, A. S. Campbell, D. Albo, and T. N. Wang, "Co-localization of macrophage inflammatory protein-3α (Mip-3α) and its receptor, CCR6, promotes pancreatic cancer cell invasion," *Cancer Journal*, vol. 10, no. 6, pp. 374–380, 2004.

[214] A. S. Campbell, D. Albo, T. F. Kimsey, S. L. White, and T. N. Wang, "Macrophage inflammatory protein-3α promotes pancreatic cancer cell invasion," *Journal of Surgical Research*, vol. 123, no. 1, pp. 96–101, 2005.

[215] J. Wang, L. Xi, J. L. Hunt et al., "Expression pattern of chemokine receptor 6 (CCR6) and CCR7 in squamous cell carcinoma of the head and neck identifies a novel metastatic phenotype," *Cancer Research*, vol. 64, no. 5, pp. 1861–1866, 2004.

[216] J. Wang, L. Xi, W. Gooding, T. E. Godfrey, and R. L. Ferris, "Chemokine receptors 6 and 7 identify a metastatic expression pattern in squamous cell carcinoma of the head and neck," *Advances in Oto-Rhino-Laryngology*, vol. 62, pp. 121–133, 2005.

[217] M. Kochetkova, S. Kumar, and S. R. McColl, "Chemokine receptors CXCR4 and CCR7 promote metastasis by preventing anoikis in cancer cells," *Cell Death and Differentiation*, vol. 16, no. 5, pp. 664–673, 2009.

[218] H. D. Cunningham, L. A. Shannon, P. A. Calloway et al., "Expression of the C-C chemokine receptor 7 mediates metastasis of breast cancer to the Lymph nodes in mice," *Translational Oncology*, vol. 3, no. 6, pp. 354–361, 2010.

[219] J. Li, R. Sun, K. Tao, and G. Wang, "The CCL21/CCR7 pathway plays a key role in human colon cancer metastasis through regulation of matrix metalloproteinase-9," *Digestive and Liver Disease*, vol. 43, no. 1, pp. 40–47, 2011.

[220] Y. Shuyi, D. Juping, Z. Zhiqun et al., "A critical role of CCR7 in invasiveness and metastasis of SW620 colon cancer cell in vitro and in vivo," *Cancer Biology & Therapy*, vol. 7, no. 7, pp. 1037–1043, 2008.

[221] M. S. Emmett, S. Lanati, D. B. A. Dunn, O. A. Stone, and D. O. Bates, "CCR7 mediates directed growth of melanomas towards lymphatics," *Microcirculation*, vol. 18, no. 3, pp. 172–182, 2011.

[222] L. Fang, V. C. Lee, E. Cha, H. Zhang, and S. T. Hwang, "CCR7 regulates B16 murine melanoma cell tumorigenesis in skin," *Journal of Leukocyte Biology*, vol. 84, no. 4, pp. 965–972, 2008.

[223] Y. Xu, L. Liu, X. Qiu et al., "CCL21/CCR7 prevents apoptosis via the ERK pathway in human non-small cell lung cancer cells," *PLoS ONE*, vol. 7, no. 3, Article ID e33262, 2012.

[224] J. Liu, L. Zhang, and C. Wang, "CCL21 modulates the migration of NSCL cancer by changing the concentration of intracellular $Ca2^{+}$," *Oncology Reports*, vol. 27, no. 2, pp. 481–486, 2012.

[225] Y. Xu, L. Liu, X. Qiu et al., "CCL21/CCR7 promotes G2/M phase progression via the ERK pathway in human non-small cell lung cancer cells," *PLoS ONE*, vol. 6, no. 6, Article ID e21119, 2011.

[226] S. Maekawa, A. Iwasaki, T. Shirakusa et al., "Association between the expression of chemokine receptors CCR7 and CXCR3, and lymph node metastatic potential in lung adenocarcinoma," *Oncology Reports*, vol. 19, no. 6, pp. 1461–1468, 2008.

[227] Z. J. Shang, K. Liu, and Z. Shao, "Expression of chemokine receptor CCR7 is associated with cervical lymph node metastasis of oral squamous cell carcinoma," *Oral Oncology*, vol. 45, no. 6, pp. 480–485, 2009.

[228] J. Sperveslage, S. Frank, C. Heneweer et al., "Lack of CCR7 expression is rate limiting for lymphatic spread of pancreatic ductal adenocarcinoma," *International Journal of Cancer*, vol. 131, no. 4, pp. E371–E381, 2012.

[229] X. Huang, K. Su, L. Zhou et al., "Hypoxia preconditioning of mesenchymal stromal cells enhances PC3 cell lymphatic metastasis accompanied by VEGFR-3/CCR7 activation," *Journal of Cellular Biochemistry*, vol. 114, no. 12, pp. 2834–2841, 2013.

[230] J. Wang, R. R. Seethala, Q. Zhang et al., "Autocrine and paracrine chemokine receptor 7 activation in head and neck cancer: implications for therapy," *Journal of the National Cancer Institute*, vol. 100, no. 7, pp. 502–512, 2008.

[231] Z. Zhao, L. Peng, F. Liu, L. Sun, and C. Sun, "PKCα take part in CCR7/NF-κB autocrine signaling loop in CCR7-positive squamous cell carcinoma of head and neck," *Molecular and Cellular Biochemistry*, vol. 357, no. 1-2, pp. 181–187, 2011.

[232] F. Liu, Z. Zhao, P. Li et al., "NF-κB participates in chemokine receptor 7-mediated cell survival in metastatic squamous cell carcinoma of the head and neck," *Oncology Reports*, vol. 25, no. 2, pp. 383–391, 2011.

[233] Z. Zhao, F. Liu, P. Li, X. Ding, Z. Zong, and C. Sun, "CCL19-induced chemokine receptor 7 activates the phosphoinositide-3 kinase-mediated invasive pathway through Cdc42 in metastatic squamous cell carcinoma of the head and neck," *Oncology Reports*, vol. 25, no. 3, pp. 729–737, 2011.

[234] P. Li, F. Liu, L. Sun et al., "Chemokine receptor 7 promotes cell migration and adhesion in metastatic squamous cell carcinoma of the head and neck by activating integrin $\alpha v\beta 3$," *International Journal of Molecular Medicine*, vol. 27, no. 5, pp. 679–687, 2011.

[235] Y. K. Mburu, K. Abe, L. K. Ferris, S. N. Sarkar, and R. L. Ferris, "Human β-defensin 3 promotes NF-kB-mediated CCR7 expression and anti-apoptotic signals in squamous cell carcinoma of the head and neck," *Carcinogenesis*, vol. 32, no. 2, pp. 168–174, 2011.

[236] P. Li, Z. Zhao, F. Liu et al., "The chemokine receptor 7 regulates cell adhesion and migration via $\beta 1$ integrin in metastatic squamous cell carcinoma of the head and neck," *Oncology Reports*, vol. 24, no. 4, pp. 989–995, 2010.

[237] J. Yang, S. Wang, G. Zhao, and B. Sun, "Effect of chemokine receptors CCR7 on disseminated behavior of human T cell lymphoma: clinical and experimental study," *Journal of Experimental and Clinical Cancer Research*, vol. 30, no. 1, article 51, 2011.

[238] C. Johnson-Holiday, R. Singh, E. Johnson et al., "CCL25 mediates migration, invasion and matrix metalloproteinase expression by breast cancer cells in a CCR9-dependent fashion," *International Journal of Oncology*, vol. 38, no. 5, pp. 1279–1285, 2011.

[239] H. J. Chen, R. Edwards, S. Tucci et al., "Chemokine 25—induced signaling suppresses colon cancer invasion and metastasis," *The Journal of Clinical Investigation*, vol. 122, no. 9, pp. 3184–3196, 2012.

[240] E. L. Johnson, R. Singh, S. Singh et al., "CCL25-CCR9 interaction modulates ovarian cancer cell migration, metalloproteinase expression, and invasion," *World Journal of Surgical Oncology*, vol. 8, article 62, 2010.

[241] X. Shen, B. Mailey, J. D. I. E. Ellenhorn, P. G. Chu, A. M. Lowy, and J. Kim, "Cc chemokine receptor 9 enhances proliferation in pancreatic intraepithelial neoplasia and pancreatic cancer cells," *Journal of Gastrointestinal Surgery*, vol. 13, no. 11, pp. 1955–1962, 2009.

[242] P. K. Sharma, R. Singh, K. R. Novakovic, J. W. Eaton, W. E. Grizzle, and S. Singh, "CCR9 mediates PI3K/AKT-dependent antiapoptotic signals in prostate cancer cells and inhibition of CCR9-CCL25 interaction enhances the cytotoxic effects of etoposide," *International Journal of Cancer*, vol. 127, no. 9, pp. 2020–2030, 2010.

[243] T. Murakami, A. R. Cardones, S. E. Finkelstein et al., "Immune evasion by murine melanoma mediated through CC chemokine receptor-10," *Journal of Experimental Medicine*, vol. 198, no. 9, pp. 1337–1347, 2003.

[244] T. Murakami, A. R. Cardones, and S. T. Hwang, "Chemokine receptors and melanoma metastasis," *Journal of Dermatological Science*, vol. 36, no. 2, pp. 71–78, 2004.

[245] J. K. Singh, G. Farnie, N. J. Bundred et al., "Targeting CXCR1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting HER2 via HER2-dependent and -independent mechanisms," *Clinical Cancer Research*, vol. 19, no. 3, pp. 643–656, 2013.

[246] L. B. Liu, F. Xie, K. K. Chang et al., "Hypoxia promotes the proliferation of cervical carcinoma cells through stimulating the secretion of IL-8," *International Journal of Clinical and Experimental Pathology*, vol. 7, no. 2, pp. 575–583, 2014.

[247] M. L. Varney, S. Singh, A. Li, R. Mayer-Ezell, R. Bond, and R. K. Singh, "Small molecule antagonists for CXCR2 and CXCR1 inhibit human colon cancer liver metastases," *Cancer Letters*, vol. 300, no. 2, pp. 180–188, 2011.

[248] M. Eck, B. Schmausser, K. Scheller, S. Brändlein, and H. K. Müller-Hermelink, "Pleiotropic effects of CXC chemokines in gastric carcinoma: differences in CXCL8 and CXCL1 expression between diffuse and intestinal types of gastric carcinoma," *Clinical & Experimental Immunology*, vol. 134, no. 3, pp. 508–515, 2003.

[249] D. W. Infanger, Y. Cho, B. S. Lopez et al., "Glioblastoma stem cells are regulated by interleukin-8 signaling in a tumoral perivascular niche," *Cancer Research*, vol. 73, no. 23, pp. 7079–7089, 2013.

[250] B. Raychaudhuri and M. A. Vogelbaum, "IL-8 is a mediator of NF-κB induced invasion by gliomas," *Journal of Neuro-Oncology*, vol. 101, no. 2, pp. 227–235, 2011.

[251] C. Gabellini, D. Trisciuoglio, M. Desideri et al., "Functional activity of CXCL8 receptors, CXCR1 and CXCR2, on human malignant melanoma progression," *European Journal of Cancer*, vol. 45, no. 14, pp. 2618–2627, 2009.

[252] S. Singh, K. C. Nannuru, A. Sadanandam, M. L. Varney, and R. K. Singh, "CXCR1 and CXCR2 enhances human melanoma tumourigenesis, growth and invasion," *British Journal of Cancer*, vol. 100, no. 10, pp. 1638–1646, 2009.

[253] S. Singh, A. Sadanandam, K. C. Nannuru et al., "Small-molecule antagonists for CXCR2 and CXCR1 inhibit human melanoma

growth by decreasing tumor cell proliferation, survival, and angiogenesis," *Clinical Cancer Research*, vol. 15, no. 7, pp. 2380–2386, 2009.

[254] C. Murphy, M. McGurk, J. Pettigrew et al., "Nonapical and cytoplasmic expression of interleukin-8, CXCR1, and CXCR2 correlates with cell proliferation and microvessel density in prostate cancer," *Clinical Cancer Research*, vol. 11, no. 11, pp. 4117–4127, 2005.

[255] W. Fang, L. Ye, L. Shen et al., "Tumor-associated macrophages promote the metastatic potential of thyroid papillary cancer by releasing CXCL8," *Carcinogenesis*, 2014.

[256] L. R. Bohrer and K. L. Schwertfeger, "Macrophages promote fibroblast growth factor receptor-driven tumor cell migration and invasion in a Cxcr2-dependent manner," *Molecular Cancer Research*, vol. 10, no. 10, pp. 1294–1305, 2012.

[257] J. L. Halpern, A. Kilbarger, and C. C. Lynch, "Mesenchymal stem cells promote mammary cancer cell migration *in vitro* via the CXCR2 receptor," *Cancer Letters*, vol. 308, no. 1, pp. 91–99, 2011.

[258] Y. Ning, M. J. Labonte, W. Zhang et al., "The CXCR2 antagonist, SCH-527123, shows antitumor activity and sensitizes cells to oxaliplatin in preclinical colon cancer models," *Molecular Cancer Therapeutics*, vol. 11, no. 6, pp. 1353–1364, 2012.

[259] Y. S. Lee, I. Choi, Y. Ning et al., "Interleukin-8 and its receptor CXCR2 in the tumour microenvironment promote colon cancer growth, progression and metastasis," *British Journal of Cancer*, vol. 106, no. 11, pp. 1833–1841, 2012.

[260] M. Kawamura, Y. Toiyama, K. Tanaka et al., "CXCL5, a promoter of cell proliferation, migration and invasion, is a novel serum prognostic marker in patients with colorectal cancer," *European Journal of Cancer*, vol. 48, no. 14, pp. 2244–2251, 2012.

[261] H. Ogata, A. Sekikawa, H. Yamagishi et al., "GROα promotes invasion of colorectal cancer cells," *Oncology Reports*, vol. 24, no. 6, pp. 1479–1486, 2010.

[262] J. Xu, C. Zhang, Y. He et al., "Lymphatic endothelial cell-secreted CXCL1 stimulates lymphangiogenesis and metastasis of gastric cancer," *International Journal of Cancer*, vol. 130, no. 4, pp. 787–797, 2012.

[263] W. L. Cheng, C. S. Wang, Y. H. Huang, M. M. Tsai, Y. Liang, and K. H. Lin, "Overexpression of CXCL1 and its receptor CXCR2 promote tumor invasion in gastric cancer," *Annals of Oncology*, vol. 22, no. 10, pp. 2267–2276, 2011.

[264] N. Baron, O. Deuster, C. Noelker et al., "Role of macrophage migration inhibitory factor in primary glioblastoma multiforme cells," *Journal of Neuroscience Research*, vol. 89, no. 5, pp. 711–717, 2011.

[265] M.-C. Lo, T.-C. Yip, K.-C. Ngan et al., "Role of MIF/CXCL8/CXCR2 signaling in the growth of nasopharyngeal carcinoma tumor spheres," *Cancer Letters*, vol. 335, no. 1, pp. 81–92, 2013.

[266] P. Saintigny, E. Massarelli, S. Lin et al., "CXCR2 expression in tumor cells is a poor prognostic factor and promotes invasion and metastasis in lung adenocarcinoma," *Cancer Research*, vol. 73, no. 2, pp. 571–582, 2013.

[267] M. P. Keane, J. A. Belperio, Y. Y. Xue, M. D. Burdick, and R. M. Strieter, "Depletion of CXCR2 inhibits tumor growth and angiogenesis in a murine model of lung cancer," *Journal of Immunology*, vol. 172, no. 5, pp. 2853–2860, 2004.

[268] G. Yang, D. G. Rosen, G. Liu et al., "CXCR2 promotes ovarian cancer growth through dysregulated cell cycle, diminished apoptosis, and enhanced angiogenesis," *Clinical Cancer Research*, vol. 16, no. 15, pp. 3875–3886, 2010.

[269] Y. Matsuo, N. Ochi, H. Sawai et al., "CXCL8/IL-8 and CXCL12/SDF-1alpha co-operatively promote invasiveness and angiogenesis in pancreatic cancer," *International Journal of Cancer*, vol. 124, no. 4, pp. 853–861, 2009.

[270] M. Balan and S. Pal, "A novel CXCR3-B chemokine receptor-induced growth-inhibitory signal in cancer cells is mediated through the regulation of Bach-1 protein and Nrf2 protein nuclear translocation," *The Journal of Biological Chemistry*, vol. 289, no. 6, pp. 3126–3137, 2014.

[271] P. Chaturvedi, D. M. Gilkes, C. C. Wong et al., "Hypoxia-inducible factor-dependent breast cancer-mesenchymal stem cell bidirectional signaling promotes metastasis," *The Journal of Clinical Investigation*, vol. 123, no. 1, pp. 189–205, 2013.

[272] A. M. Mulligan, I. Raitman, L. Feeley et al., "Tumoral lymphocytic infiltration and expression of the chemokine CXCL10 in breast cancers from the ontario familial breast cancer registry," *Clinical Cancer Research*, vol. 19, no. 2, pp. 336–346, 2013.

[273] D. Datta, J. A. Flaxenburg, S. Laxmanan et al., "Ras-induced modulation of CXCL10 and its receptor splice variant CXCR3-B in MDA-MB-435 and MCF-7 cells: relevance for the development of human breast cancer," *Cancer Research*, vol. 66, no. 19, pp. 9509–9518, 2006.

[274] T. Murakami, K. Kawada, M. Iwamoto et al., "The role of CXCR3 and CXCR4 in colorectal cancer metastasis," *International Journal of Cancer*, vol. 132, no. 2, pp. 276–287, 2013.

[275] C. Liu, D. Luo, B. A. Reynolds et al., "Chemokine receptor CXCR3 promotes growth of glioma," *Carcinogenesis*, vol. 32, no. 2, pp. 129–137, 2011.

[276] S. V. Maru, K. A. Holloway, G. Flynn et al., "Chemokine production and chemokine receptor expression by human glioma cells: Role of CXCL10 in tumour cell proliferation," *Journal of Neuroimmunology*, vol. 199, no. 1-2, pp. 35–45, 2008.

[277] S. Amatschek, R. Lucas, A. Eger et al., "CXCL9 induces chemotaxis, chemorepulsion and endothelial barrier disruption through CXCR3-mediated activation of melanoma cells," *British Journal of Cancer*, vol. 104, no. 3, pp. 469–479, 2011.

[278] T. S. Lau, T. K. Chung, T. H. Cheung et al., "Cancer cell-derived lymphotoxin mediates reciprocal tumour-stromal interactions in human ovarian cancer by inducing CXCL11 in fibroblasts," *The Journal of Pathology*, vol. 232, no. 1, pp. 43–56, 2014.

[279] Q. Wu, R. Dhir, and A. Wells, "Altered CXCR3 isoform expression regulates prostate cancer cell migration and invasion," *Molecular Cancer*, vol. 11, article 3, 2012.

[280] T. Utsumi, T. Suyama, Y. Imamura et al., "The association of CXCR3 and renal cell carcinoma metastasis," *The Journal of Urology*, 2014.

[281] D. Datta, P. Banerjee, M. Gasser, A. M. Waaga-Gasser, and S. Pal, "CXCR3-B can mediate growth-inhibitory signals in human renal cancer cells by down-regulating the expression of heme oxygenase-1," *The Journal of Biological Chemistry*, vol. 285, no. 47, pp. 36842–36848, 2010.

[282] S. Biswas, S. Sengupta, S. Roy Chowdhury et al., "CXCL13-CXCR5 co-expression regulates epithelial to mesenchymal transition of breast cancer cells during lymph node metastasis," *Breast Cancer Research and Treatment*, vol. 143, no. 2, pp. 265–276, 2014.

[283] J. Meijer, I. S. Zeelenberg, B. Sipos, and E. Roos, "The CXCR5 chemokine receptor is expressed by carcinoma cells and promotes growth of colon carcinoma in the liver," *Cancer Research*, vol. 66, no. 19, pp. 9576–9582, 2006.

[284] I. Airoldi, C. Cocco, F. Morandi, I. Prigione, and V. Pistoia, "CXCR5 may be involved in the attraction of human metastatic

neuroblastoma cells to the bone marrow," *Cancer Immunology, Immunotherapy*, vol. 57, no. 4, pp. 541–548, 2008.

[285] C. P. El-Haibi, R. Singh, P. Gupta et al., "Antibody microarray analysis of signaling networks regulated by Cxcl13 and Cxcr5 in prostate cancer," *Journal of Proteomics and Bioinformatics*, vol. 5, no. 8, pp. 177–184, 2012.

[286] C. P. El-Haibi, P. Sharma, R. Singh et al., "Differential G protein subunit expression by prostate cancer cells and their interaction with CXCR5," *Molecular Cancer*, vol. 12, no. 1, article 64, 2013.

[287] C. P. El-Haibi, R. Singh, P. K. Sharma, S. Singh, and J. W. Lillard, "CXCL13 mediates prostate cancer cell proliferation through JNK signalling and invasion through ERK activation," *Cell Proliferation*, vol. 44, no. 4, pp. 311–319, 2011.

[288] S. Singh, R. Singh, P. K. Sharma et al., "Serum CXCL13 positively correlates with prostatic disease, prostate-specific antigen and mediates prostate cancer cell invasion, integrin clustering and cell adhesion," *Cancer Letters*, vol. 283, no. 1, pp. 29–35, 2009.

[289] K. Matsushita, Y. Toiyama, K. Tanaka et al., "Soluble CXCL16 in preoperative serum is a novel prognostic marker and predicts recurrence of liver metastases in colorectal cancer patients," *Annals of Surgical Oncology*, vol. 19, supplement 3, pp. S518–S527, 2012.

[290] R. Taghizadeh, M. Noh, Y. H. Huh et al., "Cxcr6, a newly defined biomarker of tissue-specific stem cell asymmetric self-renewal, identifies more aggressive human melanoma cancer stem cells," *PLoS ONE*, vol. 5, no. 12, Article ID e15183, 2010.

[291] D. L. Ou, C. L. Chen, S. B. Lin, C. H. Hsu, and L. I. Lin, "Chemokine receptor expression profiles in nasopharyngeal carcinoma and their association with metastasis and radiotherapy," *Journal of Pathology*, vol. 210, no. 3, pp. 363–373, 2006.

[292] M. N. Wente, M. M. Gaida, C. Mayer et al., "Expression and potential function of the CXC chemokine CXCL16 in pancreatic ductal adenocarcinoma," *International Journal of Oncology*, vol. 33, no. 2, pp. 297–308, 2008.

[293] Y. Lu, J. Wang, Y. Xu et al., "CXCL16 functions as a novel chemotactic factor for prostate cancer cells *in vitro*," *Molecular Cancer Research*, vol. 6, no. 4, pp. 546–554, 2008.

[294] P. M. Hershberger, S. Peddibhotla, E. Sugarman et al., "Probing the CXCR6/CXCL16 axis: targeting prevention of prostate cancer metastasis," in *Probe Reports from the NIH Molecular Libraries Program*, National Center for Biotechnology Information (US), Bethesda, Md, USA, 2010.

[295] J. Wang, Y. Lu, J. Wang, A. E. Koch, J. Zhang, and R. S. Taichman, "CXCR6 induces prostate cancer progression by the AKT/mammalian target of rapamycin signaling pathway," *Cancer Research*, vol. 68, no. 24, pp. 10367–10376, 2008.

[296] P. Gutwein, A. Schramme, N. Sinke et al., "Tumoural CXCL16 expression is a novel prognostic marker of longer survival times in renal cell cancer patients," *European Journal of Cancer*, vol. 45, no. 3, pp. 478–489, 2009.

[297] L. Hernandez, M. A. O. Magalhaes, S. J. Coniglio, J. S. Condeelis, and J. E. Segall, "Opposing roles of CXCR4 and CXCR7 in breast cancer metastasis," *Breast Cancer Research*, vol. 13, no. 6, article R128, 2011.

[298] J. M. Burns, B. C. Summers, Y. Wang et al., "A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development," *Journal of Experimental Medicine*, vol. 203, no. 9, pp. 2201–2213, 2006.

[299] C. Liu, K. Pham, D. Luo et al., "Expression and functional heterogeneity of chemokine receptors CXCR4 and CXCR7 in primary patient-derived glioblastoma cells," *PLoS ONE*, vol. 8, no. 3, Article ID e59750, 2013.

[300] T. C. Xue, R. X. Chen, D. Han et al., "Down-regulation of CXCR7 inhibits the growth and lung metastasis of human hepatocellular carcinoma cells with highly metastatic potential," *Experimental and Therapeutic Medicine*, vol. 3, no. 1, pp. 117–123, 2012.

[301] K. Zheng, H. Y. Li, X. L. Su et al., "Chemokine receptor CXCR7 regulates the invasion, angiogenesis and tumor growth of human hepatocellular carcinoma cells," *Journal of Experimental and Clinical Cancer Research*, vol. 29, no. 1, article 31, 2010.

[302] M. Ma, J. Y. Ye, R. Deng, C. M. Dee, and G. C. Chan, "Mesenchymal stromal cells may enhance metastasis of neuroblastoma via SDF-1/CXCR4 and SDF-1/CXCR7 signaling," *Cancer Letters*, vol. 312, no. 1, pp. 1–10, 2011.

[303] M. Kim, L. Rooper, J. Xie, A. A. Kajdacsy-Balla, and M. V. Barbolina, "Fractalkine receptor CX3CR1 is expressed in epithelial ovarian carcinoma cells and required for motility and adhesion to peritoneal mesothelial cells," *Molecular Cancer Research*, vol. 10, no. 1, pp. 11–24, 2012.

[304] G. Sciumé, A. Soriani, M. Piccoli, L. Frati, A. Santoni, and G. Bernardini, "CX_3CR_1/CX_3CL_1 axis negatively controls glioma cell invasion and is modulated by transforming growth factor-beta1," *Neuro-Oncology*, vol. 12, no. 7, pp. 701–710, 2010.

[305] I. Nevo, O. Sagi-Assif, T. Meshel et al., "The involvement of the fractalkine receptor in the transmigration of neuroblastoma cells through bone-marrow endothelial cells," *Cancer Letters*, vol. 273, no. 1, pp. 127–139, 2009.

[306] T. Zhao, S. Gao, X. Wang et al., "Hypoxia-inducible factor-1α regulates chemotactic migration of pancreatic ductal adenocarcinoma cells through directly transactivating the CX3CR1 gene," *PLoS ONE*, vol. 7, no. 8, Article ID e43399, 2012.

[307] F. Marchesi, L. Piemonti, G. Fedele et al., "The chemokine receptor CX3CR1 is involved in the neural tropism and malignant behavior of pancreatic ductal adenocarcinoma," *Cancer Research*, vol. 68, no. 21, pp. 9060–9069, 2008.

[308] L. J. Xiao, Y. Y. Chen, P. Lin et al., "Hypoxia increases CX3CR1 expression via HIF-1 and NF-κB in androgen-independent prostate cancer cells," *International Journal of Oncology*, vol. 41, no. 5, pp. 1827–1836, 2012.

[309] D. Castellana, F. Zobairi, M. C. Martinez et al., "Membrane microvesicles as actors in the establishment of a favorable prostatic tumoral niche: A role for activated fibroblasts and CX3CL1-CX3CR1 axis," *Cancer Research*, vol. 69, no. 3, pp. 785–793, 2009.

[310] W. L. Jamieson, S. Shimizu, J. A. D'Ambrosio, O. Meucci, and A. Fatatis, "CX3CR1 is expressed by prostate epithelial cells and androgens regulate the levels of CX3CL1/fractalkine in the bone marrow: potential role in prostate cancer bone tropism," *Cancer Research*, vol. 68, no. 6, pp. 1715–1722, 2008.

[311] X. Yao, L. Qi, X. Chen, J. Du, Z. Zhang, and S. Liu, "Expression of CX3CR1 associates with cellular migration, metastasis, and prognosis in human clear cell renal cell carcinoma," *Urologic Oncology*, vol. 32, no. 2, pp. 162–170, 2014.

[312] M. Kim, L. Rooper, J. Xie et al., "The lymphotactin receptor is expressed in epithelial ovarian carcinoma and contributes to cell migration and proliferation," *Molecular Cancer Research*, vol. 10, no. 11, pp. 1419–1429, 2012.

[313] S. A. Khurram, S. A. Whawell, L. Bingle, C. Murdoch, B. M. McCabe, and P. M. Farthing, "Functional expression of the chemokine receptor XCR1 on oral epithelial cells," *Journal of Pathology*, vol. 221, no. 2, pp. 153–163, 2010.

[314] A. Bajetto, F. Barbieri, A. Dorcaratto et al., "Expression of CXC chemokine receptors 1-5 and their ligands in human glioma

tissues: Role of CXCR4 and SDF1 in glioma cell proliferation and migration," *Neurochemistry International*, vol. 49, no. 5, pp. 423–432, 2006.

[315] R. Bonavia, A. Bajetto, S. Barbero, P. Pirani, T. Florio, and G. Schettini, "Chemokines and their receptors in the CNS: expression of CXCL12/SDF-1 and CXCR4 and their role in astrocyte proliferation," *Toxicology Letters*, vol. 139, no. 2-3, pp. 181–189, 2003.

[316] Y. Zhou, X. Bian, Y. Le et al., "Formylpeptide receptor FPR and the rapid growth of malignant human gliomas," *Journal of the National Cancer Institute*, vol. 97, no. 11, pp. 823–835, 2005.

[317] Y. Yang, Y. Liu, X. Yao et al., "Annexin 1 released by necrotic human glioblastoma cells stimulates tumor cell growth through the formyl peptide receptor 1," *The American Journal of Pathology*, vol. 179, no. 3, pp. 1504–1512, 2011.

[318] J. Huang, J. Hu, X. Bian et al., "Transactivation of the epidermal growth factor receptor by formylpeptide receptor exacerbates the malignant behavior of human glioblastoma cells," *Cancer Research*, vol. 67, no. 12, pp. 5906–5913, 2007.

[319] S. Marsigliante, C. Vetrugno, and A. Muscella, "CCL20 induces migration and proliferation on breast epithelial cells," *Journal of Cellular Physiology*, vol. 228, no. 9, pp. 1873–1883, 2013.

[320] A. Orimo, P. B. Gupta, D. C. Sgroi et al., "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion," *Cell*, vol. 121, no. 3, pp. 335–348, 2005.

[321] K. Beider, M. Abraham, M. Begin et al., "Interaction between CXCR4 and CCL20 pathways regulates tumor growth," *PLoS ONE*, vol. 4, no. 4, Article ID e5125, 2009.

[322] S. Yaccoby, "Advances in the understanding of myeloma bone disease and tumour growth," *British Journal of Haematology*, vol. 149, no. 3, pp. 311–321, 2010.

[323] A. Valentin-Opran, S. A. Charhon, P. J. Meunier, C. M. Edouard, and M. E. Arlot, "Quantitative histology of myeloma-induced bone changes," *The British Journal of Haematology*, vol. 52, no. 4, pp. 601–610, 1982.

[324] D. J. Dairaghi, B. O. Oyajobi, A. Gupta et al., "CCR1 blockade reduces tumor burden and osteolysis in vivo in a mouse model of myeloma bone disease," *Blood*, vol. 120, no. 7, pp. 1449–1457, 2012.

[325] S. Vallet, S. Pozzi, K. Patel et al., "A novel role for CCL3 (MIP-1α) in myeloma-induced bone disease via osteocalcin downregulation and inhibition of osteoblast function," *Leukemia*, vol. 25, no. 7, pp. 1174–1181, 2011.

[326] Y. Wu, Y. Li, K. Matsushima, T. Baba, and N. Mukaida, "CCL3-CCR5 axis regulates intratumoral accumulation of leukocytes and fibroblasts and promotes angiogenesis in murine lung metastasis process," *Journal of Immunology*, vol. 181, no. 9, pp. 6384–6393, 2008.

[327] J. M. Wang, O. Chertov, P. Proost et al., "Purification and identification of chemokines potentially involved in kidney-specific metastasis by a murine lymphoma variant: induction of migration and NFkappaB activation," *International Journal of Cancer*, vol. 75, no. 6, pp. 900–907, 1998.

[328] A. Müller, B. Homey, H. Soto et al., "Involvement of chemokine receptors in breast cancer metastasis," *Nature*, vol. 410, no. 6824, pp. 50–56, 2001.

[329] N. Said and D. Theodorescu, "RhoGDI2 suppresses bladder cancer metastasis via reduction of inflammation in the tumor microenvironment," *Oncoimmunology*, vol. 1, no. 7, pp. 1175–1177, 2012.

[330] J. T. Lee, S. D. Lee, J. Z. Lee, M. K. Chung, and H. K. Ha, "Expression analysis and clinical significance of CXCL16/CXCR6 in patients with bladder cancer," *Oncology Letters*, vol. 5, no. 1, pp. 229–235, 2013.

[331] A. Biragyn, M. Bodogai, P. B. Olkhanud et al., "Inhibition of lung metastasis by chemokine CCL17-mediated in vivo silencing of genes in CCR4+ Tregs," *Journal of Immunotherapy*, vol. 36, no. 4, pp. 258–267, 2013.

[332] Y. Zhang, F. Y. Meng, W. L. Li, C. X. Zhou, Z. Guan, and H. Y. Fan, "Association of chemotactic factor receptor 5 gene with breast cancer," *Genetics and Molecular Research*, vol. 12, no. 4, pp. 5289–5300, 2013.

[333] F. Andre, N. Cabioglu, H. Assi et al., "Expression of chemokine receptors predicts the site of metastatic relapse in patients with axillary node positive primary breast cancer," *Annals of Oncology*, vol. 17, no. 6, pp. 945–951, 2006.

[334] A. Issa, T. X. Le, A. N. Shoushtari, J. D. Shields, and M. A. Swartz, "Vascular endothelial growth factor-C and C-C chemokine receptor 7 in tumor cell-lymphatic cross-talk promote invasive phenotype," *Cancer Research*, vol. 69, no. 1, pp. 349–357, 2009.

[335] M. Pan, M. Hou, H. Chang, and W. Hung, "Cyclooxygenase-2 up-regulates CCR7 via EP2/EP4 receptor signaling pathways to enhance lymphatic invasion of breast cancer cells," *Journal of Biological Chemistry*, vol. 283, no. 17, pp. 11155–11163, 2008.

[336] Y. Liu, R. Ji, J. Li et al., "Correlation effect of EGFR and CXCR4 and CCR7 chemokine receptors in predicting breast cancer metastasis and prognosis," *Journal of Experimental and Clinical Cancer Research*, vol. 29, no. 1, article 16, 2010.

[337] N. Cabioglu, M. S. Yazici, B. Arun et al., "CCR7 and CXCR4 as novel biomarkers predicting axillary lymph node metastasis in T1 breast cancer," *Clinical Cancer Research*, vol. 11, no. 16, pp. 5686–5693, 2005.

[338] A. Kamalakar, M. S. Bendre, C. L. Washam et al., "Circulating interleukin-8 levels explain breast cancer osteolysis in mice and humans," *Bone*, vol. 61, pp. 176–185, 2014.

[339] M. S. Bendre, D. C. Montague, T. Peery, N. S. Akel, D. Gaddy, and L. J. Suva, "Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease," *Bone*, vol. 33, no. 1, pp. 28–37, 2003.

[340] B. Sharma, D. M. Nawandar, K. C. Nannuru, M. L. Varney, and R. K. Singh, "Targeting CXCR2 enhances chemotherapeutic response, inhibits mammary tumor growth, angiogenesis, and lung metastasis," *Molecular Cancer Therapeutics*, vol. 12, no. 5, pp. 799–808, 2013.

[341] S. Bersini, J. S. Jeon, G. Dubini et al., "A microfluidic 3D *in vitro* model for specificity of breast cancer metastasis to bone," *Biomaterials*, vol. 35, no. 8, pp. 2454–2461, 2014.

[342] T. C. Walser, X. Ma, N. Kundu, R. Dorsey, O. Goloubeva, and A. M. Fulton, "Immune-mediated modulation of breast cancer growth and metastasis by the chemokine Mig (CXCL9) in a murine model," *Journal of Immunotherapy*, vol. 30, no. 5, pp. 490–498, 2007.

[343] H. Bu, B. Shu, F. Gao et al., "Spinal IFN-γ-induced protein-10 (CXCL10) mediates metastatic breast cancer-induced bone pain by activation of microglia in rat models," *Breast Cancer Research and Treatment*, vol. 143, no. 2, pp. 255–263, 2014.

[344] X. Ma, K. Norsworthy, N. Kundu et al., "CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model," *Molecular Cancer Therapeutics*, vol. 8, no. 3, pp. 490–498, 2009.

[345] A. Markiewicz, M. Ksiazkiewicz, M. Welnicka-Jaskiewicz et al., "Mesenchymal phenotype of CTC-enriched blood fraction and

lymph node metastasis formation potential," *PLoS ONE*, vol. 9, no. 4, Article ID e93901, 2014.

[346] L. D. Roy, M. Sahraei, J. L. Schettini, H. E. Gruber, D. M. Besmer, and P. Mukherjee, "Systemic neutralization of IL-17A significantly reduces breast cancer associated metastasis in arthritic mice by reducing CXCL12/SDF-1 expression in the metastatic niches," *BMC Cancer*, vol. 14, article 225, 2014.

[347] C. S. Hung, H. Y. Su, H. H. Liang et al., "High-level expression of CXCR4 in breast cancer is associated with early distant and bone metastases," *Tumor Biology*, vol. 35, no. 2, pp. 1581–1588, 2014.

[348] K. Subik, L. Shu, C. Wu et al., "The ubiquitin E3 ligase WWP1 decreases CXCL12-mediated MDA231 breast cancer cell migration and bone metastasis," *Bone*, vol. 50, no. 4, pp. 813–823, 2012.

[349] J. B. Shepard, R. A. Wilkinson, J. R. Starkey, and M. Teintze, "Novel guanide-substituted compounds bind to CXCR4 and inhibit breast cancer metastasis," *Anti-Cancer Drugs*, vol. 25, no. 1, pp. 8–16, 2014.

[350] S. Lin, L. Sun, J. Hu et al., "Chemokine C-X-C motif receptor 6 contributes to cell migration during hypoxia," *Cancer Letters*, vol. 279, no. 1, pp. 108–117, 2009.

[351] K. E. Luker, S. A. Lewin, L. A. Mihalko et al., "Scavenging of CXCL12 by CXCR7 promotes tumor growth and metastasis of CXCR4-positive breast cancer cells," *Oncogene*, vol. 31, no. 45, pp. 4750–4758, 2012.

[352] N. Chaudary, H. Mujcic, B. G. Wouters, and R. P. Hill, "Hypoxia and metastasis in an orthotopic cervix cancer xenograft model," *Radiotherapy and Oncology*, vol. 108, no. 3, pp. 506–510, 2013.

[353] J. Kodama, T. Kusumoto, N. Seki et al., "Association of CXCR4 and CCR7 chemokine receptor expression and lymph node metastasis in human cervical cancer," *Annals of Oncology*, vol. 18, no. 1, pp. 70–76, 2007.

[354] Y. C. Yang, Z. Y. Lee, C. C. Wu, T. C. Chen, C. L. Chang, and C. P. Chen, "CXCR4 expression is associated with pelvic lymph node metastasis in cervical adenocarcinoma," *International Journal of Gynecological Cancer*, vol. 17, no. 3, pp. 676–686, 2007.

[355] Y. Itatani, K. Kawada, T. Fujishita et al., "Loss of SMAD4 from colorectal cancer cells promotes CCL15 expression to recruit $CCR1^+$ myeloid cells and facilitate liver metastasis," *Gastroenterology*, vol. 145, no. 5, pp. 1064–1075, 2013.

[356] Y. B. Cho, W. Y. Lee, S. J. Choi et al., "CC chemokine ligand 7 expression in liver metastasis of colorectal cancer," *Oncology Reports*, vol. 28, no. 2, pp. 689–694, 2012.

[357] T. Kitamura, T. Fujishita, P. Loetscher et al., "Inactivation of chemokine (C-C motif) receptor 1 (CCR1) suppresses colon cancer liver metastasis by blocking accumulation of immature myeloid cells in amouse model," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 29, pp. 13063–13068, 2010.

[358] S. Y. Lim, A. N. Gordon-Weeks, L. Zhao et al., "Recruitment of myeloid cells to the tumor microenvironment supports liver metastasis," *Oncoimmunology*, vol. 2, article e23187, no. 3, 2013.

[359] M. J. Wolf, A. Hoos, J. Bauer et al., "Endothelial CCR2 signaling induced by colon carcinoma cells enables extravasation via the JAK2-Stat5 and p38MAPK pathway," *Cancer Cell*, vol. 22, no. 1, pp. 91–105, 2012.

[360] P. Ghadjar, S. E. Coupland, I. K. Na et al., "Chemokine receptor CCR6 expression level and liver metastases in colorectal cancer," *Journal of Clinical Oncology*, vol. 24, no. 12, pp. 1910–1916, 2006.

[361] T. Higashiguchi, T. Hotta, K. Takifuji et al., "Clinical impact of matrix metalloproteinase-7 mRNA expression in the invasive front and inner surface of tumor tissues in patients with colorectal cancer," *Diseases of the Colon and Rectum*, vol. 50, no. 10, pp. 1585–1593, 2007.

[362] M. Yamamoto, H. Kikuchi, M. Ohta et al., "TSU68 prevents liver metastasis of colon cancer xenografts by modulating the premetastatic niche," *Cancer Research*, vol. 68, no. 23, pp. 9754–9762, 2008.

[363] O. Kollmar, B. Junker, K. Rupertus, M. D. Menger, and M. K. Schilling, "Studies on MIP-2 and CXCR2 expression in a mouse model of extrahepatic colorectal metastasis," *European Journal of Surgical Oncology*, vol. 33, no. 6, pp. 803–811, 2007.

[364] K. Kawada, H. Hosogi, M. Sonoshita et al., "Chemokine receptor CXCR3 promotes colon cancer metastasis to lymph nodes," *Oncogene*, vol. 26, no. 32, pp. 4679–4688, 2007.

[365] B. Cambien, B. F. Karimdjee, P. Richard-Fiardo et al., "Organ-specific inhibition of metastatic colon carcinoma by CXCR3 antagonism," *British Journal of Cancer*, vol. 100, no. 11, pp. 1755–1764, 2009.

[366] J. Kee, A. Ito, S. Hojo et al., "Chemokine CXCL16 suppresses liver metastasis of colorectal cancer via augmentation of tumor-infiltrating natural killer T cells in a murine model," *Oncology Reports*, vol. 29, no. 3, pp. 975–982, 2013.

[367] C. Rubie, V. O. Frick, P. Ghadjar et al., "CXC receptor-4 mRNA silencing abrogates CXCL12-induced migration of colorectal cancer cells," *Journal of Translational Medicine*, vol. 9, article 22, 2011.

[368] L. J. Drury, J. J. Ziarek, S. Gravel et al., "Monomeric and dimeric $CXCL_{12}$ inhibit metastasis through distinct $CXCR_4$ interactions and signaling pathways," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 43, pp. 17655–17660, 2011.

[369] F. Abedini, M. Ismail, H. Hosseinkhani et al., "Effects of CXCR4 siRNA/dextran-spermine nanoparticles on CXCR4 expression and serum LDH levels in a mouse model of colorectal cancer metastasis to the liver," *Cancer Management and Research*, vol. 3, no. 1, pp. 301–309, 2011.

[370] L. Ma, H. Qiao, C. He et al., "Modulating the interaction of CXCR4 and CXCL12 by low-molecular-weight heparin inhibits hepatic metastasis of colon cancer," *Investigational New Drugs*, vol. 30, no. 2, pp. 508–517, 2012.

[371] J. Silinsky, C. Grimes, T. Driscoll et al., "$CD133^+$ and $CXCR4^+$ colon cancer cells as a marker for lymph node metastasis," *Journal of Surgical Research*, vol. 185, no. 1, pp. 113–118, 2013.

[372] S. C. Chang, P. C. Lin, S. H. Yang, H. S. Wang, A. F. Li, and J. K. Lin, "SDF-1α G801A polymorphism predicts lymph node metastasis in stage T3 colorectal cancer," *Annals of Surgical Oncology*, vol. 16, no. 8, pp. 2323–2330, 2009.

[373] J. P. Mongan, C. E. Fadul, B. F. Cole et al., "Brain metastases from colorectal cancer: risk factors, incidence, and the possible role of chemokines," *Clinical Colorectal Cancer*, vol. 8, no. 2, pp. 100–105, 2009.

[374] Y. Ding, Y. Shimada, M. Maeda et al., "Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma," *Clinical Cancer Research*, vol. 9, no. 9, pp. 3406–3412, 2003.

[375] K. Ishida, M. Iwahashi, M. Nakamori et al., "High CCR7 mRNA expression of cancer cells is associated with lymph node involvement in patients with esophageal squamous cell carcinoma," *International Journal of Oncology*, vol. 34, no. 4, pp. 915–922, 2009.

[376] Y. Song, Z. Wang, X. Liu, W. Jiang, and M. Shi, "CCR7 and VEGF-C: molecular indicator of lymphatic metastatic recurrence in pn0 esophageal squamous cell carcinoma after ivor-lewis esophagectomy?" *Annals of Surgical Oncology*, vol. 19, no. 11, pp. 3606–3612, 2012.

[377] P. Sui, P. Hu, T. Zhang, X. Zhang, Q. Liu, and J. Du, "High expression of CXCR-2 correlates with lymph node metastasis and predicts unfavorable prognosis in resected esophageal carcinoma," *Medical Oncology*, vol. 31, article 809, 2013.

[378] S. J. Gros, N. Kurschat, A. Drenckhan et al., "Involvement of CXCR4 chemokine receptor in metastastic HER2-positive esophageal cancer," *PLoS ONE*, vol. 7, no. 10, Article ID e47287, 2012.

[379] S. J. Gros, H. Graeff, A. Drenckhan et al., "CXCR4/SDF-1α-mediated chemotaxis in an in vivo model of metastatic esophageal carcinoma," *In Vivo*, vol. 26, no. 4, pp. 711–718, 2012.

[380] K. Sasaki, S. Natsugoe, S. Ishigami et al., "Expression of CXCL12 and its receptor CXCR4 correlates with lymph node metastasis in submucosal esophageal cancer," *Journal of Surgical Oncology*, vol. 97, no. 5, pp. 433–438, 2008.

[381] S. Futagami, A. Tatsuguchi, T. Hiratsuka et al., "Monocyte chemoattractant protein 1 and CD40 ligation have a synergistic effect on vascular endothelial growth factor production through cyclooxygenase 2 upregulation in gastric cancer," *Journal of Gastroenterology*, vol. 43, no. 3, pp. 216–224, 2008.

[382] T. Arigami, S. Natsugoe, Y. Uenosono et al., "CCR7 and CXCR4 expression predicts lymph node status including micrometastasis in gastric cancer," *International Journal of Oncology*, vol. 35, no. 1, pp. 19–24, 2009.

[383] W. Wang, Y. Chen, Y. Zhang, and T. Hu, "The regulatory mechanism of CCR7 gene expression and its involvement in the metastasis and progression of gastric cancer," *Tumor Biology*, vol. 34, no. 3, pp. 1865–1871, 2013.

[384] B. Feng, K. Li, H. Zhong et al., "RhoE promotes metastasis in gastric cancer through a mechanism dependent on enhanced expression of CXCR4," *PLoS ONE*, vol. 8, no. 11, Article ID e81709, 2013.

[385] B. C. Zhao, Z. J. Wang, W. Z. Mao, H. C. Ma, J. G. Han, and H. Xu, "CXCR4/SDF-1 axis is involved in lymph node metastasis of gastric carcinoma," *World Journal of Gastroenterology*, vol. 17, no. 19, pp. 2389–2396, 2011.

[386] J. Ying, Q. Xu, G. Zhang, B. Liu, and L. Zhu, "The expression of CXCL12 and CXCR4 in gastric cancer and their correlation to lymph node metastasis," *Medical Oncology*, vol. 29, no. 3, pp. 1716–1722, 2012.

[387] S. Iwasa, T. Yanagawa, J. Fan, and R. Katoh, "Expression of CXCR4 and its ligand SDF-1 in intestinal-type gastric cancer is associated with lymph node and liver metastasis," *Anticancer Research*, vol. 29, no. 11, pp. 4751–4758, 2009.

[388] K. Koizumi, S. Kato, H. Sakurai, I. Hashimoto, K. Yasumoto, and I. Saiki, "Therapeutics target of CXCR4 and its downstream in peritoneal carcinomatosis of gastric cancer," *Frontiers in Bioscience*, vol. 4, no. 1, pp. 269–276, 2012.

[389] I. Hashimoto, K. Koizumi, M. Tatematsu et al., "Blocking on the CXCR4/mTOR signalling pathway induces the anti-metastatic properties and autophagic cell death in peritoneal disseminated gastric cancer cells," *European Journal of Cancer*, vol. 44, no. 7, pp. 1022–1029, 2008.

[390] K. Yasumoto, K. Koizumi, A. Kawashima et al., "Role of the CXCL12/CXCR4 axis in peritoneal carcinomatosis of gastric cancer," *Cancer Research*, vol. 66, no. 4, pp. 2181–2187, 2006.

[391] K. Hattermann and R. Mentlein, "An Infernal Trio: The chemokine CXCL12 and its receptors CXCR4 and CXCR7 in tumor biology," *Annals of Anatomy*, vol. 195, no. 2, pp. 103–110, 2013.

[392] R. Kesselring, A. Thiel, R. Pries, T. Trenkle, and B. Wollenberg, "Human Th17 cells can be induced through head and neck cancer and have a functional impact on HNSCC development," *British Journal of Cancer*, vol. 103, no. 8, pp. 1245–1254, 2010.

[393] M. Ueda, T. Shimada, Y. Goto et al., "Expression of CC-chemokine receptor 7 (CCR7) and CXC-chemokine receptor 4 (CXCR4) in head and neck squamous cell carcinoma," *Auris Nasus Larynx*, vol. 37, no. 4, pp. 488–495, 2010.

[394] K. A. Warner, M. Miyazawa, M. M. R. Cordeiro et al., "Endothelial cells enhance tumor cell invasion through a crosstalk mediated by CXC chemokine signaling," *Neoplasia*, vol. 10, no. 2, pp. 131–139, 2008.

[395] L. Han, B. Jiang, H. Wu et al., "High expression of CXCR2 is associated with tumorigenesis, progression, and prognosis of laryngeal squamous cell carcinoma," *Medical Oncology*, vol. 29, no. 4, pp. 2466–2472, 2012.

[396] S. Albert, M. E. Riveiro, C. Halimi et al., "Focus on the role of the CXCL12/CXCR4 chemokine axis in head and neck squamous cell carcinoma," *Head and Neck*, vol. 35, no. 12, pp. 1819–1828, 2013.

[397] D. Uchida, N. Kuribayashi, M. Kinouchi et al., "Expression and function of CXCR4 in human salivary gland cancers," *Clinical and Experimental Metastasis*, vol. 30, no. 2, pp. 133–142, 2013.

[398] Y. Yoon, Z. Liang, X. Zhang et al., "CXC chemokine receptor-4 antagonist blocks both growth of primary tumor and metastasis of head and neck cancer in xenograft mouse models," *Cancer Research*, vol. 67, no. 15, pp. 7518–7524, 2007.

[399] Y. Sambandam, K. Sundaram, A. Liu, K. L. Kirkwood, W. L. Ries, and S. V. Reddy, "CXCL13 activation of c-Myc induces RANK ligand expression in stromal/preosteoblast cells in the oral squamous cell carcinoma tumor-bone microenvironment," *Oncogene*, vol. 32, no. 1, pp. 97–105, 2013.

[400] C. C. Schimanski, R. Bahre, I. Gockel et al., "Chemokine receptor CCR7 enhances intrahepatic and lymphatic dissemination of human hepatocellular cancer," *Oncology Reports*, vol. 16, no. 1, pp. 109–113, 2006.

[401] K. A. Manu, M. K. Shanmugam, T. H. Ong et al., "Emodin suppresses migration and invasion through the modulation of CXCR4 expression in an orthotopic model of human hepatocellular carcinoma," *PLoS ONE*, vol. 8, no. 3, Article ID e57015, 2013.

[402] Z.-L. Xiang, Z.-C. Zeng, J. Fan et al., "A clinicopathological model to predict bone metastasis in hepatocellular carcinoma," *Journal of Cancer Research and Clinical Oncology*, vol. 137, no. 12, pp. 1791–1797, 2011.

[403] Z. Xiang, Z. Zeng, Z. Tang et al., "Chemokine receptor CXCR4 expression in hepatocellular carcinoma patients increases the risk of bone metastases and poor survival," *BMC Cancer*, vol. 9, article 176, 2009.

[404] Z. Xiang, Z. Zeng, Z. Tang et al., "Increased expression of vascular endothelial growth factor-C and nuclear CXCR4 in hepatocellular carcinoma is correlated with lymph node metastasis and poor outcome," *Cancer Journal*, vol. 15, no. 6, pp. 519–525, 2009.

[405] T. C. Xue, D. Han, R. X. Chen et al., "High expression of CXCR7 combined with alpha fetoprotein in hepatocellular carcinoma correlates with extra-hepatic metastasis to lung after

hepatectomy," *Asian Pacific Journal of Cancer Prevention*, vol. 12, no. 3, pp. 657–663, 2011.

[406] J. Juarez, A. Dela Pena, R. Baraz et al., "CXCR4 antagonists mobilize childhood acute lymphoblastic leukemia cells into the peripheral blood and inhibit engraftment," *Leukemia*, vol. 21, no. 6, pp. 1249–1257, 2007.

[407] F. Rondepierre, B. Bouchon, J. Papon et al., "Proteomic studies of B16 lines: involvement of annexin A1 in melanoma dissemination," *Biochimica et Biophysica Acta*, vol. 1794, no. 1, pp. 61–69, 2009.

[408] R. R. Braeuer, M. Zigler, G. J. Villares, A. S. Dobroff, and M. Bar-Eli, "Transcriptional control of melanoma metastasis: the importance of the tumor microenvironment," *Seminars in Cancer Biology*, vol. 21, no. 2, pp. 83–88, 2011.

[409] V. O. Melnikova, G. J. Villares, and M. Bar-Eli, "Emerging roles of PAR-1 and PAFR in melanoma metastasis," *Cancer Microenvironment*, vol. 1, no. 1, pp. 103–111, 2008.

[410] H. W. van Deventer, D. A. Palmieri, Q. P. Wu, E. C. McCook, and J. S. Serody, "Circulating fibrocytes prepare the lung for cancer metastasis by recruiting Ly-6C$^+$ monocytes via CCL2," *The Journal of Immunology*, vol. 190, no. 9, pp. 4861–4867, 2013.

[411] A. Klein, O. Sagi-Assif, S. Izraely et al., "The metastatic microenvironment: brain-derived soluble factors alter the malignant phenotype of cutaneous and brain-metastasizing melanoma cells," *International Journal of Cancer*, vol. 131, no. 11, pp. 2509–2518, 2012.

[412] H. W. Van Deventer, P. W. Qing, D. T. Bergstralh et al., "C-C chemokine receptor 5 on pulmonary fibrocytes facilitates migration and promotes metastasis via matrix metalloproteinase 9," *The American Journal of Pathology*, vol. 173, no. 1, pp. 253–264, 2008.

[413] H. W. van Deventer, W. O'Connor Jr., W. J. Brickey, R. M. Aris, J. P. Ting, and J. S. Serody, "C–C chemokine receptor 5 on stromal cells promotes pulmonary metastasis," *Cancer Research*, vol. 65, no. 8, pp. 3374–3379, 2005.

[414] S. Lanati, D. B. Dunn, M. Roussigné et al., "Chemotrap-1: an engineered soluble receptor that blocks chemokine-induced migration of metastatic cancer cells in vivo," *Cancer Research*, vol. 70, no. 20, pp. 8138–8148, 2010.

[415] T. van den, A. E. Bosch, J. Vaarwater, M. van den Berg, A. de Klein, and R. M. Verdijk, "Chemokine receptor CCR7 expression predicts poor outcome in uveal melanoma and relates to liver metastasis whereas expression of CXCR4 is not of clinical relevance," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 12, pp. 7354–7361, 2013.

[416] A. Letsch, U. Keilholz, D. Schadendorf et al., "Functional CCR9 expression is associated with small intestinal metastasis," *Journal of Investigative Dermatology*, vol. 122, no. 3, pp. 685–690, 2004.

[417] F. F. Amersi, A. M. Terando, Y. Goto et al., "Activation of CCR9/CCL25 in cutaneous melanoma mediates preferential metastasis to the small intestine," *Clinical Cancer Research*, vol. 14, no. 3, pp. 638–645, 2008.

[418] S. Singh, M. Varney, and R. K. Singh, "Host CXCR2-dependent regulation of melanoma growth, angiogenesis, and experimental lung metastasis," *Cancer Research*, vol. 69, no. 2, pp. 411–415, 2009.

[419] K. Kawada and M. M. Taketo, "Significance and mechanism of lymph node metastasis in cancer progression," *Cancer Research*, vol. 71, no. 4, pp. 1214–1218, 2011.

[420] K. Kawada, M. Sonoshita, H. Sakashita et al., "Pivotal role of CXCR3 in melanoma cell metastasis to lymph nodes," *Cancer Research*, vol. 64, no. 11, pp. 4010–4017, 2004.

[421] J. H. Lee, H. N. Kim, K. O. Kim et al., "CXCL10 promotes osteolytic bone metastasis by enhancing cancer outgrowth and osteoclastogenesis," *Cancer Research*, vol. 72, no. 13, pp. 3175–3186, 2012.

[422] T. Murakami, W. Maki, A. R. Cardones et al., "Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells," *Cancer Research*, vol. 62, no. 24, pp. 7328–7334, 2002.

[423] T. Takekoshi, J. J. Ziarek, B. F. Volkman, and S. T. Hwang, "A locked, dimeric CXCL12 variant effectively inhibits pulmonary metastasis of CXCR4-expressing melanoma cells due to enhanced serum stability," *Molecular Cancer Therapeutics*, vol. 11, no. 11, pp. 2516–2525, 2012.

[424] A. Fusi, Z. Liu, V. Kümmerlen, A. Nonnemacher, J. Jeske, and U. Keilholz, "Expression of chemokine receptors on circulating tumor cells in patients with solid tumors," *Journal of Translational Medicine*, vol. 10, no. 1, article 52, 2012.

[425] L. Goldberg-Bittman, O. Sagi-Assif, T. Meshel et al., "Cellular characteristics of neuroblastoma cells: regulation by the ELR—CXC chemokine CXCL10 and expression of a CXCR3-like receptor," *Cytokine*, vol. 29, no. 3, pp. 105–117, 2005.

[426] H. Zhao, W. Cai, S. Li et al., "Characterization of neuroblastoma bone invasion/metastasis in established bone metastatic model of SY5Y and KCNR cell lines," *Child's Nervous System*, vol. 29, no. 7, pp. 1097–1105, 2013.

[427] H. Zhao, W. Cai, S. Li et al., "Establishment and characterization of xenograft models of human neuroblastoma bone metastasis," *Child's Nervous System*, vol. 28, no. 12, pp. 2047–2054, 2012.

[428] H. V. Russell, J. Hicks, M. F. Okcu, and J. G. Nuchtern, "CXCR4 expression in neuroblastoma primary tumors is associated with clinical presentation of bone and bone marrow metastases," *Journal of Pediatric Surgery*, vol. 39, no. 10, pp. 1506–1511, 2004.

[429] R. Meier, A. Mühlethaler-Mottet, M. Flahaut et al., "The chemokine receptor CXCR4 strongly promotes neuroblastoma primary tumour and metastatic growth, but not invasion," *PLoS ONE*, vol. 2, no. 10, Article ID e1016, 2007.

[430] L. Zhang, H. Yeger, B. Das, M. S. Irwinz, and S. Baruchel, "Tissue microenvironment modulates CXCR4 expression and tumor metastasis in neuroblastoma," *Neoplasia*, vol. 9, no. 1, pp. 36–46, 2007.

[431] E. S. Nakamura, K. Koizumi, M. Kobayashi et al., "RANKL-induced CCL22/macrophage-derived chemokine produced from osteoclasts potentially promotes the bone metastasis of lung cancer expressing its receptor CCR4," *Clinical and Experimental Metastasis*, vol. 23, no. 1, pp. 9–18, 2006.

[432] Q. Zhang, L. Sun, L. Yin et al., "CCL19/CCR7 upregulates heparanase via specificity protein-1 (Sp1) to promote invasion of cell in lung cancer," *Tumor Biology*, vol. 34, no. 5, pp. 2703–2708, 2013.

[433] Y. H. Choi, M. D. Burdick, B. A. Strieter, B. Mehrad, and R. M. Strieter, "CXCR4, but not CXCR7, discriminates metastatic behavior in non-small cell lung cancer cells," *Molecular Cancer Research*, vol. 12, no. 1, pp. 38–47, 2014.

[434] C. L. Yang, S. S. Liu, Y. G. Ma, Y. Liu, Y. Xue, and B. Huang, "The influence of intraoperative pleural perfusion with matrine-cisplatin or cisplatin on stromal cell-derived factor-1 in non-small cell lung cancer patients with subclinical pleural metastasis," *Medical Oncology*, vol. 29, no. 2, pp. 574–581, 2012.

[435] G. Chen, Z. Wang, X. Liu, and F. Liu, "High-level CXCR4 expression correlates with brain-specific metastasis of non-small cell lung cancer," *World Journal of Surgery*, vol. 35, no. 1, pp. 56–61, 2011.

[436] F. A. Mauri, D. J. Pinato, P. Trivedi, R. Sharma, and R. J. Shiner, "Isogeneic comparison of primary and metastatic lung cancer identifies CX3CR1 as a molecular determinant of site-specific metastatic diffusion," *Oncology Reports*, vol. 28, no. 2, pp. 647–653, 2012.

[437] E. Pradelli, B. Karimdjee-Soilihi, J. Michiels et al., "Antagonism of chemokine receptor CXCR3 inhibits osteosarcoma metastasis to lungs," *International Journal of Cancer*, vol. 125, no. 11, pp. 2586–2594, 2009.

[438] P. Brennecke, M. J. Arlt, C. Campanile et al., "CXCR4 antibody treatment suppresses metastatic spread to the lung of intratibial human osteosarcoma xenografts in mice," *Clinical & Experimental Metastasis*, vol. 31, no. 3, pp. 339–49, 2014.

[439] E. Goguet-Surmenian, P. Richard-Fiardo, E. Guillemot et al., "CXCR7-mediated progression of osteosarcoma in the lungs," *British Journal of Cancer*, vol. 109, no. 6, pp. 1579–1585, 2013.

[440] R. Singh, C. R. Stockard, W. E. Grizzle, J. W. Lillard Jr., and S. Singh, "Expression and histopathological correlation of CCR9 and CCL25 in ovarian cancer," *International Journal of Oncology*, vol. 39, no. 2, pp. 373–381, 2011.

[441] Q. X. Qu, Q. Huang, J. Xu, L. T. Duan, Y. B. Zhu, and X. G. Zhang, "CD40 signal regulates CXCR4 mediating ovarian carcinoma cell migration: implications for extrapelvic metastastic factors," *Oncology Research*, vol. 20, no. 9, pp. 383–392, 2013.

[442] J. Wang, J. Cai, F. Han et al., "Silencing of CXCR4 blocks progression of ovarian cancer and depresses canonical Wnt signaling pathway," *International Journal of Gynecological Cancer*, vol. 21, no. 6, pp. 981–987, 2011.

[443] L. Guo, Z. Cui, J. Zhang, and Y. Huang, "Chemokine axes CXCL12/CXCR4 and CXCL16/CXCR6 correlate with lymph node metastasis in epithelial ovarian carcinoma," *Chinese Journal of Cancer*, vol. 30, no. 5, pp. 336–343, 2011.

[444] H. Kajiyama, K. Shibata, M. Terauchi, K. Ino, A. Nawa, and F. Kikkawa, "Involvement of SDF-1α/CXCR4 axis in the enhanced peritoneal metastasis of epithelial ovarian carcinoma," *International Journal of Cancer*, vol. 122, no. 1, pp. 91–99, 2008.

[445] J. B. Mitchem, D. J. Brennan, B. L. Knolhoff et al., "Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses," *Cancer Research*, vol. 73, no. 3, pp. 1128–1141, 2013.

[446] J. Guo, W. Lou, Y. Ji, and S. Zhang, "Effect of CCR7, CXCR4 and VEGF-C on the lymph node metastasis of human pancreatic ductal adenocarcinoma," *Oncology Letters*, vol. 5, no. 5, pp. 1572–1578, 2013.

[447] I. Roy, N. P. Zimmerman, A. C. Mackinnon, S. Tsai, D. B. Evans, and M. B. Dwinell, "CXCL12 chemokine expression suppresses human pancreatic cancer growth and metastasis," *PLoS ONE*, vol. 9, no. 3, Article ID e90400, 2014.

[448] D. Saur, B. Seidler, G. Schneider et al., "CXCR4 expression increases liver and lung metastasis in a mouse model of pancreatic cancer," *Gastroenterology*, vol. 129, no. 4, pp. 1237–1250, 2005.

[449] W. Zhong, W. Chen, D. Zhang et al., "CXCL12/CXCR4 axis plays pivotal roles in the organ-specific metastasis of pancreatic adenocarcinoma: a clinical study," *Experimental and Therapeutic Medicine*, vol. 4, no. 3, pp. 363–369, 2012.

[450] Y. Lu, Q. Chen, E. Corey et al., "Activation of MCP-1/CCR2 axis promotes prostate cancer growth in bone," *Clinical and Experimental Metastasis*, vol. 26, no. 2, pp. 161–169, 2009.

[451] S. J. Kim, H. Uehara, T. Karashima, M. Mccarty, N. Shih, and I. J. Fidler, "Expression of interleukin-8 correlates with angiogenesis, tumorigenicity, and metastasis of human prostate cancer cells implanted orthotopically in nude mice," *Neoplasia*, vol. 3, no. 1, pp. 33–42, 2001.

[452] X. Yang, Z. Xu, D. Li et al., "Cell surface nucleolin is crucial in the activation of the CXCL12/CXCR4 signaling pathway," *Tumor Biology*, vol. 35, no. 1, pp. 333–338, 2014.

[453] W. Hu, X. Zhen, B. Xiong, B. Wang, W. Zhang, and W. Zhou, "CXCR6 is expressed in human prostate cancer in vivo and is involved in the in vitro invasion of PC3 and LNCap cells," *Cancer Science*, vol. 99, no. 7, pp. 1362–1369, 2008.

[454] S. L. Kominsky, S. M. Abdelmagid, M. Doucet, K. Brady, and K. L. Weber, "Macrophage inflammatory protein-1δ: A novel osteoclast stimulating factor secreted by renal cell carcinoma bone metastasis," *Cancer Research*, vol. 68, no. 5, pp. 1261–1266, 2008.

[455] P. L. Wagner, T. A. Moo, N. Arora et al., "The chemokine receptors CXCR4 and CCR7 are associated with tumor size and pathologic indicators of tumor aggressiveness in papillary thyroid carcinoma," *Annals of Surgical Oncology*, vol. 15, no. 10, pp. 2833–2841, 2008.

[456] M. Sancho, J. M. Vieira, C. Casalou et al., "Expression and function of the chemokine receptor CCR7 in thyroid carcinomas," *Journal of Endocrinology*, vol. 191, no. 1, pp. 229–238, 2006.

[457] N. Wang, H. J. Luo, G. B. Yin et al., "Overexpression of HIF-2$\boldsymbol{\alpha}$, TWIST, and CXCR4 is associated with lymph node metastasis in papillary thyroid carcinoma," *Clinical and Developmental Immunology*, vol. 2013, Article ID 589423, 9 pages, 2013.

[458] H. Yasuoka, R. Kodama, M. Hirokawa et al., "CXCR4 expression in papillary thyroid carcinoma: induction by nitric oxide and correlation with lymph node metastasis," *BMC Cancer*, vol. 8, article 274, 2008.

[459] S. Dang, Y. Peng, L. Ye et al., "Stimulation of TLR4 by LMW-HA induces metastasis in human papillary thyroid carcinoma through CXCR7," *Clinical and Developmental Immunology*, vol. 2013, Article ID 712561, 11 pages, 2013.

[460] H. Spring, T. Schüler, B. Arnold, G. J. Hämmerling, and R. Ganss, "Chemokines direct endothelial progenitors into tumor neovessels," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 50, pp. 18111–18116, 2005.

[461] Y. Shih, M. C. Wang, H. Peng et al., "Modulation of chemotactic and pro-inflammatory activities of endothelial progenitor cells by hepatocellular carcinoma," *Cellular Signalling*, vol. 24, no. 3, pp. 779–793, 2012.

[462] A. Li, X. J. Cheng, A. Moro, R. K. Singh, O. J. Hines, and G. Eibl, "CXCR2-dependent endothelial progenitor cell mobilization in pancreatic cancer growth," *Translational Oncology*, vol. 4, no. 1, pp. 20–28, 2011.

[463] Z. Liu, R. Tian, Y. Li et al., "Inhibition of tumor angiogenesis and melanoma growth by targeting vascular E-selectin," *Annals of Surgery*, vol. 254, no. 3, pp. 450–457, 2011.

[464] X. Yang, P. Lu, C. Fujii et al., "Essential contribution of a chemokine, CCL3, and its receptor, CCR1, to hepatocellular carcinoma progression," *International Journal of Cancer*, vol. 118, no. 8, pp. 1869–1876, 2006.

[465] M. P. Rodero, C. Auvynet, L. Poupel, B. Combadière, and C. Combadière, "Control of both myeloid cell infiltration and

angiogenesis by ccr1 promotes liver cancer metastasis development in mice," *Neoplasia*, vol. 15, no. 6, pp. 641–648, 2013.

[466] E. Menu, E. De Leenheer, H. De Raeve et al., "Role of CCR1 and CCR5 in homing and growth of multiple myeloma and in the development of osteolytic lesions: a study in the 5TMM model," *Clinical and Experimental Metastasis*, vol. 23, no. 5-6, pp. 291–300, 2006.

[467] S. Wang, M. Xu, F. Li et al., "Ethanol promotes mammary tumor growth and angiogenesis: the involvement of chemoattractant factor MCP-1," *Breast Cancer Research and Treatment*, vol. 133, no. 3, pp. 1037–1048, 2012.

[468] S. M. Potter, R. M. Dwyer, M. C. Hartmann et al., "Influence of stromal-epithelial interactions on breast cancer in vitro and in vivo," *Breast Cancer Research and Treatment*, vol. 131, no. 2, pp. 401–411, 2012.

[469] M. Ohta, Y. Kitadai, S. Tanaka et al., "Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas," *International Journal of Cancer*, vol. 102, no. 3, pp. 220–224, 2002.

[470] M. Koga, H. Kai, K. Egami et al., "Mutant MCP-1 therapy inhibits tumor angiogenesis and growth of malignant melanoma in mice," *Biochemical and Biophysical Research Communications*, vol. 365, no. 2, pp. 279–284, 2008.

[471] S. Araki, Y. Omori, D. Lyn et al., "Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer," *Cancer Research*, vol. 67, no. 14, pp. 6854–6862, 2007.

[472] K. J. Sales, J. R. Sutherland, H. N. Jabbour, and A. A. Katz, "Seminal plasma induces angiogenic chemokine expression in cervical cancer cells and regulates vascular function," *Biochimica et Biophysica Acta*, vol. 1823, no. 10, pp. 1789–1795, 2012.

[473] J. Dwyer, J. K. Hebda, A. Le Guelte et al., "Glioblastoma cell-secreted interleukin-8 induces brain endothelial cell permeability via CXCR2," *PLoS ONE*, vol. 7, no. 9, Article ID e45562, 2012.

[474] S. Tazzyman, S. T. Barry, S. Ashton et al., "Inhibition of neutrophil infiltration into A549 lung tumors *in vitro* and *in vivo* using a CXCR2-specific antagonist is associated with reduced tumor growth," *International Journal of Cancer*, vol. 129, no. 4, pp. 847–858, 2011.

[475] Y. Saijo, M. Tanaka, M. Miki et al., "Proinflammatory cytokine IL-1β promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: in vivo analysis of tumor-stromal interaction," *Journal of Immunology*, vol. 169, no. 1, pp. 469–475, 2002.

[476] L. W. Horton, Y. Yu, S. Zaja-Milatovic, R. M. Strieter, and A. Richmond, "Opposing roles of murine duffy antigen receptor for chemokine and murine CXC chemokine receptor-2 receptors in murine melanoma tumor growth," *Cancer Research*, vol. 67, no. 20, pp. 9791–9799, 2007.

[477] M. N. Wente, M. P. Keane, M. D. Burdick et al., "Blockade of the chemokine receptor CXCR2 inhibits pancreatic cancer cell-induced angiogenesis," *Cancer Letters*, vol. 241, no. 2, pp. 221–227, 2006.

[478] Y. Matsuo, M. Raimondo, T. A. Woodward et al., "CXC-chemokine/CXCR2 biological axis promotes angiogenesis in vitro and in vivo in pancreatic cancer," *International Journal of Cancer*, vol. 125, no. 5, pp. 1027–1037, 2009.

[479] A. Li, J. King, A. Moro et al., "Overexpression of CXCL5 is associated with poor survival in patients with pancreatic cancer," *The American Journal of Pathology*, vol. 178, no. 3, pp. 1340–1349, 2011.

[480] H. Shen, R. Schuster, B. Lu, S. E. Waltz, and A. B. Lentsch, "Critical and opposing roles of the chemokine receptors CXCR2 and CXCR3 in prostate tumor growth," *Prostate*, vol. 66, no. 16, pp. 1721–1728, 2006.

[481] J. Mestas, M. D. Burdick, K. Reckamp, A. Pantuck, R. A. Figlin, and R. M. Strieter, "The role of CXCR2/CXCR2 ligand biological axis in renal cell carcinoma," *The Journal of Immunology*, vol. 175, no. 8, pp. 5351–5357, 2005.

[482] I. Desbaillets, A.-C. Diserens, N. de Tribolet, M.-F. Hamou, and E. G. van Meir, "Upregulation of interleukin 8 by oxygen-deprived cells in glioblastoma suggests a role in leukocyte activation, chemotaxis, and angiogenesis," *The Journal of Experimental Medicine*, vol. 186, no. 8, pp. 1201–1212, 1997.

[483] M. Kline, K. Donovan, L. Wellik et al., "Cytokine and chemokine profiles in multiple myeloma; significance of stromal interaction and correlation of IL-8 production with disease progression," *Leukemia Research*, vol. 31, no. 5, pp. 591–598, 2007.

[484] A. Agarwal, S. L. Tressel, R. Kaimal et al., "Identification of a metalloprotease-chemokine signaling system in the ovarian cancer microenvironment: implications for antiangiogenic therapy," *Cancer Research*, vol. 70, no. 14, pp. 5880–5890, 2010.

[485] K. S. Hill, I. Gaziova, L. Harrigal et al., "Met receptor tyrosine kinase signaling induces secretion of the angiogenic chemokine interleukin-8/CXCL8 in pancreatic cancer," *PLoS ONE*, vol. 7, no. 7, Article ID e40420, 2012.

[486] C. Charalambous, L. B. Pen, Y. S. Su, J. Milan, T. C. Chen, and F. M. Hofman, "Interleukin-8 differentially regulates migration of tumor-associated and normal human brain endothelial cells," *Cancer Research*, vol. 65, no. 22, pp. 10347–10354, 2005.

[487] Z. Liang, J. Brooks, M. Willard et al., "CXCR4/CXCL12 axis promotes VEGF-mediated tumor angiogenesis through Akt signaling pathway," *Biochemical and Biophysical Research Communications*, vol. 359, no. 3, pp. 716–722, 2007.

[488] O. Kollmar, K. Rupertus, C. Scheuer et al., "CXCR4 and CXCR7 regulate angiogenesis and CT26.WT tumor growth independent from SDF-1," *International Journal of Cancer*, vol. 126, no. 6, pp. 1302–1315, 2010.

[489] D. A. Margolin, J. Silinsky, C. Grimes et al., "Lymph node stromal cells enhance drug-resistant colon cancer cell tumor formation through SDF-1α/CXCR4 paracrine signaling," *Neoplasia*, vol. 13, no. 9, pp. 874–886, 2011.

[490] B. Ingold, E. Simon, U. Ungethüm et al., "Vascular CXCR4 expression—a novel antiangiogenic target in gastric cancer?" *PLoS ONE*, vol. 5, no. 4, Article ID e10087, 2010.

[491] A. Salmaggi, M. Gelati, B. Pollo et al., "CXCL12 in malignant glial tumors: a possible role in angiogenesis and cross-talk between endothelial and tumoral cells," *Journal of Neuro-Oncology*, vol. 67, no. 3, pp. 305–317, 2004.

[492] S. A. Rempel, S. Dudas, S. Ge, and J. A. Gutiérrez, "Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma," *Clinical Cancer Research*, vol. 6, no. 1, pp. 102–111, 2000.

[493] Y. Ping, X. Yao, J. Jiang et al., "The chemokine CXCL12 and its receptor CXCR4 promote glioma stem cell-mediated VEGF production and tumour angiogenesis via PI3K/AKT signalling," *Journal of Pathology*, vol. 224, no. 3, pp. 344–354, 2011.

[494] W. Li, E. Gomez, and Z. Zhang, "Immunohistochemical expression of stromal cell-derived factor-1 (SDF-1) and CXCR4 ligand

receptor system in hepatocellular carcinoma," *Journal of Experimental and Clinical Cancer Research*, vol. 26, no. 4, pp. 527–533, 2007.

[495] I. Kryczek, A. Lange, P. Mottram et al., "CXCL12 and vascular endothelial growth factor synergistically induce neonaniogenisis in human ovarian cancers," *Cancer Research*, vol. 65, no. 2, pp. 465–472, 2005.

[496] T. Koshiba, R. Hosotani, Y. Miyamoto et al., "Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression," *Clinical Cancer Research*, vol. 6, no. 9, pp. 3530–3535, 2000.

[497] J. Wang, J. Wang, Y. Sun et al., "Diverse signaling pathways through the SDF-1/CXCR4 chemokine axis in prostate cancer cell lines leads to altered patterns of cytokine secretion and angiogenesis," *Cellular Signalling*, vol. 17, no. 12, pp. 1578–1592, 2005.

[498] M. Hao, J. Zheng, K. Hou et al., "Role of chemokine receptor CXCR7 in bladder cancer progression," *Biochemical Pharmacology*, vol. 84, no. 2, pp. 204–214, 2012.

[499] Z. Miao, K. E. Luker, B. C. Summers et al., "CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 40, pp. 15735–15740, 2007.

[500] J. Wang, Y. Shiozawa, Y. Wang et al., "The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer," *The Journal of Biological Chemistry*, vol. 283, no. 7, pp. 4283–4294, 2008.

[501] N. Maishi, N. Ohga, Y. Hida et al., "CXCR7: a novel tumor endothelial marker in renal cell carcinoma," *Pathology International*, vol. 62, no. 5, pp. 309–317, 2012.

[502] T. Ren, Q. Chen, Z. Tian, and H. Wei, "Down-regulation of surface fractalkine by RNA interference in B16 melanoma reduced tumor growth in mice," *Biochemical and Biophysical Research Communications*, vol. 364, no. 4, pp. 978–984, 2007.

[503] D. M. Smadja, I. Bièche, G. Uzan et al., "PAR-1 activation on human late endothelial progenitor cells enhances angiogenesis in vitro with upregulation of the SDF-1/CXCR4 system," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 11, pp. 2321–2327, 2005.

[504] D. J. Nolan, A. Ciarrocchi, A. S. Mellick et al., "Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization," *Genes and Development*, vol. 21, no. 12, pp. 1546–1558, 2007.

[505] A. S. Arbab, S. D. Pandit, S. A. Anderson et al., "Magnetic resonance imaging and confocal microscopy studies of magnetically labeled endothelial progenitor cells trafficking to sites of tumor angiogenesis," *Stem Cells*, vol. 24, no. 3, pp. 671–678, 2006.

[506] D. J. Brat and T. B. Mapstone, "Malignant glioma physiology: cellular response to hypoxia and its role in tumor progression," *Annals of Internal Medicine*, vol. 138, no. 8, pp. 659–668, 2003.

[507] L. M. Campbell, P. J. Maxwell, and D. J. J. Waugh, "Rationale and means to target pro-inflammatory interleukin-8 (CXCL8) signaling in cancer," *Pharmaceuticals*, vol. 6, no. 8, pp. 929–959, 2013.

[508] S. Futagami, T. Hiratsuka, T. Shindo et al., "COX-2 and CCR2 induced by CD40 ligand and MCP-1 are linked to VEGF production in endothelial cells," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 78, no. 2, pp. 137–146, 2008.

[509] J. M. Brown, "Vasculogenesis: a crucial player in the resistance of solid tumours to radiotherapy," *The British Journal of Radiology*, vol. 87, no. 1035, Article ID 20130686, 2014.

[510] K. Tobinai, T. Takahashi, and S. Akinaga, "Targeting chemokine receptor CCR4 in adult T-cell leukemia-lymphoma and other T-cell lymphomas," *Current Hematologic Malignancy Reports*, vol. 7, no. 3, pp. 235–240, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 453497, 9 pages
http://dx.doi.org/10.1155/2014/453497

Research Article

Effects of Single or Combined Treatments with Radiation and Chemotherapy on Survival and Danger Signals Expression in Glioblastoma Cell Lines

Francesca Pasi,[1] Alessandro Paolini,[1,2] Rosanna Nano,[1] Riccardo Di Liberto,[2] and Enrica Capelli[3]

[1] *Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, Via Ferrata 9, 27100 Pavia, Italy*
[2] *Department of Medical Physics, IRCCS Policlinico S. Matteo Foundation, Viale Golgi 19, 27100 Pavia, Italy*
[3] *Department of Earth and Environmental Sciences, University of Pavia, Via Ferrata 1, 27100 Pavia, Italy*

Correspondence should be addressed to Francesca Pasi; francesca.pasi@gmail.com

Received 5 April 2014; Accepted 10 June 2014; Published 1 July 2014

Academic Editor: Zhen Chen

The success of chemo- and radiotherapy in glioblastoma multiforme, the most common and lethal primary brain tumour, could rely on the induction of immunogenic tumour cell death and on the induction of anticancer immune response. In this study we investigated cell survival to single treatments or combination of X-rays and temozolomide in glioblastoma cell lines (T98G and U251MG) and we attempted to identify danger signals (HMGB1 and HSP70) released by dying cells in the microenvironment that could activate antitumour immunity contributing to the therapeutic efficacy of conventional treatments. Our data suggest that HSP70 translocates from cytoplasm to extracellular environment after an increase in radiation dose and HMGB1 translocates from the nucleus to the cytoplasm and subsequently is released into the extracellular space, confirming a role of these proteins as signals released after radiation-induced damage in glioblastoma cells. We also could state that TMZ had limited effectiveness in activating HMGB1 and HSP70 signalling and, instead, an adjuvant effect was observed in some combined treatments, depending on schedule, cell line, and timing. A big challenge in tumour therapy is, therefore, to identify the most beneficial combination and chronology of multiple treatment options to contribute to the improvement of the therapeutic outcome.

1. Introduction

Glioblastoma multiforme (GBM, WHO grade IV) is the most common and lethal primary malignant brain tumour that continues to have poor prognosis and a high likelihood of recurrence [1]. The median survival time from the time of diagnosis without any treatment is 3 months and despite the recommended treatment regimen of aggressive surgical resection, radiation, and chemotherapy, it remains approximately 9–11 months [2]. Thus, these tumours continue to present an enormous therapeutic challenge. Since most of the patients develop also a relapse [3], a deeper knowledge is urgently needed in order to find out how the applied therapies can modulate the tumour cells. For this reason, to win the fight against cancer, it is necessary not only to develop strategies to kill all cancer cells efficiently but also to attempt to stimulate an immune response so that the immune system can keep residual tumour cells in check [4]. In particular, combined tumour therapies of radiation and chemotherapy should on the one hand kill the cancer cells and on the other hand induce antigen release and danger signals expression from the tumour. Consequently these signals released in the microenvironment could activate both the innate and adaptive immune system of the host. Hence, the cellular stress induced by treatments dictates the immunological response to dying cells and the activation of immune system might contribute to the therapeutic efficacy of conventional cancer treatments determining synergistic effects of radiation and immunotherapy increases [5].

However, the underlying biological pathways are only partially understood. In particular, the immunogenic potential of different tumour cell types, the number of different

danger signals, their molecular identity, their different biological effects, the receptors, and the pathways that sense the release of these signals are still not known. It could be necessary to define their roles in both health and disease and it might be possible to use these molecules, expressed on cell surface or released in the microenvironment, to manipulate immune responses or to inhibit them to treat malignant brain tumour [6].

In the present study, to gain new insights into the mechanisms of radiation and/or chemotherapeutic effect in malignant gliomas we investigated cell survival to single treatment or combination of radiation and temozolomide (TMZ), a chemotherapeutic drug currently used in clinics for the treatments of these tumours. Moreover, to better elucidate target molecules involved in malignant glioma responses, we attempted to identify danger signals like high mobility group box-1 (HMGB1) protein and heat-shock protein 70 (HSP70) in glioblastoma using cell culture system. These proteins are released as danger signals by dying cells and could activate dendritic cells and stimulate antigen processing and presentation to T cells [7]. HSP70 is taken up from dendritic cells for cross-presentation via HSP receptors (e.g., CD91 and CD14) and HMGB1 released by necrotic cells is a potent adjuvant *in vivo* that triggers a protective immune response through activation of TLR4 on DCs [8, 9].

Stimulation by these danger signals, uptake, and presentation of dead tumour cell-derived peptides by mature dendritic cell as well as their consecutive contact with T cells may lead to specific and, most importantly, long-lasting antitumour immunity that might contribute to the therapeutic efficacy of conventional cancer treatments.

For this purpose, glioblastoma cell lines (T98G and U251MG) were exposed at different doses of radiation (X-rays), for example, 2 Gy (common single fraction in tumour therapy) and 10 Gy (weekly fraction). We performed combined treatments with additional application of TMZ for 2 hours before irradiation and we evaluated the survival of cells and the expression and release of HMGB1 and HSP70 after single or combined treatments.

2. Materials and Methods

2.1. Cell Culture. Human glioblastoma T98G and U251MG cells were obtained from the European Collection of Cell Cultures (Porton Down, Salisbury, UK). T98G cells were cultured in Eagle's minimum essential medium (EMEM; Euroclone SpA, MI, Italy) supplemented with 10% foetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 units/mL penicillin/streptomycin (Euroclone SpA, MI, Italy), 2 mM L-glutamine (Euroclone SpA, MI, Italy), and 0.01% sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in an atmosphere of 5% CO_2 and 90% humidity. U251MG cells were grown in Eagle's minimum essential medium (EMEM; Euroclone SpA, MI, Italy) supplemented with 10% foetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 1% sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 2 mM glutamine (Euroclone SpA, MI, Italy), 100 U/mL of penicillin (Euroclone SpA, MI, Italy), 100 μg/mL of streptomycin (Euroclone SpA, MI, Italy), and 1% nonessential amino acids (Euroclone SpA, MI, Italy) at 37°C in 5% CO_2 and 90% humidity. Stock cultures were maintained in exponential growth as monolayers in 75 cm^2 Corning plastic tissue-culture flasks (VWR International PBI Srl, MI, Italy).

2.2. Irradiation and Chemotherapic Treatments. Cells were irradiated at doses from 2 Gy to 10 Gy with X-rays at room temperature using a Raycell Mk2 (Clinica Pediatrica, IRCCS Policlinico San Matteo, Pavia, Italy) with a dose rate of 8 Gy/min. Sham irradiated cells (0 Gy) were performed as control. Before irradiation the medium was removed from the flasks and fresh medium was added in the cells.

For combined applications, cells were treated with TMZ used at concentrations of 20 μM and added to the culture medium. Cells were incubated at 37°C for 2 hours with TMZ before irradiation treatment.

2.3. Clonogenic Survival Assay. The clonogenic assay was performed on single-cell suspension of exponentially growing cells. Cells were counted and plated in growth medium into T25 flasks and 24 h after plating they were irradiated and treated with TMZ or with combined treatments. After 10–14 days cells were stained with crystal violet solution for 6 min; colonies > 50 cells were counted. Calculation of survival fractions (SF) was performed using the equation SF = colonies counted/cells seeded × (PE/100), taking the individual plating efficiency (PE) into consideration. All experiments were repeated at least three times.

2.4. Immunocytochemistry Analysis. The presence and localization of danger signals (HMGB1 and HSP70) in glioblastoma cells were evaluated also with immunocytochemistry technique. Irradiated cells or combined treated cells were kept in an incubator at 37°C for 20 hours. Subsequently, cells were fixed for 10 minutes with cold 70% ethanol. As primary antibody, a polyclonal rabbit anti-HMGB1 antibody (dilution 1 : 100; Upstate, New York, USA) and a polyclonal mouse anti-HSP70 antibody (dilution 1 : 1000, BD Biosciences, NJ, USA) were used, whereas the detection was performed with the EnVision+ System-HRP (AEC) kit (Dako, Glostrup, Denmark). A semiquantitative analysis of Hsp70 and HMGB1 expression on the immunostained cells with a computer-assisted analysis was performed. In detail, the image acquisition was carried out with a digital camera (Olympus) coupled to an Olympus BX 41 optic microscope (Olympus, Milano, Italy). The digital images acquired were then processed using the ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA) that gives a measure of the intensity of the immunostaining.

2.5. Enzyme-Linked-Immunosorbent Assay (ELISA). The quantification of free-proteins HSP70 and HMGB1 in supernatants was performed with an enzyme-linked-immunosorbent assay (ELISA) (resp., EIAab, China, and Uscn Life Science Inc., Houston, USA) according to the manufacturer's instructions. The minimum detectable dose of Hsp70 with our test system is less than 0.039 ng/mL and

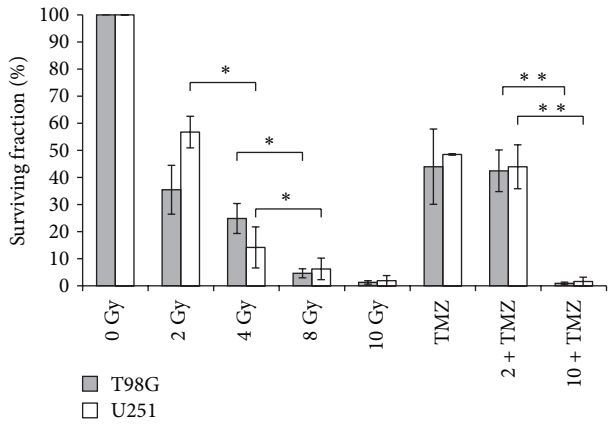


Figure 1: Clonogenic survival of human T98G and U251MG glioblastoma cells after single or combined treatments with radiation and temozolomide (TMZ). Means of three independent experiments ± standard deviation ($^{*}P < 0.05$, $^{**}P < 0.01$).

the minimum detectable dose of HMGB1 is typically less than 5.1 pg/mL.

2.6. Statistical Analyses. Data are obtained from four independent experiments, each performed in duplicate. Statistical analyses were performed using Student's t-test. A P value of <0.05 was considered as significant (∗).

3. Results and Discussion

In this work we evaluated clonogenic potential of T98G and U251MG cells after radiation treatments in comparison to radiation plus chemotherapy with TMZ. Cells treated with radiation alone showed a decrease in cell survival in a dose-dependent manner compared to control. A significant tumour growth retardation was observed, as expected, when the cells were irradiated with 8 and 10 Gy. The treatment with TMZ induced a decrease in clonogenicity compared to control but did not appear to be more effective in terms of decrease of cell survival in comparison to radiation alone. Also the combination of TMZ plus 2 or 10 Gy did not affect the percentage of surviving fraction compared to radiation alone in our experimental conditions (Figure 1).

Our data suggested that the exposure of TMZ 2 hours before irradiation did not induce an increase of radiosensitivity of T98G and U251MG cell lines. In fact, for primary malignant cell lines that express MGMT, such as T98G cells, an acute preexposure to TMZ was reported not to influence radiation cell death [10], whereas U251MG cells that are MGMT negative showed probably a radiosensitization by higher concentration of TMZ as reported by Kil and collegueas [11].

These results suggested that TMZ did not seem to have an additional effect of radiation in our experimental conditions. However, combination schedule and cell lines are important to determine an enhancement of cytotoxic effects on tumour cells and could have important implications for developing strategies to improve outcomes of patients.

Subsequently, we focus our attention on two immunogenic signals, HMGB1 and HSP70, that may lead to the activation of an immune response if released by dying cells in the microenvironment.

We studied the expression and release into the medium of these free-proteins in T98G and U251MG cells after irradiation and TMZ treatments. After radiation-induced cellular stress, cells responded initially with a gradual increase of intracellular HSP70 that is produced in higher quantities with the increase of the dose up to 4 Gy and 8 Gy and is then no longer produced after 10 Gy (Tables 1 and 2, Figures 2 and 3). The increase of intracellular HSP70 continued over 48 hours and is joined to an increase in the extracellular release of the protein after high doses such as 8 and 10 Gy. These results are consistent with the externalization of HSPs that takes place in the late phases of apoptotic cell death and after a serious stress such as the one due to high doses of radiation. These results support and promote the idea about the role of HSPs as signal of damage, demonstrated to be involved in the late phases of apoptotic cell death and in stress conditions like high doses of radiation [12].

T98G cells treated with TMZ alone showed an increase in the production of the protein compared to the control and irradiated cells (Table 1, Figure 2) and also an increase of the release at 48 hours compared to the control and 2 Gy treatment (Figure 4). As we can see from literature, HSP70 that is released from tumour cells undergoing chemotherapy may mediate a danger signal to the host's immune system [13, 14].

After combined treatment with TMZ and low doses of radiation, an additive effect of radiation and the drug was observed in the release of the protein in the microenvironment where HSP70 could play its immunogenic role. Extracellular HSP70 binds to high-affinity receptors on antigen presenting cells (APC), leading to activation and representation of the peptide antigen cargo by the APC. HSP70-peptide complexes coordinately activate innate immune responses and deliver antigens for representation by MHC classes I and II molecules on the APC cell surface, leading to specific antitumor immunity [15].

At higher doses (10 Gy), the effect is minor compared to radiation alone. In this case HSP70 may have a cytoprotective effect and be involved in preventing or hindering cell death. High levels of HSP70 in cytoplasm, in fact, prevent stress-induced apoptosis [16] and may induce resistance to chemotherapy and radiotherapy [15].

As occurred in T98G and in U87 cells analysed by us previously [17], even in U251MG the production of HSP70 increased in the first 24 hours with the increase of the dose up to 4 Gy and decreased at higher doses (Table 2, Figures 2 and 3). In this case, however, this decrease of intracellular expression is not accompanied by a release into the extracellular space (Figure 5). Probably the release occurs after a period of time longer for this cell line. In fact, after 48 hours it was detectable in the controls and after low doses. It is possible that later it occurs at higher doses and increases again at low doses.

After treatment with TMZ, there was no difference to 24 hours compared to the control or with regard to the release or the cytoplasmic expression, while at 48 hours there was a decrease of the release. The cell probably responds to TMZ

Table 1: Intracellular expression of HSP70 in T98G cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ).

HSP70	0 Gy	2 Gy	4 Gy	8 Gy	10 Gy	TMZ	2 Gy + TMZ	10 Gy + TMZ
24 h	0/+	++/+++	++	+/++	+	+++	++/+++	+/++
48 h	++	+/++	++/+++	++/+++	+	+++	+++	++/+++

0: no expression of protein; +: low expression of protein; ++: moderate expression of protein; +++: high expression of protein.

Table 2: Intracellular expression of HSP70 in U251MG cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ).

HSP70	0 Gy	2 Gy	4 Gy	8 Gy	10 Gy	TMZ	2 Gy + TMZ	10 Gy + TMZ
24 h	+/++	+/++	++/+++	++	++	+/++	++	+++
48 h	+++	++/+++	++	++/+++	0/+	+++	+/++	++

0: no expression of protein; +: low expression of protein; ++: moderate expression of protein; +++: high expression of protein.

with another mechanism and HSP70 pathway is not involved in TMZ-induced stress response.

The combination of TMZ and radiation induced instead an increase of the production of the protein at 24 hours followed by a release at 48 hours that was more substantial than the one after drug and radiation alone. So the drug alone did not seem to have a significant effect on the production and release of HSP70 in U251MG, probably due to the fact that this cell line has low MGMT level and TMZ tolerance [18]. However, when TMZ was combined with radiation, it induced an effect on the release of HSP70. The pathway of this protein may be triggered by combined treatments and not by single treatment with drug, probably because the radiation causes more damage pathway targeted to cell death and hence involved in the immunogenicity phenomenon, while the drug induced a mechanism more directed to the cytoprotection without activating the expression and release of damage signals.

Data obtained from immunocytochemical analysis showed that in both cell lines HMGB1 had a nuclear localization in controls and in cells exposed to doses of 2 and 4 Gy (Tables 3 and 4, Figure 6). HMGB1 is ubiquitously present in the nucleus of almost all mammalian cells [19]. Within nucleus HMGB1 stabilizes nucleosomes and regulates transcription of many genes [20, 21]. At higher doses (8 Gy and 10 Gy) the expression of this protein appeared paler and the protein localization became cytoplasmatic (Figures 6 and 7). Moreover, a dose-dependent increase of HMGB1 release was observed in T98G cells after 24 hours, with the exception of the dose of 8 Gy that did not seem to induce an increase of the release (Figure 8). At this dose and this time after treatments, the cells could carry out the translocation of the protein and not its release. In U251MG the release was affected after 8 Gy exposure where it was higher compared to control. Probably this cell line needs more time to activate the release at low doses. After exposure of 10 Gy, instead, the protein was neither produced nor released (Figure 9), suggesting that this dose is that high to induce a huge damage that involves other pathways rather than the expression of DAMPs (damage-associated molecular pattern molecules). These data could demonstrate that radiation induces an increase of HMGB1 and a translocation from nucleus to cytoplasm at low doses, whereas at higher doses radiation could stimulate the release of the protein in the extracellular environment. The release of HMGB1 into the extracellular space occurs during cell death induced by radiation, both necrosis and apoptosis [22, 23], when the protein plays a role as a DAMP which activates local antigen presenting cells to become stimulatory to the immune system response [6]. The release of HMGB1 from the nucleus of dying tumour cells to their cytoplasm and subsequently to the extracellular space during cell death constitutes a crucial step in the activation of antigen presenting cells [24].

After 24 hours from treatment with 20 μM of TMZ T98G, cells showed a nuclear expression of HMGB1 accompanied by an extracellular release higher than that observed in control cells (Figure 6). After 48 hours from treatment a translocation of HMGB1 in the cytoplasmic compartment was observed followed by a release of the protein that seemed to be higher than control cells but similar to the one obtained after treatment with 4 Gy (Figure 8). On the contrary, U251MG cells treated with TMZ showed no difference in the protein expression and release compared to 0 and 2 Gy treatments (Figure 9), as demonstrated previously also in U87 cells [17]. Probably the incubation time period and the drug concentration are too low to show significant changes in HMGB1 expression and localization in this cell line. Another explanation could be that TMZ did not induce a cell death type able to cause the concomitant release of this protein in this cell line. In fact, the release process may vary with cell type; generally it occurs in necrotic cells [22] but in some cell types HMGB1 could be released also by apoptotic or secondary necrotic cells [23].

Combined treatments with 2 Gy plus TMZ caused a cytoplasmatic production of HMGB1 after 24 hours, which is highly expressed in T98G and slightly in U251MG cells (Tables 3 and 4). In this condition a high release of HMGB1 compared to control was induced after short time in the first cell line, whereas in the second cell lines the values obtained are lower than those observed after single treatments. However the protein tends to be no longer produced nor released over time. On the contrary, in the condition 10 Gy plus TMZ, both after 24 and after 48 hours from treatment, a lesser presence of HMGB1 in the cytoplasm was observed due to a persistent release of the protein. At 10 Gy plus TMZ the situation is quite similar to that obtained

Table 3: Intracellular expression of HMGB1 in T98G cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ).

HMGB1	0 Gy	2 Gy	4 Gy	8 Gy	10 Gy	TMZ	2 Gy + TMZ	10 Gy + TMZ
24 h	+ (n)	+/++ (n)	++ (n)	+/++ (c)	+/++ (c)	+++ (n)	++/+++ (c)	+ (c)
48 h	++/+++ (n/p)	+++ (n)	++/+++ (n)	+/++ (c)	+ (c)	+/++ (p)	+/++ (c)	+/++ (c)

0: no expression of protein; +: low expression of protein; ++: moderate expression of protein; +++: high expression of protein; n: nuclear expression; p: perinuclear expression; c: cytoplasmatic expression.

Table 4: Intracellular expression of HMGB1 in U251MG cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ).

HMGB1	0 Gy	2 Gy	4 Gy	8 Gy	10 Gy	TMZ	2 Gy + TMZ	10 Gy + TMZ
24 h	+/++ (n)	++/+++ (p)	+ (n)	+++ (c)	+/++ (c)	++ (n)	+ (c)	+/++ (c)
48 h	+/++ (n)	++ (p)	+++ (p)	++/+++ (c)	+ (c)	++/+++ (p)	+ (c)	++ (p)

0: no expression of protein; +: low expression of protein; ++: moderate expression of protein; +++: high expression of protein; n: nuclear expression; p: perinuclear expression; c: cytoplasmatic expression.

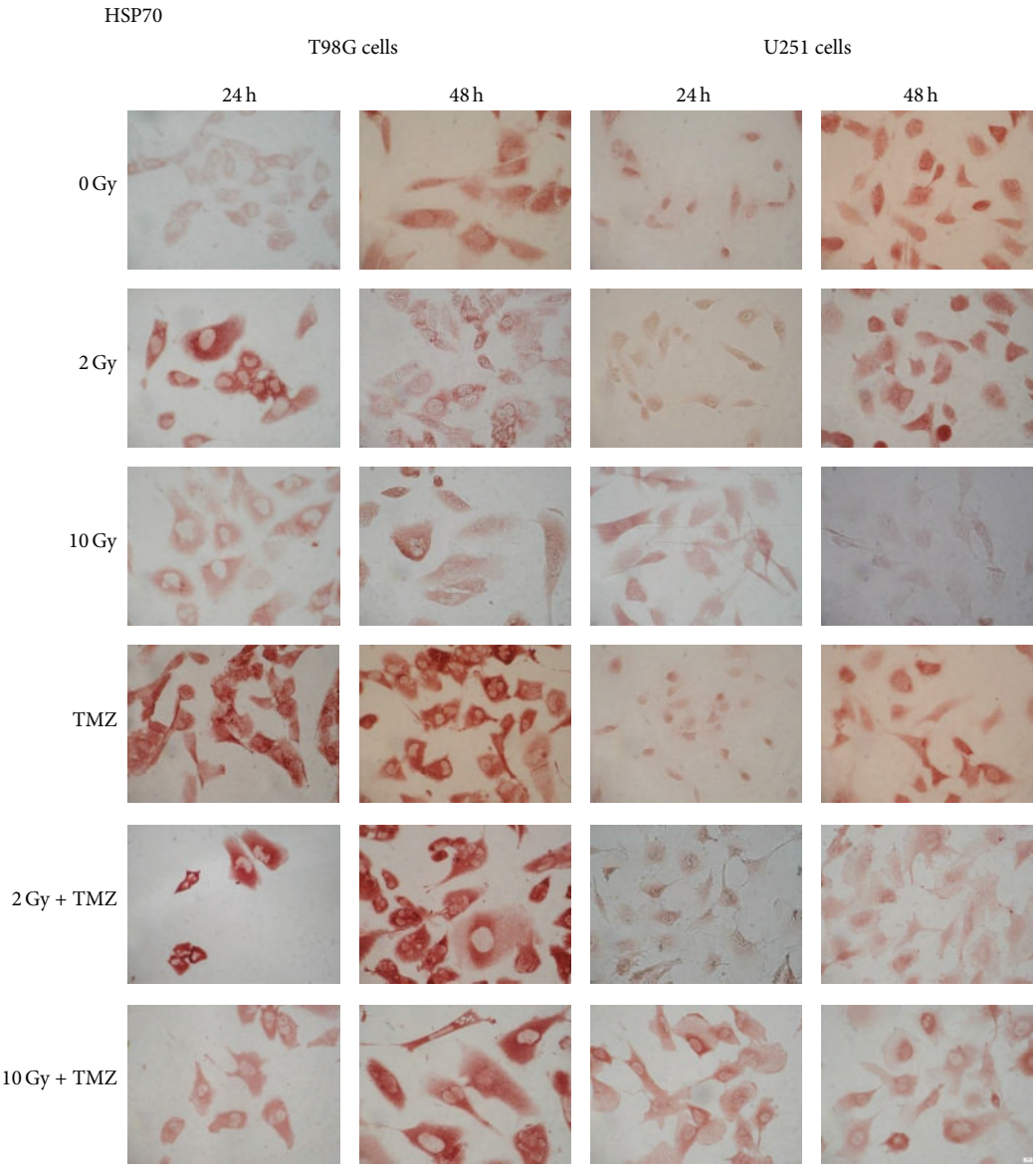


Figure 2: Intracellular HSP70 expression detected by immunocytochemical technique in T98G and U251MG cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ) (400x).

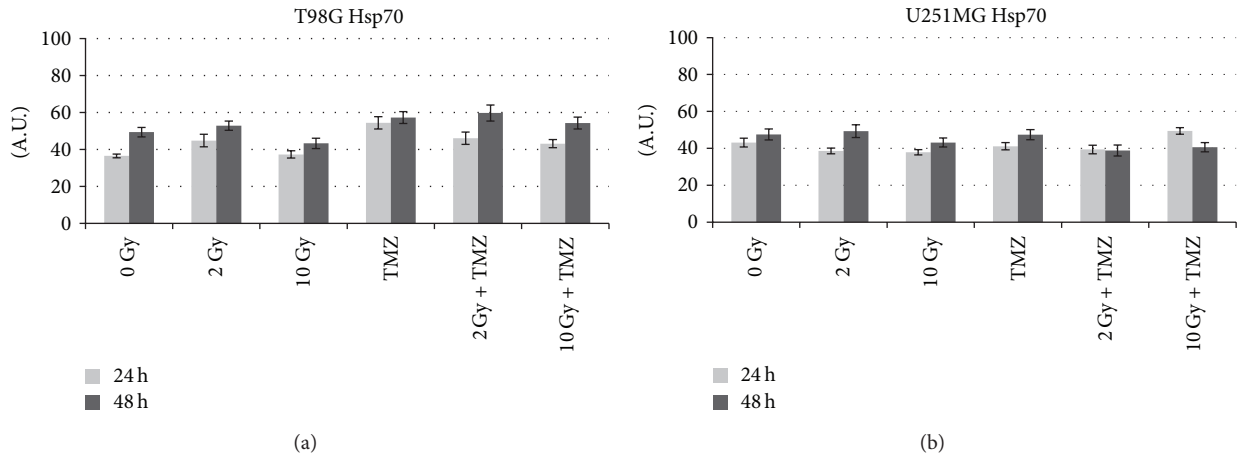


Figure 3: Quantitative analysis with ImageJ of Hsp70 profile in comparison with sham irradiated (0 Gy) in T98G and U251MG cells. The intensity of immunostaining is expressed as arbitrary units (A.U.) from 0 to 100.

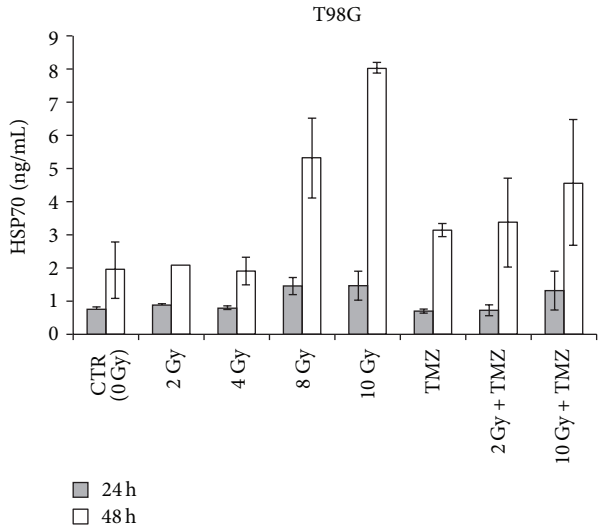


Figure 4: Extracellular HSP70 in T98G glioblastoma cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ) detected by ELISA. Data from 3 independent experiments are expressed as mean ± standard deviation.

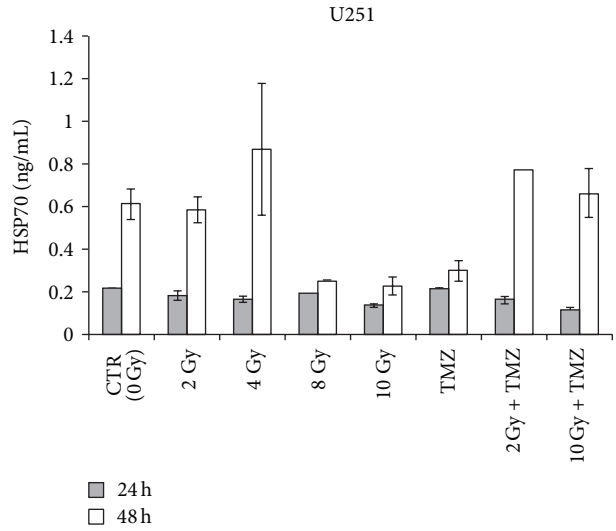


Figure 5: Extracellular HSP70 in U251MG glioblastoma cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ) detected by ELISA. Data from 3 independent experiments are expressed as mean ± standard deviation.

in the single although the release of HMGB1, after 24 hours, is different for each cell line: lower in T98G and slightly greater in U251MG in comparison to that obtained by 10 Gy alone (Figures 8 and 9). Results obtained from combined treatments did not suggest a beneficial use of combined treatments in comparison with radiation alone to activate signalling of immunogenic proteins able to stimulate an antitumoral response.

4. Conclusions

The success of some chemo- and radiotherapeutic regimens could rely on the induction of immunogenic tumour cell death and on the induction of anticancer immune response. In glioblastoma conventional cancer treatments are destined to fail because of dormant micrometastases or tumour (stem) cells resistant to therapy and able to induce relapse and therapeutic failure. One possible strategy consists in stimulating the immunogenicity of tumour cells, resulting in the expose, release, or active specific immunogenic factors and molecules that change microenvironment and deliver a stimulatory signal to the immune system [12]. Only few data exist dealing with the immune sensitizing effects of TMZ when added to radiation in human glioma and the identity of danger signals are still not better known in this kind of tumours.

In this work we studied two danger signals HSP70 and HMGB1 that are expressed and released in response to single or combined treatments of radiation and temozolomide in glioblastoma cells.

In particular, our data suggested that HSP70 translocates from cytoplasm to extracellular environment after an increase of radiation dose. When cell undergoes cellular

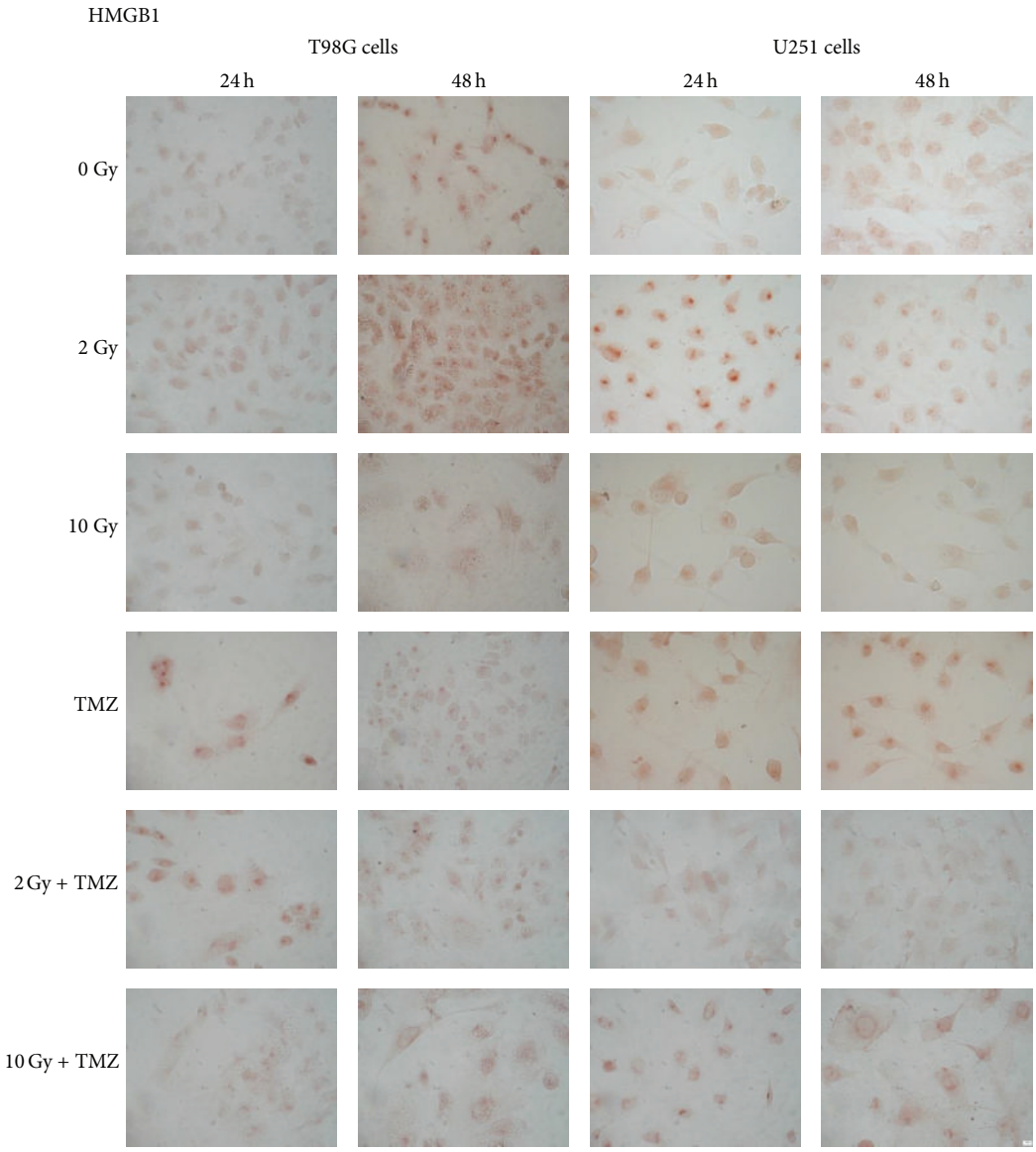


Figure 6: Intracellular HMGB1 expression detected by immunocytochemical technique in T98G and U251MG cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ) (400x).

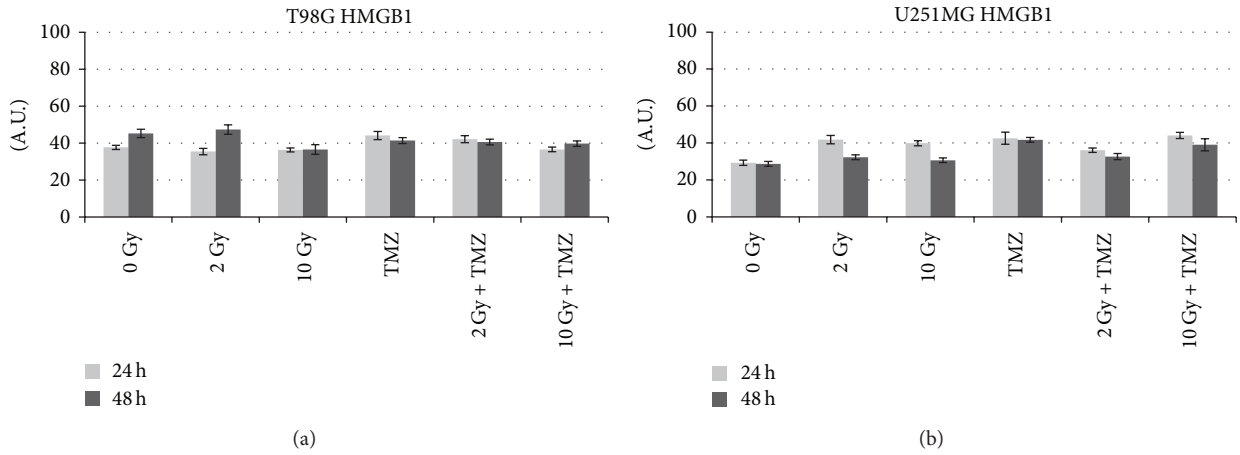


Figure 7: Quantitative analysis with ImageJ of HMGB1 profile in comparison with sham irradiated (0 Gy) in T98G and U251MG cells. The intensity of immunostaining is expressed as arbitrary units (A.U.) from 0 to 100.

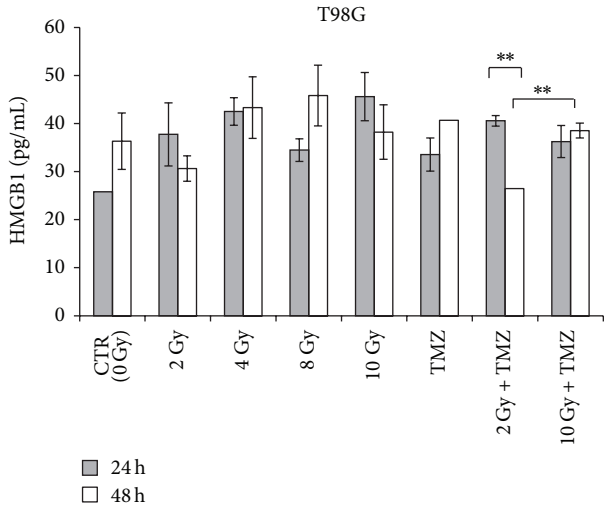


Figure 8: Extracellular HMGB1 in T98G glioblastoma cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ) detected by ELISA. Data from 3 independent experiments are expressed as mean ± standard deviation ($^{**}P < 0.01$).

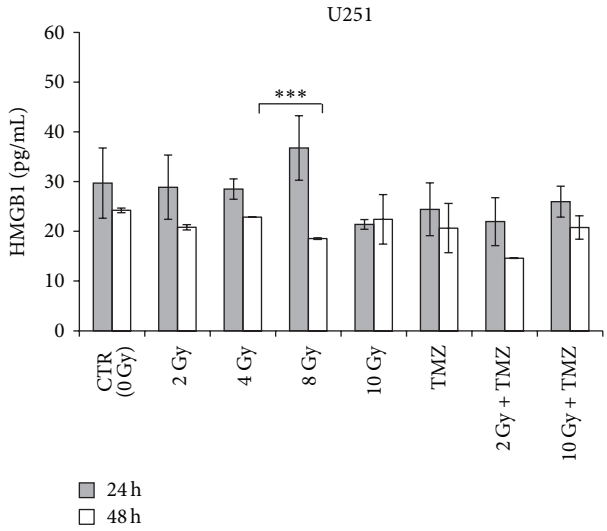


Figure 9: Extracellular HMGB1 in U251MG glioblastoma cells after 24 and 48 hours from single or combined treatments with radiation and temozolomide (TMZ) detected by ELISA. Data from 3 independent experiments are expressed as mean ± standard deviation ($^{***}P < 0.001$).

stress, such as exposure to radiation, many HSPs are overexpressed in the cytoplasm. Some of these proteins such as HSP70 are also able to move from cytoplasm to plasmatic membrane and subsequently outside the cells in order to carry out a potent immune-stimulatory activity [25]. Our results reported a HMGB1 translocation from the nucleus to the cytoplasm and subsequent release into the extracellular space after irradiation in glioblastoma cells. It is likely that HMGB1 released into the microenvironment as a result of tumour cells induced by radiation facilitates the activation of dendritic cells within the tumour [9]. However, the increase of HMGB1 in the extracellular space could drive cancer progression because of its activity as autocrine factor capable of promoting the growth and migration of tumour cells [26]. Further studies should be carried on to better understand the mechanism of action of HMGB1 and HSP70 on glioblastoma cells and on immune system cells to activate an immune response against tumour.

We also could state that TMZ had limited effectiveness in activating HMGB1 and HSP70 signalling and an adjuvant effect in their expression and release, instead, was observed in some combined treatments, but it depends on schedule, cell line, and timing, as reported by Chalmers and colleagues [27]. A big challenge in tumour therapy is, therefore, to identify the most beneficial combination and chronology of multiple treatment options to contribute to the improvement of the therapeutic outcome. Moreover, future work should focus on the modification of brain microenvironment through the enhancing or triggering of DAMPs signalling that is necessary to stimulate antitumour response and elicit tumour regression and long-term immunological memory.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors are grateful to Dr. F. Corbella of Struttura Complessa di Radioterapia, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy, for his assistance and useful expertise at the X-rays source, and Professor Udo Gaipl of Department of Radiation Oncology, Radiation Immunology, University Hospital Erlangen, Friedrich-Alexander University of Erlangen-Nurnberg, Erlangen, Germany, for his contribution to the design of this study. Paolini Alessandro is supported by a scholarship mandate of IRCCS Policlinico San Matteo Foundation, Pavia, Italy.

References

[1] J. J. Virrey, E. B. Golden, W. Sivakumar et al., "Glioma-associated endothelial cells are chemoresistant to temozolomide," *Journal of Neuro-Oncology*, vol. 95, no. 1, pp. 13–22, 2009.

[2] K. Ichimura, H. Ohgaki, P. Kleihues, and V. P. Collins, "Molecular pathogenesis of astrocytic tumours," *The Journal of Neuro-Oncology*, vol. 70, no. 2, pp. 137–160, 2004.

[3] W. Han, S. Chen, K. N. Yu, and L. Wu, "Nitric oxide mediated DNA double strand breaks induced in proliferating bystander cells after α-particle irradiation," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 684, no. 1-2, pp. 81–89, 2010.

[4] L. Zitvogel, L. Apetoh, F. Ghiringhelli, F. André, A. Tesniere, and G. Kroemer, "The anticancer immune response: indispensable for therapeutic success?" *The Journal of Clinical Investigation*, vol. 118, no. 6, pp. 1991–2001, 2008.

[5] C. Fonseca and G. Dranoff, "Capitalizing on the immunogenicity of dying tumor cells," *Clinical Cancer Research*, vol. 14, no. 6, pp. 1603–1608, 2008.

[6] H. Kono and K. L. Rock, "How dying cells alert the immune system to danger," *Nature Reviews Immunology*, vol. 8, no. 4, pp. 279–289, 2008.

[7] J. W. Hodge, C. Guha, J. Neefjes, and J. L. Gulley, "Synergizing radiation therapy and immunotherapy for curing incurable cancers: opportunities and challenges," *Oncology*, vol. 22, no. 9, pp. 1064–1084, 2008.

[8] T. Hagemann, F. Balkwill, and T. Lawrence, "Inflammation and cancer: a double-edged sword," *Cancer Cell*, vol. 12, no. 4, pp. 300–301, 2007.

[9] J. F. Curtin, N. Liu, M. Candolfi et al., "HMGB1 mediates endogenous TLR2 activation and brain tumor regression," *PLoS Medicine*, vol. 6, no. 1, article e10, 2009.

[10] S. R. Wedge, J. K. Porteous, M. G. Glaser, K. Marcus, and E. S. Newlands, "In vitro evaluation of temozolomide combined with X-irradiation," *Anti-Cancer Drugs*, vol. 8, no. 1, pp. 92–97, 1997.

[11] W. J. Kil, D. Cerna, W. E. Burgan et al., "In vitro and in vivo radiosensitization induced by the DNA methylating agent temozolomide," *Clinical Cancer Research*, vol. 14, no. 3, pp. 931–938, 2008.

[12] O. Kepp, L. Galluzzi, I. Martins et al., "Molecular determinants of immunogenic cell death elicited by anticancer chemotherapy," *Cancer and Metastasis Reviews*, vol. 30, no. 1, pp. 61–69, 2011.

[13] S. K. Calderwood, J. R. Theriault, and J. Gong, "Message in a bottle: role of the 70-kDa heat shock protein family in anti-tumor immunity," *European Journal of Immunology*, vol. 35, no. 9, pp. 2518–2527, 2005.

[14] D. R. Ciocca, V. R. Rozados, F. D. Cuello Carrion, S. I. Gervasoni, P. Matar, and O. G. Scharovsky, "Hsp25 and Hsp70 in rodent tumors treated with doxorubicin and lovastatin," *Cell Stress and Chaperones*, vol. 8, pp. 26–36, 2003.

[15] M. Gehrmann, J. Radons, M. Molls, and G. Multhoff, "The therapeutic implications of clinically applied modifiers of heat shock protein 70 (Hsp70) expression by tumor cells," *Cell Stress and Chaperones*, vol. 13, no. 1, pp. 1–10, 2008.

[16] A. Brondani Da Rocha, A. Regner, I. Grivicich et al., "Radioresistance is associated to increased Hsp70 content in human glioblastoma cell lines," *International Journal of Oncology*, vol. 25, no. 3, pp. 777–785, 2004.

[17] A. Paolini, F. Pasi, A. Facoetti et al., "Cell death forms and HSP70 expression in U87 cells after ionizing radiation and/or chemotherapy," *Anticancer Research*, vol. 31, no. 11, pp. 3727–3731, 2011.

[18] J. van Rijn, J. J. Heimans, J. van den Berg, P. van der Valk, and B. J. Slotman, "Survival of human glioma cells treated with various combination of temozolomide and X-rays," *International Journal of Radiation Oncology, Biology, Physics*, vol. 47, no. 3, pp. 779–84, 2000.

[19] J. E. Ellerman, C. K. Brown, M. de Vera et al., "Masquerader: high mobility group box-1 and cancer," *Clinical Cancer Research*, vol. 13, no. 10, pp. 2836–2848, 2007.

[20] J. S. Park, J. Arcaroli, H. K. Yum et al., "Activation of gene expression in human neutrophils by high mobility group box 1 protein," *The American Journal of Physiology: Cell Physiology*, vol. 284, no. 4, pp. C870–C879, 2003.

[21] M. Stros, T. Ozaki, A. Bacikova, H. Kageyama, and A. Nakagawara, "HMGB1 and HMGB2 cell-specifically down-regulate the p53- and p73-dependent sequence-specific transactivation from the human Bax gene promoter," *The Journal of Biological Chemistry*, vol. 277, no. 9, pp. 7157–7164, 2002.

[22] P. Rovere-Querini, A. Capobianco, P. Scaffidi et al., "HMGB1 is an endogenous immune adjuvant released by necrotic cells," *EMBO Reports*, vol. 5, no. 8, pp. 825–830, 2004.

[23] C. W. Bell, W. Jiang, C. F. Reich III, and D. S. Pisetsky, "The extracellular release of HMGB1 during apoptotic cell death," *American Journal of Physiology—Cell Physiology*, vol. 291, no. 6, pp. C1318–C1325, 2006.

[24] L. Apetoh, F. Ghiringhelli, A. Tesniere et al., "The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy," *Immunological Reviews*, vol. 220, no. 1, pp. 47–59, 2007.

[25] A. Tesniere, L. Apetoh, F. Ghiringhelli et al., "Immunogenic cancer cell death: a key-lock paradigm," *Current Opinion in Immunology*, vol. 20, no. 5, pp. 504–511, 2008.

[26] R. Bassi, P. Giussani, V. Anelli et al., "HMGB1 as an autocrine stimulus in human T98G glioblastoma cells: role in cell growth and migration," *Journal of Neuro-Oncology*, vol. 87, no. 1, pp. 23–33, 2008.

[27] A. J. Chalmers, E. M. Ruff, C. Martindale, N. Lovegrove, and S. C. Short, "Cytotoxic effects of TMZ and radiation are additive- and schedule-dependent," *International Journal of Radiation Oncology Biology Physics*, vol. 75, no. 5, pp. 1511–1519, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 759791, 10 pages
http://dx.doi.org/10.1155/2014/759791

Research Article

Metabolic Effects of Hypoxia in Colorectal Cancer by ^{13}C NMR Isotopomer Analysis

Ana M. Abrantes,[1,2] Ludgero C. Tavares,[3] Salomé Pires,[1,4] João Casalta-Lopes,[1,5] Cândida Mendes,[6] Marta Simões,[6] Manuela M. Grazina,[6,7] Rui A. Carvalho,[3,7] and Maria Filomena Botelho[1,2]

[1] *Biophysics Unit, IBILI, Faculty of Medicine, University of Coimbra, 3000-548 Coimbra, Portugal*
[2] *Centre of Investigation on Environment, Genetics and Oncobiology (CIMAGO), Faculty of Medicine, University of Coimbra, 3001-301 Coimbra, Portugal*
[3] *Life Sciences Department, Faculty of Sciences and Technology, University of Coimbra, 3000-456 Coimbra, Portugal*
[4] *Faculty of Sciences and Technology, University of Coimbra, 3000-456 Coimbra, Portugal*
[5] *Radiotherapy Department, CHUC, 3000-075 Coimbra, Portugal*
[6] *Laboratory of Biochemical Genetics (CNC/UC), Faculty of Medicine, University of Coimbra, 3000-548 Coimbra, Portugal*
[7] *Center for Neuroscience and Cell Biology (CNC), 3004-517 Coimbra, Portugal*

Correspondence should be addressed to Ana M. Abrantes; mabrantes@fmed.uc.pt and Rui A. Carvalho; carvalho@ci.uc.pt

Received 28 February 2014; Accepted 27 May 2014; Published 1 July 2014

Academic Editor: Zhiqiang Meng

^{13}C NMR isotopomer analysis was used to characterize intermediary metabolism in three colorectal cancer cell lines (WiDr, LS1034, and C2BBe1) and determine the "metabolic remodeling" that occurs under hypoxia. Under normoxia, the three colorectal cancer cell lines present high rates of lactate production and can be seen as "Warburg" like cancer cells independently of substrate availability, since such profile was dominant at both high and low glucose media contents. The LS1034 was the less glycolytic of the three cell lines and was the most affected by the event of hypoxia, raising abruptly glucose consumption and lactate production. The other two colorectal cell lines, WiDr and C2BBe1, adapted better to hypoxia and were able to maintain their oxidative fluxes even at the very low levels of oxygen. These differential metabolic behaviors of the three colorectal cell lines show how important an adequate knowledge of the "metabolic remodeling" that follows a given cancer treatment is towards the correct (re)design of therapeutic strategies against cancer.

1. Introduction

Cancer cell alters its metabolism in response to a challenging environment by promoting cell growth and proliferation, diverging significantly from normal tissues. According to Otto Warburg, cancer cells maintain high aerobic glycolytic rates and produce high levels of lactate and pyruvate [1–3] to sustain cell proliferation and its high energy demands. When the pO_2 is normal, the oxidative phosphorylation process occurs and pyruvate is directed towards the Krebs cycle. Thus, metabolism in "Warburg" like tumor cells could at first be seen as "wasteful" when compared to normal cells, or we could say that tumor cells use a disproportionate nutrient exchange with its environment. This metabolic profile is in fact prevalent in many cancer cells and grants them advantage over normal cells by allowing them to proliferate at much higher rates. By avoiding oxidative phosphorylation even when oxygen is plentiful [4] and adopting aerobic glycolysis, carbon skeletons build up considerably in their cytosol and biosynthetic pathways are efficiently activated. Several studies have demonstrated an increase in the contents of several glycolytic enzymes such as 6-phosphofructo-2-kinase/fructose 2,6-biphosphatases. The regulation of glycolysis by these enzymes allows the alterations in glycolytic fluxes required to fulfill cancer cells bioenergetics and biosynthetic demands. The glycolytic pathway is in fact becoming an increasing

target in cancer therapy both by itself and in combination with other therapies such as immunotherapy. This advance helps overcoming drug resistance issues and improves the efficacy of current anticancer agents [3, 5–7].

Besides that, and having into account tumor microenvironment, due to the temporal and spatial heterogeneity of oxygenation that occurs in solid tumors, the adaptation to the variability of its microenvironment is critical. Oxygen supply is impaired in many tumors because there is imbalance between tissue growth and the development of new vasculature. In solid tumors hypoxia is thus a common characteristic/microenvironment of tumoral cells, becoming a key factor for tumor progression and resistance to anticancer therapy [8]. This decrease in oxygen pO_2 influences compensatory physiological events involving adaptations at all levels in order to maintain homeostasis between cells energetic requirements and supplies [8]. Thus, it appears that aerobic glycolysis is an adaptive mechanism that involves several metabolic pathways coordinates, which maintain the morphological characteristics of tumor cells, including the ability to survive hypoxic conditions, the capacity of metastasis, and evasion of death by apoptosis [5–8].

Solid tumors have also heterogeneous populations of cells due, in part, to a limited blood supply that provides reduced levels of oxygen and prompts for acidic conditions and avidity of glucose [9]. These changes in the tumor microenvironment may represent physiological signals that activate cell survival or death by apoptosis, affecting the balance between growth and tumor suppression. The mechanisms by which tumor cells adapt or die in the presence of low levels of oxygen are not well studied and understood. However, it is known that the expression of several transcription factors as well as the modification of metabolic pathways interfere with the response to the lack of oxygen and nutrients by tumor cells [10].

The aim of this study is to characterize the metabolic profile, namely, glycolysis and Krebs cycle fluxes, of three colorectal cancer cell lines using carbon-13 (^{13}C) tracers and nuclear magnetic resonance (NMR) spectroscopy. With this approach central metabolic pathways will be evaluated and major metabolic changes resulting from hypoxia and glucose availability will be determined towards depicting the possible involvement of metabolic mechanisms [11] in some processes of chemotherapeutic resistance in colon cancer [12].

2. Materials and Methods

2.1. Cell Lines. Colorectal cancer cell lines (WiDr, LS1034, and C2BBe1) purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) were used to perform *in vitro* studies. The three cell lines are from different colon localizations: WiDr from rectosigmoid location, C2BBe1 clone of the cell line Caco-2 (ATCC) from descending colon, and LS1034 resistant to chemotherapy concerning P-glycoprotein overexpression from ascending colon [9–13]. WiDr and C2BBe1 cell lines were maintained in Dulbecco's Modified Eagle's cell culture medium (Sigma) and LS1034 cell line was maintained in Roswell Park Memorial Institute (RPMI) medium. All cell lines were cultured in high (25 mM) and low (5 mM) glucose concentrations, supplemented with 10% fetal calf serum (Gibco) in 5% CO_2 atmosphere, at 37°C. To perform hypoxia studies, cells were incubated at 37°C in 93% N_2, 2% O_2, and 5% CO_2 using a controlled-environment cabinet (PlasLabs, USA). Each of the media was acquired without glucose in order to be supplemented with [U-^{13}C]glucose for performing ^{13}C NMR isotopomer analysis.

2.2. Cell Perchloric Acid Extracts. Perchloric acid extracts were obtained using published methods [14, 15]. Briefly, to the cell pellet is added the equivalent (v/w) of 2 mL ice-cold perchloric acid 7.2%/gram wet weight of cells. The resulting supernatant is subsequently neutralized using ice-cold KOH solutions before lyophilization. This procedure allows precipitation of $KClO_4$ and a considerable reduction of solution ionic strength towards the acquisition of high quality NMR spectra. For kinetic analysis of lactate production, aliquots of cell culture media (160 μL) were drawn at specific time intervals (0, 4, and 8 hours). To each of these aliquots 40 μL of an internal standard consisting of sodium fumarate (10 mM) in phosphate buffer (100 mM) was added for absolute quantification purposes.

2.3. NMR Analyses of Intermediary Metabolism

2.3.1. Glycolytic Fluxes by Analysis of Cell Culture Media. Proton (^1H) nuclear magnetic resonance (NMR) spectra of cell culture media were acquired on a 600 MHz Varian NMR spectrometer using a 3 mm indirect detection probe. Typical acquisition parameters included a 3 s acquisition time, a radiofrequency pulse of 45°, and an interpulse delay of 10 seconds to ensure full relaxation of all nuclei in the sample. Due to direct ($^1J_{HC}$) and long range ($^2J_{HC}$ and $^3J_{HC}$) heteronuclear scalar coupling it is possible to distinguish the resonances of [U-^{13}C]lactate from those due to nonenriched [U-^{12}C]lactate (Figure 1). [U-^{13}C]lactate originates from [U-^{13}C]glucose as a result of glycolysis followed by reduction of pyruvate by lactate dehydrogenase (LDH). Levels of [U-^{13}C]lactate are thus used as an indirect measure of glycolytic activity.

2.3.2. Krebs Cycle Kinetics by ^1H and ^{13}C NMR Analysis of Cell Extracts. ^{1}H- and ^{13}C-NMR spectra of cell extracts were acquired in a 600 MHz Varian NMR spectrometer using a 3 mm indirect detection and a 3 mm broadband NMR probe, respectively. ^{1}H-NMR spectra of cell extracts allow the monitoring of the levels of intracellular metabolic intermediates while ^{13}C-NMR spectra allows tackling the dynamics of ^{13}C incorporation in metabolic intermediates thus warranting a dynamic vision of cell metabolism. Rates of ^{13}C incorporation in glycolytic and Krebs cycle intermediates are used as a measure of metabolic coupling between these two major metabolic pathways and provide valuable clues about the "metabolic remodeling" associated with the tumorigenic transformation and treatment. ^{13}C isotopomers of intracellular glutamate provide a window for monitoring

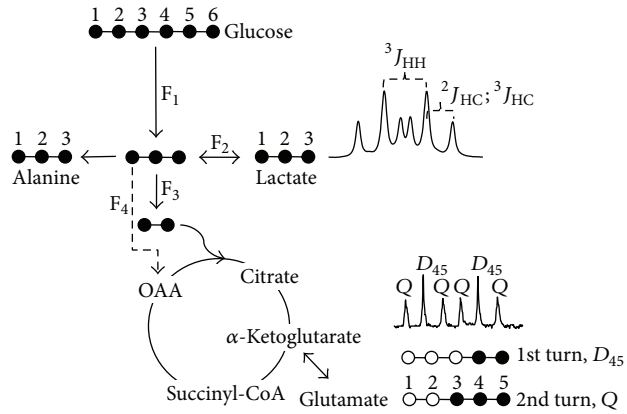


Figure 1: [U-^{13}C]glucose as a carbon tracer for monitoring glycolysis and Krebs cycle. Through glycolysis and lactic fermentation [U-^{13}C]lactate is produced which is distinguishable (multiplets in each ^{13}C satellite due to $^{2}J_{HC}$, $^{3}J_{HC}$, and $^{3}J_{HH}$—duplet of triplets since $^{2}J_{HC}$ and $^{3}J_{HC}$ have similar magnitude) from tissue lactate or lactate originating from unenriched carbon sources. [1,2-$^{13}C_2$]acetil-CoA originating from [U-^{13}C]pyruvate leads to incorporation of ^{13}C atoms in Krebs cycle intermediates and other metabolites that exchange with those (e.g., glutamate). It is possible to follow Krebs cycle kinetics by monitoring the ^{13}C multiplet due to carbon 4 of glutamate. The duplet component (D_{45}) results from the simple entrance of [1,2-$^{13}C_2$]acetil-CoA but the quartet requires further cycling and combination of [1,2-$^{13}C_2$]acetil-CoA with previously enriched oxaloacetate (OAA). A simple ratio of Q/D_{45} is proportional to Krebs cycle flux and denotes higher rates of oxidative metabolism in tissues as opposed to glycolysis. Metabolic fluxes F_{1-4} denote the major pathways for [U-^{13}C]glucose use.

Krebs cycle kinetics (Figure 1) since the appearance of multi-^{13}C-labeled species clearly indicate cycle turnover [14, 16, 17] and oxidative efficiency.

2.4. Citrate Synthase Enzyme Activity. The citrate synthase enzyme (CS) is used as a marker enzyme of mitochondrial preparations due to its stability and its regulatory role in the Krebs cycle. Its activity can provide an estimate of the number of mitochondria in a cell suspension and can be used in order to standardize the results of the activities of the enzymes of the mitochondrial respiratory chain [15, 18]. The evaluation of the activity of CS was performed by spectrophotometry at a λ of 412 nm. This assay consists of the condensation reaction of acetyl-CoA (0.2 mM) with oxaloacetate (8 mM), catalyzed by CS. This reaction results in coenzyme A (CoA) release that reacts with 5,5′-dithiobis(-nitrobenzoic acid) (DNTB) added to the medium at a concentration of 2 mM, thus enabling the reading at 412 nm. To allow the access of substrates to the enzyme Triton X-100 was used at 0.1%.

2.5. Complex IV Activity. To measure complex IV activity, 50 μg of protein was added to 1 mL of buffer composed by 10 mM KH_2PO_4, 300 mM sucrose, and 5 mg/mL BSA with pH 6.5, which was in a cuvette. The analysis started with the completion of a baseline tracing at λ 550 nm and then 10 μL was added of detergent n-dodecyl-β-D-maltoside, which promotes the formation of pores in the outer membrane of mitochondria that allow the entry of reduced cytochrome c, resulting in a concentration of 125 mM. This detergent transfers its electrons to cytochrome c from the mitochondrial respiratory chain located in the inner mitochondrial membrane, without the inner membrane commitment. Upon addition of reduced cytochrome c solution to make up the concentration of 10 mM, it becomes possible to record changes in absorbance at 550 nm. At the end, 2 μL of a solution of potassium cyanide (KCN) 80 mM was added to inhibit complex IV. Measuring the ratio of complex IV activity in the presence and absence of detergent allows the analysis of mitochondrial fraction quality.

2.6. Statistical Analysis. Statistical analysis of the different results was performed using the IBM SPSS software v. 20.0 (IBM Corporation, Armonk, New York, USA). Doubling time results were analyzed by nonparametric Kruskal-Wallis test, with multiple comparisons using Bonferroni correction. NMR results were analysed using parametric Student t-test. Complex IV activity results were analyzed using nonparametric Kruskal-Wallis test. A significance of 5% was considered for all comparisons.

3. Results and Discussion

3.1. Rates of Glycolysis. Levels of lactate in cell culture media were monitored by ^{1}H-NMR spectroscopy. Figure 2 shows the temporal evolution of lactate resonances for one of the cell lines (LS1034). This multiplet (duplet of triplets) represents one of the ^{13}C-satellites of the methyl resonance of lactate and constitutes half of the signal due to [U-^{13}C]lactate. Figure 3 shows the lactate production for all cell lines under all experimental conditions, high/low glucose and normoxia/hypoxia. A linear evolution is seen for all experimental conditions and for all colorectal cancer cell lines for the first 8 h of incubation. After this period there is a considerable reduction in lactate production which is also consistent with a significant reduction in glucose levels in the culture media. Under normoxia, the three cell lines exhibit a very pronounced glycolytic metabolic profile, exporting considerable amounts of lactate to the culture media. This "Warburg" like metabolic behavior is characteristic of many tumor cells and was expectable for the colorectal cancer cell lines under study. From the three, LS1034 is the less glycolytic

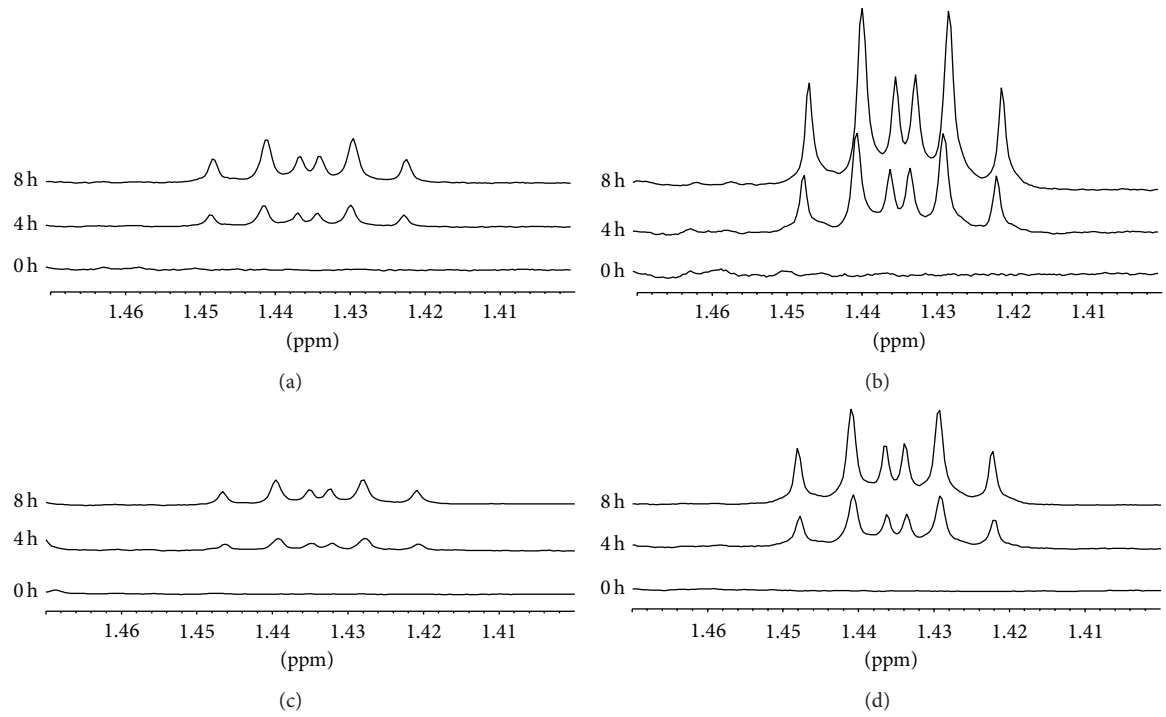


Figure 2: Expansions from ^{1}H-NMR spectra of culture media of LS1034 cells, showing the region of one of the satellites due to [U-^{13}C]lactate at 0, 4, and 8 hours of incubation in culture media: (a) normoxia/high glucose; (b) hypoxia/high glucose; (c) normoxia/low glucose; (d) hypoxia/low glucose.

followed by C2BBe1 and ultimately WiDr appears as the most glycolytic at both high and low glucose normoxic conditions. This metabolic profile is dramatically altered under hypoxia. This "insult" has completely differential effects in the three cell lines and shows as well a dependence on the glucose content of the cell culture media. The LS1034 cell line is the most susceptible to hypoxia (Figure 3(b)). During the first 8 hours of incubation, lactate production rates increase significantly, more than triplicate, while for other cell lines the increases in those rates are much less notorious. The colorectal cancer cell line with the lowest dependence on glycolysis/lactic fermentation under normoxia becomes the most glycolytic under hypoxia. The amount of glucose in the cell culture media also influences the rates of lactate production by the three cell lines. The combination of normoxia/hypoxia and high/low glucose forms a matrix that seemingly affects differently each of the colorectal cancer cell lines. LS1034 shows a significant increase in lactate production due to hypoxia at both high and low glucose contents while WiDr behaves opposingly for the two glucose levels, increasing glycolysis at high glucose but reducing glycolysis with significant decrease for low glucose, assuming an adaptive behavior. The C2BBe1 is the less affected by the hypoxic insult at both high and low glucose levels, however, with significance at low levels of glucose.

3.2. Glycolytic Flux versus Proliferative Activities. A significant glycolytic activity is expectable in high proliferative tissues for the sake of availability of carbons skeletons for biosynthetic routes. On this basis one would expect that the more proliferative a given cell is, the more glycolytic its metabolic profile should be. Figure 4 shows the duplication times for the three colorectal cell lines. WiDr is the most proliferative followed by the C2BBe1 and LS1034 appears as the cancer line with the lowest proliferation rate, in agreement with its lowest glycolytic activity as demonstrated by the lactate production rates measured under normoxia with either high or low glucose in culture media. Duplication times and glycolytic activities fully match the concept that there is no "wasteful" metabolic behavior by the cancer cell lines but solely an adaptive behavior that grants them advantage over normal neighboring cells.

3.3. Krebs Cycle Kinetics. Krebs cycle kinetics was evaluated by ^{13}C-NMR isotopomer analysis using cell perchloric acid extracts. Figure 5 shows representative ^{13}C-NMR spectra of perchloric acid extracts derived from the three colorectal cancer cell lines grown in normoxic conditions in the presence of high glucose. Some of the resonances are identified and the expansions show the multiplet of glutamate C4 resonance (C4-Glu; 34.2 ppm), a crucial carbon for performing Krebs cycle kinetics analysis [14, 16]. Immediately perceptible is the fact that the resonance due to lactate methyl carbon (C3-Lac; 20.8 ppm) dominates all three spectra, corroborating the robust glycolytic/fermentative character of these colorectal cancer cell lines even under normoxia, as outlined above from the rates of lactate production. Other major differences between cell lines are the amounts of alanine (C3-Ala; 17.0 ppm) present in cell extracts, much higher in LS1034, and

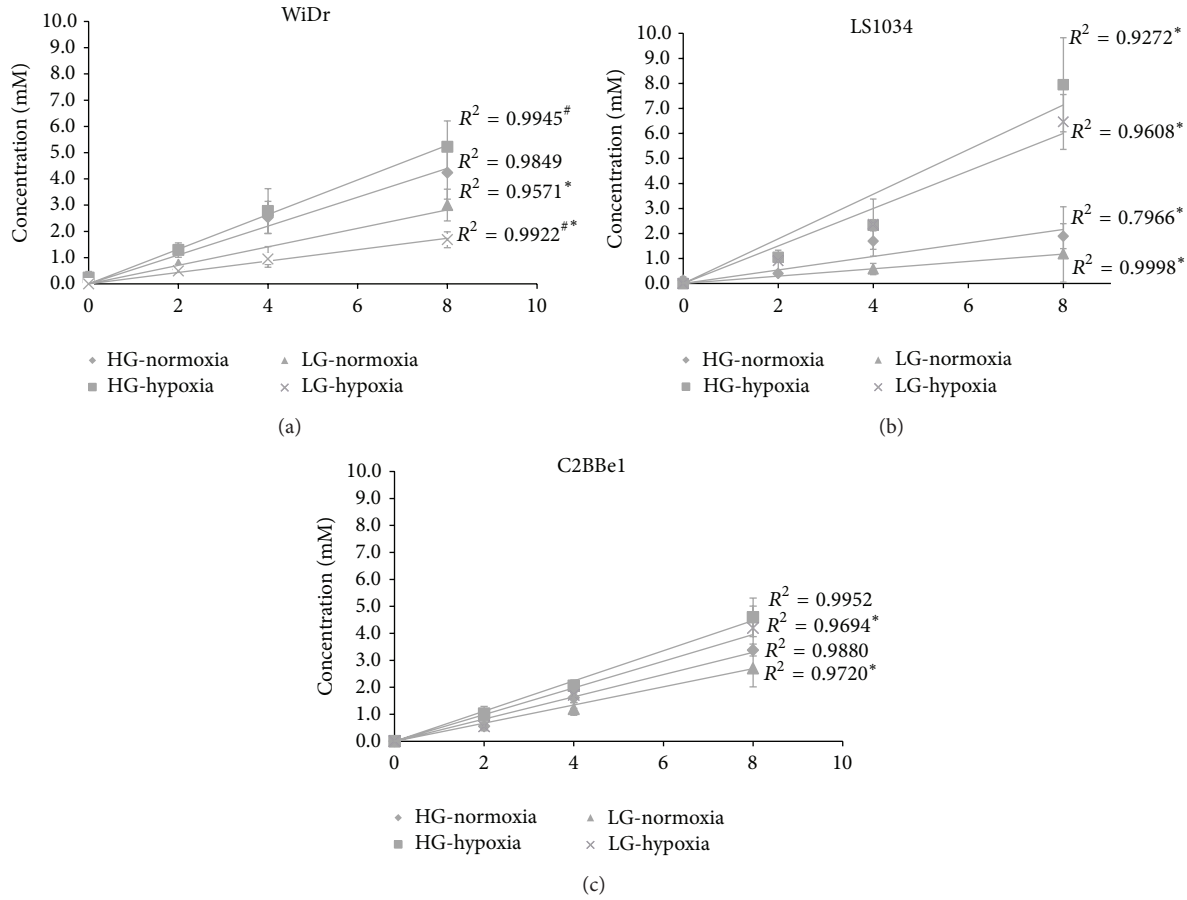


Figure 3: [U-^{13}C]lactate exported by colon cancer cell lines to the incubation media during the first 8 hours of incubation under normoxia and hypoxia and low and high glucose media: (a) WiDr; (b) LS1034; (c) C2BBe1. Significant differences are indicated by $*$ corresponding to $P < 0.05$ (comparison between normoxia and hypoxia conditions) and # corresponding to $P < 0.05$ (comparison between high and low cell media glucose concentrations (5 and 25 mM)).

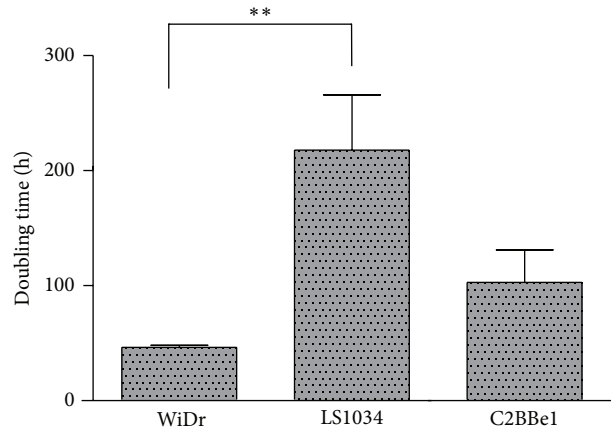


Figure 4: Duplication times (h) for the three colorectal cancer cell lines.

the composition of the C4-Glu multiplet in terms of duplet 45 (D_{45}) and quartet (Q) multiplets, which as mentioned correlate with Krebs cycle turnover rates. The levels of alanine are intimately associated with the levels of pyruvate in the cells and can be used instead for deriving information concerning the cytosolic redox status [19]. In fact, C3-Lac/C3-Ala is frequently used as an indirect measure of NADH/NAD$^+$ ratio [20, 21]. The more the NADH/H$^+$ exists in cytosol the more extensive the conversion of pyruvate is to lactate through lactic dehydrogenase (LDH) and the higher the ratio is of those two metabolites. In terms of C4-Glu, the appearance of the Q multiplet is only possible after multiple

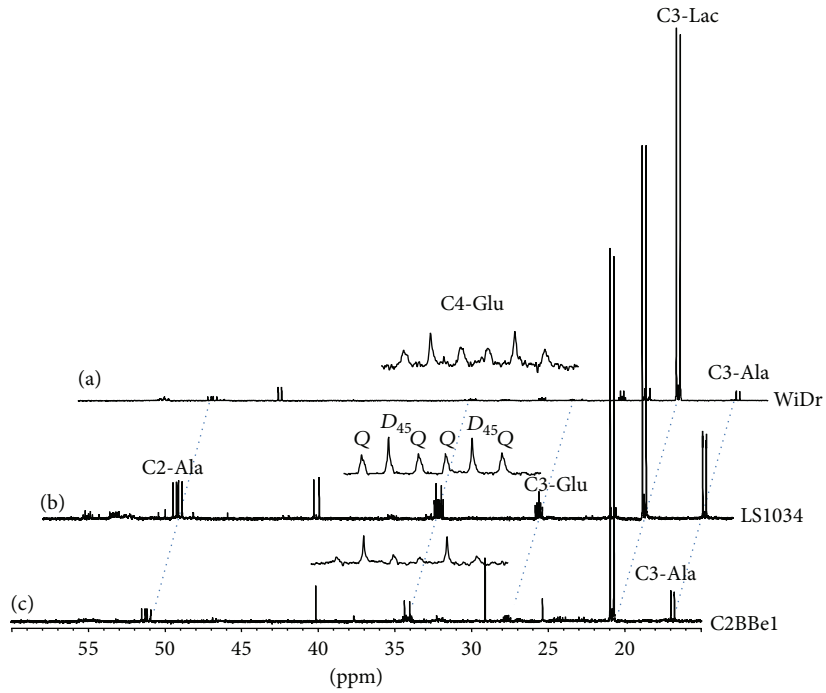


Figure 5: ^{13}C NMR spectra of perchloric acid extracts for the three colorectal cancer cell lines under normoxia and high glucose media: (a) WiDr; (b) LS1034; (c) C2BBe1. Expanded is the multiplet due to glutamate carbon 4 (C4-Glu; 34.2 ppm), composed by two major multiplets, duplet 45 (D_{45}), due to glutamate isotopomers labeled in carbons 4 and 5 but not carbon 3, and quartet (Q) representing all glutamate isotopomers with those three carbons enriched simultaneously. Other resonances easily seen are the methyl carbons of lactate (C3-Lac; 20.8 ppm) and alanine (C3-Ala; 17.0 ppm) and the carbon 3 of glutamate (C3-Glu; 27.8 ppm).

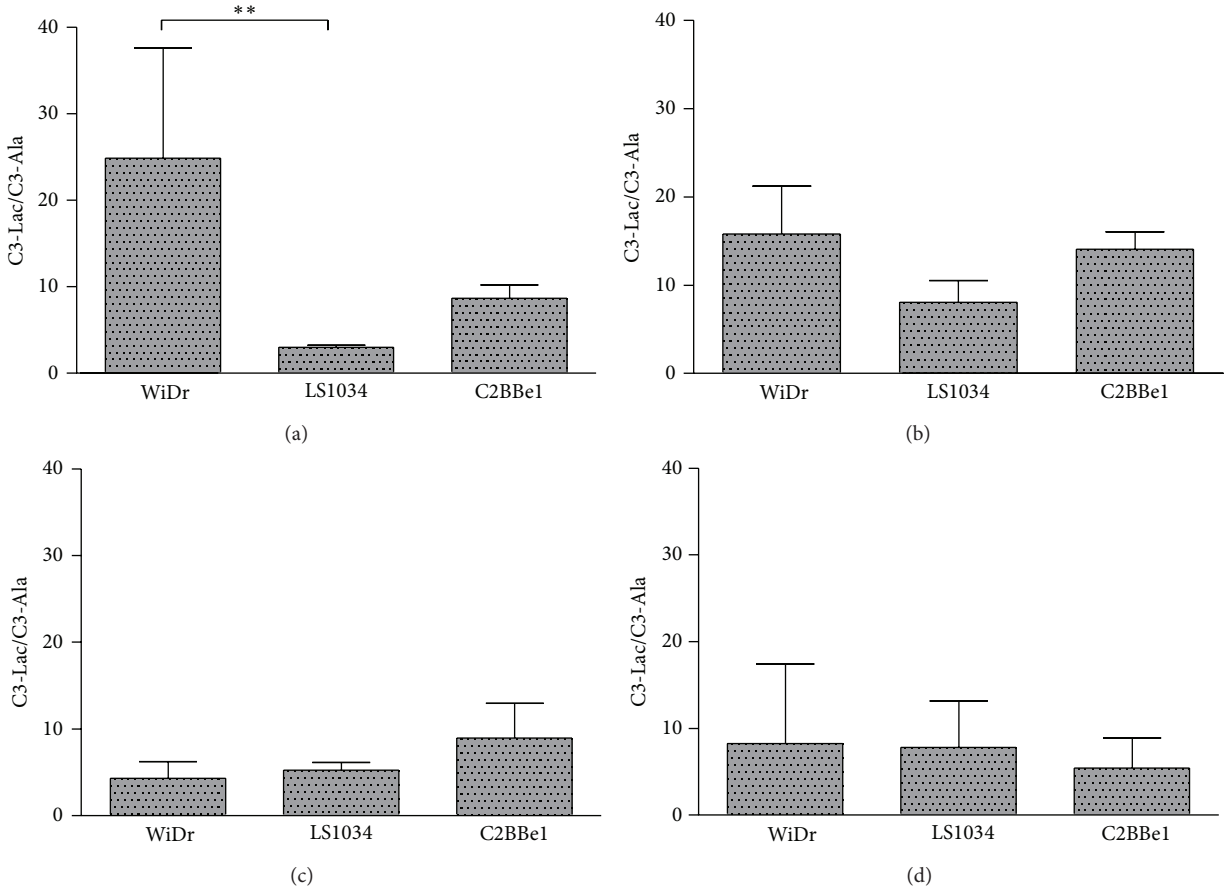


Figure 6: Ratios of the methyl carbon of lactate (C3-Lac) to the methyl carbon of alanine (C3-Ala) for the three colorectal cancer cell lines under all experimental conditions: (a) normoxia/high glucose; (b) hypoxia/high glucose; (c) normoxia/low glucose; (d) hypoxia/low glucose.

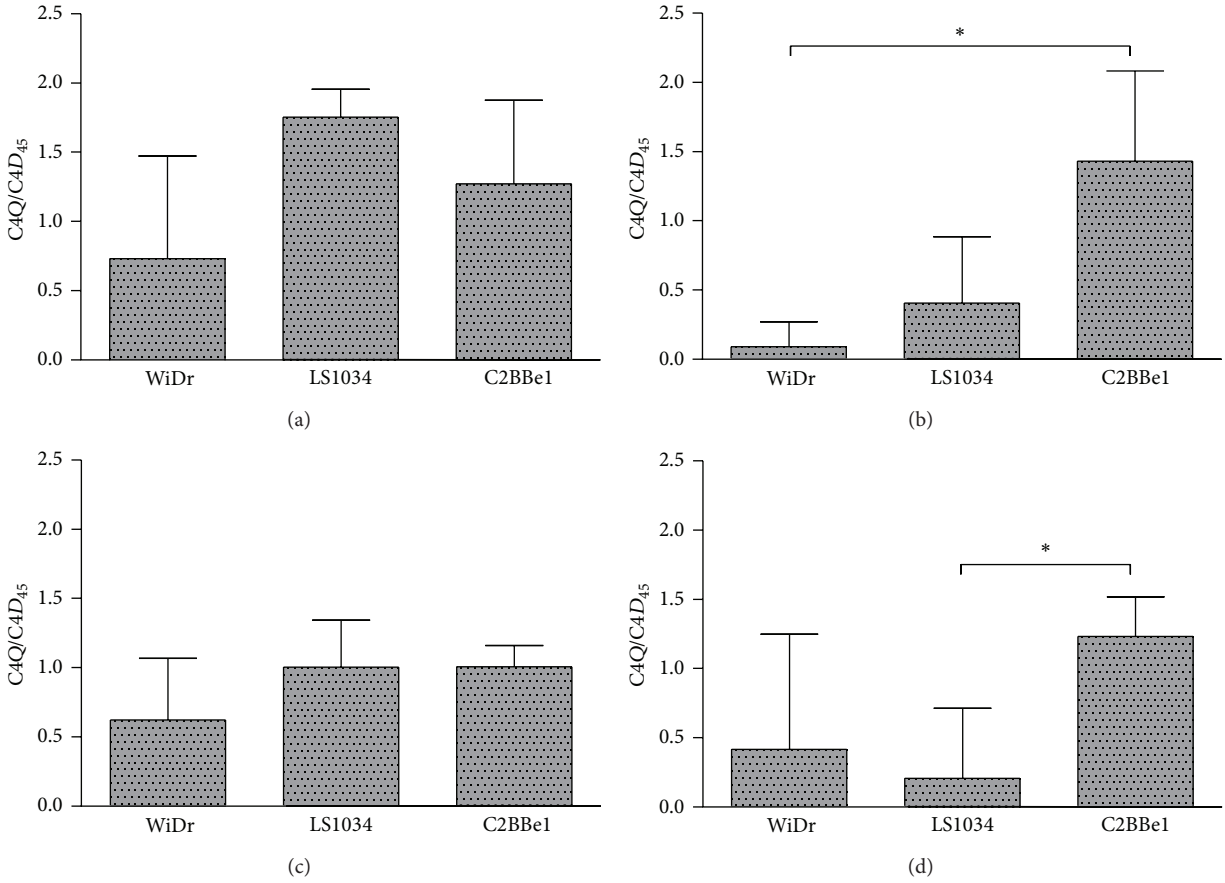


Figure 7: Ratios of the glutamate C4 quartet (C4Q) to the duplet 45 (D_{45}) multiplets for the three colorectal cancer cell lines under all experimental conditions: (a) normoxia/high glucose; (b) hypoxia/high glucose; (c) normoxia/low glucose; (d) hypoxia/low glucose.

turns of the Krebs cycle (Figure 1) and its contribution to the overall C4-Glu multiplet is higher in cells with a more active oxidative metabolism. The ratio C4Q/C4D_{45} provides a way to probe Krebs cycle kinetics and to compare different cell lines in terms of their glycolytic/oxidative metabolic characters. Another ratio of metabolic interest is the C3-Lac/C4-Glu, since it reports how enriched the glycolytic/oxidative metabolite pools are and in such a way provides a measure of the metabolic coupling between glycolysis and Krebs cycle. The higher the ratio is the more uncoupled those two central metabolic pathways are and the more dependent the cell line is on glycolysis for its bioenergetics requirements. This ratio is frequently high in "Warburg" like cancer cell lines like the ones used in this study.

Figure 6 shows the ratios of C3-Lac/C3-Ala for the three colon cancer cell lines in the four experimental conditions. This ratio is significantly higher in WiDr under normoxic/high glucose conditions than in the other two cell lines. This correlates with a more pronounced glycolytic flux in this cancer cell line under such conditions. Under hypoxia and high glucose, there is a considerable attenuation of C3-Lac/C3-Ala ratio in WiDr, only explainable by an overall metabolic inhibition in these cells. For LS1034 and C2BBe1 there is an increase in the C3-Lac/C3-Ala ratio under hypoxia, explainable by a higher dependence on glycolysis for bioenergetics under anaerobic conditions.

Figure 7 shows the ratios C4Q/C4D_{45} for the three colorectal cancer cell lines in the four experimental conditions chosen. This ratio clearly emphasizes that the three cell lines have distinct Krebs cycle turnovers under hypoxia. WiDr and LS1034 cell lines suffer a considerable reduction in such ratio at both high and low glucose conditions. The same does not hold however for the C2BBe1, which essentially shows no effect in such ratio due to hypoxia. This distinctive behavior emphasizes a distinct capacity of the cells for usage of oxygen at low concentration levels such as the ones prompted by the hypoxic episode and concomitantly justifies the distinctive behavior of cells in tumors when subjected to hypoxia [22].

Figure 8 shows the C3-Lac/C4-Glu ratio for the three colon cancer cell lines in the four experimental conditions. As expected, this ratio is higher for more glycolytic cell lines, like WiDr, and suffers a slight increase under hypoxia. Under normoxia and high glucose the LS1034 has the lowest C3-Lac/C4-Glu ratio in accordance with a much more pronounced oxidative profile. Upon hypoxia the LS1034 is the one suffering the more severe metabolic shift since the oxidative pathway reduces its contribution to cell bioenergetics and has to be compensated by further glycolytic activity.

3.4. Complex IV Activities. In order to corroborate the metabolic alterations mentioned above for each of the colorectal cancer cell lines and strengthen our findings

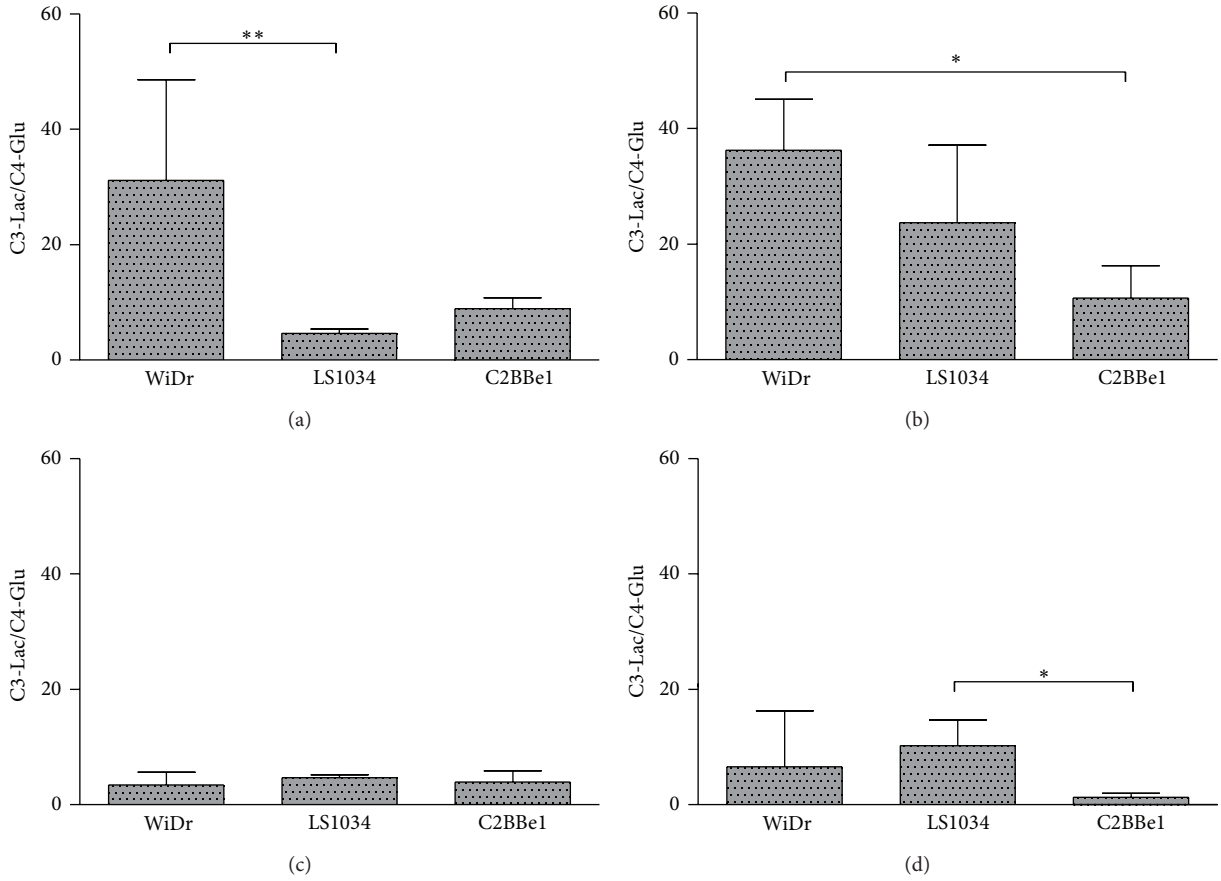


Figure 8: Ratios of the methyl carbon of lactate (C3-Lac) to the carbon 4 of glutamate (C4-Glu) for the three colorectal cancer cell lines under all experimental conditions: (a) normoxia/high glucose; (b) hypoxia/high glucose; (c) normoxia/low glucose; (d) hypoxia/low glucose.

concerning the metabolic (un)coupling observed for each of the cell lines in each of the metabolic conditions, the activity of complex IV from the electron transport chain was also evaluated. This activity, adequately normalized for citrate synthase activity, is presented in Figure 9. Under normoxia and high glucose, LS1034 cell line is the one possessing the most active complex IV. This is in accordance with all presented data, which pointed these cells as the most oxidative/less glycolytic under such incubation conditions. Under normoxia and low glucose no significant changes were detected in complex IV activities in accordance with the absence of significant differences on the ratios C3-Ala/C4-Glu and C4Q/C4D_{45} discussed above for this experimental condition. Low substrate concentration does not seem by itself to be able to distinguish the three colorectal cell lines under analysis. Hypoxia on the other hand prompts for distinction even at low glucose levels, thus emphasizing the distinctive metabolic character of each colorectal cancer cell line. This distinct responsiveness is a crucial parameter to be evaluated aiming at the implementation of more efficient anticancer therapeutic strategies.

The implemented methodology for analysis of both culture media and perchloric acid cellular extracts provides unique and complimentary metabolic information, which affords a more complete picture of the metabolic transformations associated with each of the specific actions undertaken for challenging cancer cell metabolism. This methodology can naturally be extended to other metabolic pathways and metabolic tracers towards widening the scopes of the current metabolic analysis.

4. Conclusions

The three colorectal cancer cell lines show very distinct metabolic profiles in the four experimental conditions tested. Glycolysis is in every circumstance the most active metabolic pathway denoting the "Warburg" like metabolic behavior that characterizes many cancer cells. Nevertheless, oxidative flux through the Krebs cycle assumes its importance in whole cellular bioenergetics and its extent is itself very distinct among the three colorectal cancer cell lines. The interplay of oxygen (normoxia versus hypoxia) and nutrient (high versus low glucose) availability also plays a crucial role in the definition of cell capacity to adapt to distinct microenvironments. In this study the (in)sensibility to the three colorectal cancer cell lines was surprisingly different. The less glycolytic under normoxia, LS1034, turns into the most glycolytic in hypoxia while the most glycolytic barely respond to the hypoxic insult.

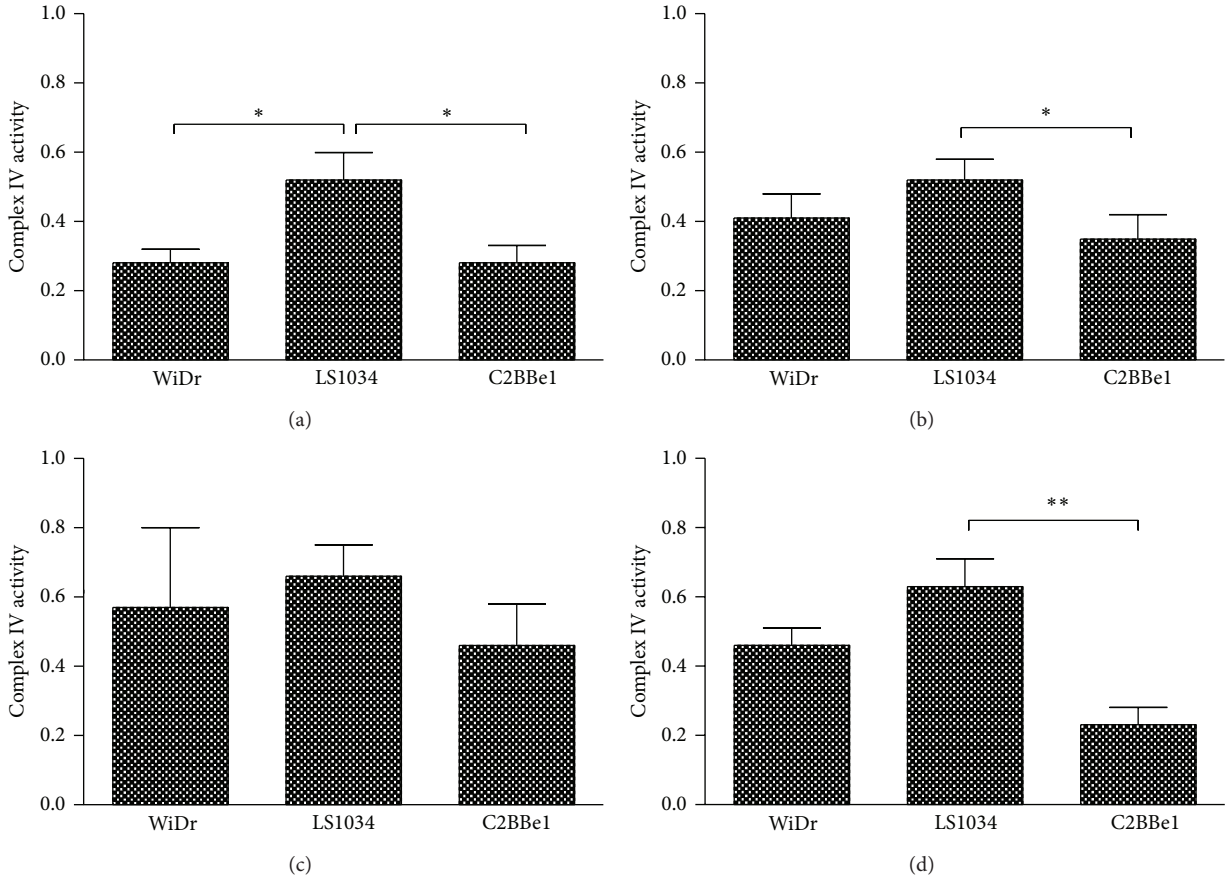


Figure 9: Complex IV activities for the three colorectal cancer cell lines under all experimental conditions: (a) normoxia/high glucose; (b) hypoxia/high glucose; (c) normoxia/low glucose; (d) hypoxia/low glucose.

Also, the alteration in nutrient availability interferes in the metabolic coupling between glycolysis and Krebs cycle and demonstrates the distinct capacity that cells have to react to such interferences. We understand that the whole picture is much more complex but would like to point out that the simple management of O_2 and glucose constitutes certainly a mechanism to take into account to tackle tumor proliferation and devise more efficient therapeutic strategies. The adequate knowledge of the metabolic behavior of cancer cells is thus a must on this "crusade" against cancer and the methodology here described is certainly pivotal for deriving this much-needed information.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ana M. Abrantes, Ludgero C. Tavares, Salomé Pires, and Cândida Mendes performed the experiments. Manuela Grazina, Rui A. Carvalho, and Maria Filomena Botelho analyzed results and made the figures. Ana M. Abrantes, Manuela Grazina, Rui A. Carvalho, and Maria Filomena Botelho designed the research and wrote the paper. João Casalta-Lopes performed statistical analyses.

Acknowledgments

The authors are grateful to FCT (Fundação para a Ciência e Tecnologia—Portugal) for financial support (PTDC/EBB-EBI/115810/2009). The authors also acknowledge the "Rede Nacional de RMN" (REDE/1517/RMN/2005) for access to the NMR spectrometer and facilities at Coimbra University. The authors thank Centre of Investigation on Environment Genetics and Oncobiology (CIMAGO) for financial support. Salomé Pires and Ludgero Tavares are recipients of fellowships from the Portuguese Foundation for Science and Technology (SFRH/BD/75300/2010) and (SFRH/BD/66600/2009), respectively.

References

[1] O. Warburg, "Origin of cancer cells," *Oncologia*, vol. 9, no. 2, pp. 75–83, 1956 (German).

[2] S. Weinhouse, O. Warburg, D. Burk, and A. L. Schade, "On respiratory impairment in cancer cells," *Science*, vol. 124, no. 3215, pp. 267–272, 1956.

[3] Y. Zhao, E. B. Butler, and M. Tan, "Targeting cellular metabolism to improve cancer therapeutics," *Cell Death and Disease*, vol. 4, no. 3, article e532, 2013.

[4] C. V. Dang, "Links between metabolism and cancer," *Genes & Development*, vol. 26, no. 9, pp. 877–890, 2012.

[5] M. Talekar, S. R. Boreddy, A. Singh, and M. Amiji, "Tumor aerobic glycolysis: new insights into therapeutic strategies with targeted delivery," *Expert Opinion on Biological Therapy*, 2014.

[6] R. Xu, H. Pelicano, Y. Zhou et al., "Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia," *Cancer Research*, vol. 65, no. 2, pp. 613–621, 2005.

[7] S. Ros and A. Schulze, "Balancing glycolytic flux: the role of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatases in cancer metabolism," *Cancer & Metabolism*, vol. 1, article 8, 2013.

[8] E. Roudier and A. Perrin, "Considering the role of pyruvate in tumor cells during hypoxia," *Biochimica et Biophysica Acta*, vol. 1796, no. 2, pp. 55–62, 2009.

[9] K. J. Williams, B. A. Telfer, R. E. Airley et al., "A protective role for HIF-1 in response to redox manipulation and glucose deprivation: implications for tumorigenesis," *Oncogene*, vol. 21, no. 2, pp. 282–290, 2002.

[10] M. Achison and T. R. Hupp, "Hypoxia attenuates the p53 response to cellular damage," *Oncogene*, vol. 22, no. 22, pp. 3431–3440, 2003.

[11] H. L. Kotze, E. G. Armitage, K. J. Sharkey, J. W. Allwood, W. B. Dunn, and K. J. Williams, "A novel untargeted metabolomics correlation-based network analysis incorporating human metabolic reconstructions," *BMC Systems Biology*, vol. 7, article 107, 2013.

[12] Y.-A. Wen, P. D. Stevens, M. L. Gasser, R. Andrei, and T. Gao, "Downregulation of PHLPP expression contributes to hypoxia-induced resistance to chemotherapy in colon cancer cells," *Molecular and Cellular Biology*, vol. 33, no. 22, pp. 4594–4605, 2013.

[13] B. Garcia-Barreno, T. Delgado, B. Akerlind-Stopner, E. Norrby, and J. A. Melero, "Location of the epitope recognized by monoclonal antibody 63G on the primary structure of human respiratory syncytial virus G glycoprotein and the ability of synthetic peptides containing this epitope to induce neutralizing antibodies," *Journal of General Virology*, vol. 73, no. 10, pp. 2625–2630, 1992.

[14] I. F. Duarte, J. Marques, A. F. Ladeirinha et al., "Analytical approaches toward successful human cell metabolome studies by NMR spectroscopy," *Analytical Chemistry*, vol. 81, no. 12, pp. 5023–5032, 2009.

[15] S. L. Pereira, J. Ramalho-Santos, A. F. Branco, V. A. Sardão, P. J. Oliveira, and R. A. Carvalho, "Metabolic remodeling during H9c2 myoblast differentiation: relevance for in vitro toxicity studies," *Cardiovascular Toxicology*, vol. 11, no. 2, pp. 180–190, 2011.

[16] M. D. Basson, I. M. Modlin, and J. A. Madri, "Human enterocyte (Caco-2) migration is modulated in vitro by extracellular matrix composition and epidermal growth factor," *Journal of Clinical Investigation*, vol. 90, no. 1, pp. 15–23, 1992.

[17] M. D. Peterson and M. S. Mooseker, "Characterization of the enterocyte-like brush border cytoskeleton of the C2BBe clones of the human intestinal cell line, Caco-2," *Journal of Cell Science*, vol. 102, no. 3, pp. 581–600, 1992.

[18] E. M. Chance, S. H. Seeholzer, K. Kobayashi, and J. R. Williamson, "Mathematical analysis of isotope labeling in the citric acid cycle with applications to 13C NMR studies in perfused rat hearts," *Journal of Biological Chemistry*, vol. 258, no. 22, pp. 13785–13794, 1983.

[19] D. H. Williamson, P. Lund, and H. A. Krebs, "The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver," *Biochemical Journal*, vol. 103, no. 2, pp. 514–527, 1967.

[20] L. Suardet, A. C. Gaide, J. M. Calmès et al., "Responsiveness of three newly established human colorectal cancer cell lines to transforming growth factors β1 and β21," *Cancer Research*, vol. 52, no. 13, pp. 3705–3712, 1992.

[21] R. A. Carvalho, T. B. Rodrigues, P. Zhao, F. M. H. Jeffrey, C. R. Malloy, and A. D. Sherry, "A ^{13}C isotopomer kinetic analysis of cardiac metabolism: influence of altered cytosolic redox and $[Ca^{2+}]_o$," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 287, no. 2, pp. H889–H895, 2004.

[22] A. M. Abrantes, M. E. S. Serra, A. C. Gonçalves et al., "Hypoxia-induced redox alterations and their correlation with 99mTc-MIBI and 99mTc-HL-91 uptake in colon cancer cells," *Nuclear Medicine and Biology*, vol. 37, no. 2, pp. 125–132, 2010.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 974309, 8 pages
http://dx.doi.org/10.1155/2014/974309

Research Article

Targeting the Neddylation Pathway to Suppress the Growth of Prostate Cancer Cells: Therapeutic Implication for the Men's Cancer

Xiaofang Wang,[1,2,3] Lihui Li,[1,3] Yupei Liang,[1,3] Chunjie Li,[1,3] Hu Zhao,[4] Dingwei Ye,[5] Menghong Sun,[6] Lak Shin Jeong,[7] Yan Feng,[2] Shen Fu,[2] Lijun Jia,[1,3] and Xiaomao Guo[2,3]

[1] *Cancer Institute, Fudan University Shanghai Cancer Center, Shanghai 200032, China*
[2] *Department of Radiation Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China*
[3] *Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China*
[4] *Department of Laboratory Medicine, Laboratory of Molecular Biology of Huadong Hospital affiliated to Fudan University, Shanghai 200040, China*
[5] *Department of Urology, Fudan University Shanghai Cancer Center, Shanghai 200032, China*
[6] *Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai 200032, China*
[7] *College of Pharmacy, Seoul National University, Seoul, 151-742, Republic of Korea*

Correspondence should be addressed to Lijun Jia; ljjia@fudan.edu.cn and Xiaomao Guo; guoxm1800@126.com

Received 10 May 2014; Accepted 14 May 2014; Published 29 June 2014

Academic Editor: Zhen Chen

The neddylation pathway has been recognized as an attractive anticancer target in several malignancies, and its selective inhibitor, MLN4924, has recently advanced to clinical development. However, the anticancer effect of this compound against prostate cancer has not been well investigated. In this study, we demonstrated that the neddylation pathway was functional and targetable in prostate cancer cells. Specific inhibition of this pathway with MLN4924 suppressed the proliferation and clonogenic survival of prostate cancer cells. Mechanistically, MLN4924 treatment inhibited cullin neddylation, inactivated Cullin-RING E3 ligases (CRLs), and led to accumulation of tumor-suppressive CRLs substrates, including cell cycle inhibitors (p21, p27, and WEE1), NF-κB signaling inhibitor IκBα, and DNA replication licensing proteins (CDT1 and ORC1). As a result, MLN4924 triggered DNA damage, G2 phase cell cycle arrest, and apoptosis. Taken together, our results demonstrate the effectiveness of targeting the neddylation pathway with MLN4924 in suppressing the growth of prostate cancer cells, implicating a potentially new therapeutic approach for the men's cancer.

1. Introduction

Prostate cancer is the most common malignancy among elderly men, representing the second leading cause of male cancer death in developed countries [1, 2]. With the aging population in the coming year, the incidence of prostate cancer will be annually rising. Although prostate cancer patients own favorable 5-year overall survival in general [3], a substantial proportion of patients with an initial response to medical or surgical castration suffers from treatment failure due to acquired hormone resistance [4]. Chemotherapeutic options for prostate cancer patients are historically limited largely because prostate cancer is insensitive to most chemotherapeutics. In order to overcome such limitations, safer and more effective therapeutic agents are needed.

Protein neddylation is a newly characterized protein posttranslational modification in eukaryotic cells by adding NEDD8, an ubiquitin-like molecule, to target proteins [5–8]. Similar to protein ubiquitination, NEDD8 is firstly activated by NEDD8-activating enzyme E1 (also known as NAE, a heterodimer composed of NAE1 and UBA3 subunits), the activated NEDD8 is then transferred to NEDD8-conjugating enzyme E2 (UBC12 or UBE2F), and finally NEDD8-conjugating enzyme E2 collaborates with substrate-specific NEDD8-E3 ligase to covalently conjugate the NEDD8 to its target substrates [9]. The best-characterized NEDD8

substrates are the cullin family proteins that serve as the scaffold components of cullin-RING E3 ligases (CRLs) [10, 11]. CRLs, the largest cellular ubiquitin ligase family, mediate proteasomal degradation of a variety of cellular proteins that function in diverse biological processes whereas their dysfunction leads to carcinogenesis [12–14].

Neddylation-CRLs axis has emerged as a novel anticancer strategy, as evidenced by the antitumor activity of the NEDD8-activating enzyme (NAE) inhibitor MLN4924 [14–16]. This small molecule binds to NAE at the active site and forms a covalent NEDD8-MLN4924 adduct that blocks the subsequent enzymatic cascades for protein neddylation [17, 18]. By doing so, MLN4924 blocks cullin neddylation, inactivates CRLs, and thus leads to accumulation of CRLs substrates and growth suppression of cancer cells [14, 19, 20]. Due to its significant anticancer efficacy and well-tolerated toxicity in preclinical studies, MLN4924 has been advanced into phase I clinical trials for several malignancies [21, 22]. In the present study, we reported that neddylation pathway was activated in prostate cancer cells whereas inhibition of this pathway by MLN4924 exerted significant anticancer efficacy in prostate cancer cells. Our findings lay the foundation for the future development of MLN4924 as a potential treatment of prostate cancer.

2. Materials and Methods

2.1. Cell Lines and Drug Solutions. Prostate cancer cell lines DU145, LNCap, and PC3 were purchased from American Type Culture Collection and grown in RPMI1640 with 10% fetal bovine serum. Neddylation pathway inhibitor MLN4924, proteasome inhibitors Bortezomib, and MG132 were each dissolved in dimethyl sulfoxide (DMSO) and kept in −20°C before use [23].

2.2. Cell Proliferation Assay. Cells were seeded in 96-well plates in triplicate (3000 cells per well) and treated with MLN4924 at various doses. After treatment for 96 hours, cell viability was measured with ATPlite kit (Perkin Elmer), according to the manufacturer's instructions [24].

2.3. Clonogenic Cell Survival Assay. Cells were seeded into 6-well plates in triplicate (250 cells per well). Twenty-four hours later, the old culture media were replaced with fresh media in the presence or absence of MLN4924, followed by incubation at 37°C for 12 days. After fixation with 0.2% crystal violet, colonies containing more than 50 individual cells were counted [23].

2.4. Immunoblotting (IB). Cells were harvested and cell lysates were extracted for immunoblotting as described [25], using antibodies against NAE1, UBA3, UBC12, UBE2F, DCN-1, CDT1, ROC1, NEDD8, cullin1, p21, p27, WEE1, phospho-histone H3 (p-H3), total-histone H3 (t-H3), phospho-H2AX (p-H2AX), total H2AX (t-H2AX), total-IκBα (t-IκBα), phospho-IκBα (p-IκBα), cleaved Caspase-3 (c-Caspase-3), cleaved PARP (c-PARP), and GAPDH.

2.5. Fluorescence Activated Cell Sorting (FACS) Analysis. After treatment with MLN4924, cells were trypsinized, washed with PBS, and fixed in ice-cold 70% ethanol. For cell cycle analysis, cells were stained with propidium iodide and analyzed by CyAn ADP (Beckman Coulter). Data were analyzed with ModFit LT software [23].

2.6. Statistical Analysis. Data are presented as mean ± standard error of the mean (SEM). GraphPad Prism5 software was adopted to assess the statistical differences. The unpaired 2-tailed t test was performed for the comparison of two groups, and the level of significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$.

3. Results

3.1. The Neddylation Pathway Was Activated and Targetable in Prostate Cancer Cells. To evaluate the activation status of the neddylation pathway in prostate cancer cells, the expression of key components of the neddylation pathway was examined. As shown in Figure 1(a), NEDD8-activating enzyme E1 (NAE1 and UBA3), NEDD8-conjugating enzyme E2 (UBC12 and UBE2F), and NEDD8-E3 ligases (DCN-1 and ROC1) were expressed in high levels, suggesting the activation of neddylation pathway in prostate cancer cells. In addition, both conjugated and free NEDD8 were revealed to be highly expressed in prostate cancer cells (Figure 1(b)).

The activation of neddylation pathway renders it a potential anticancer target in prostate cancer cells. To test this hypothesis, an effort to suppress neddylation pathway was made by using NAE inhibitor MLN4924. As shown in Figure 1(b), global protein neddylation was obviously suppressed by MLN4924 while free NEDD8 accumulated dramatically in treated cells, demonstrating the functional and targetable status of neddylation pathway in prostate cancer cells. Furthermore, we determined the specificity of MLN4924 for inhibition of the neddylation pathway when compared to Bortezomib (originally codenamed PS-341) and MG132, two classical proteasome inhibitors, and found that MLN4924, but neither Bortezomib nor MG132, specifically inhibited global protein neddylation and cullin neddylation (Figure 1(c)). These results demonstrate that MLN4924 specifically blocks protein neddylation in prostate cancer cells.

3.2. MLN4924 Inhibited the Growth of Prostate Cancer Cells. Next we determined the sensitivity of two prostate cancer cell lines to MLN4924. Morphological observations showed that MLN4924 significantly inhibited the proliferation of prostate cancer cells. Moreover, MLN4924-treated cells were shrunk and became round, indicating that the cells were undergoing the apoptosis (Figure 2(a)). Consistently, cell viability assay revealed that MLN4924 induced a dose-dependent impairment of cell viability (Figure 2(b)). In addition, MLN4924 effectively suppressed colony formation in a standard clonogenic survival assay in prostate cancer cells (Figure 2(c)). These data demonstrate that MLN4924 is a potent inhibitor of cell proliferation and survival in prostate cancer cell lines.

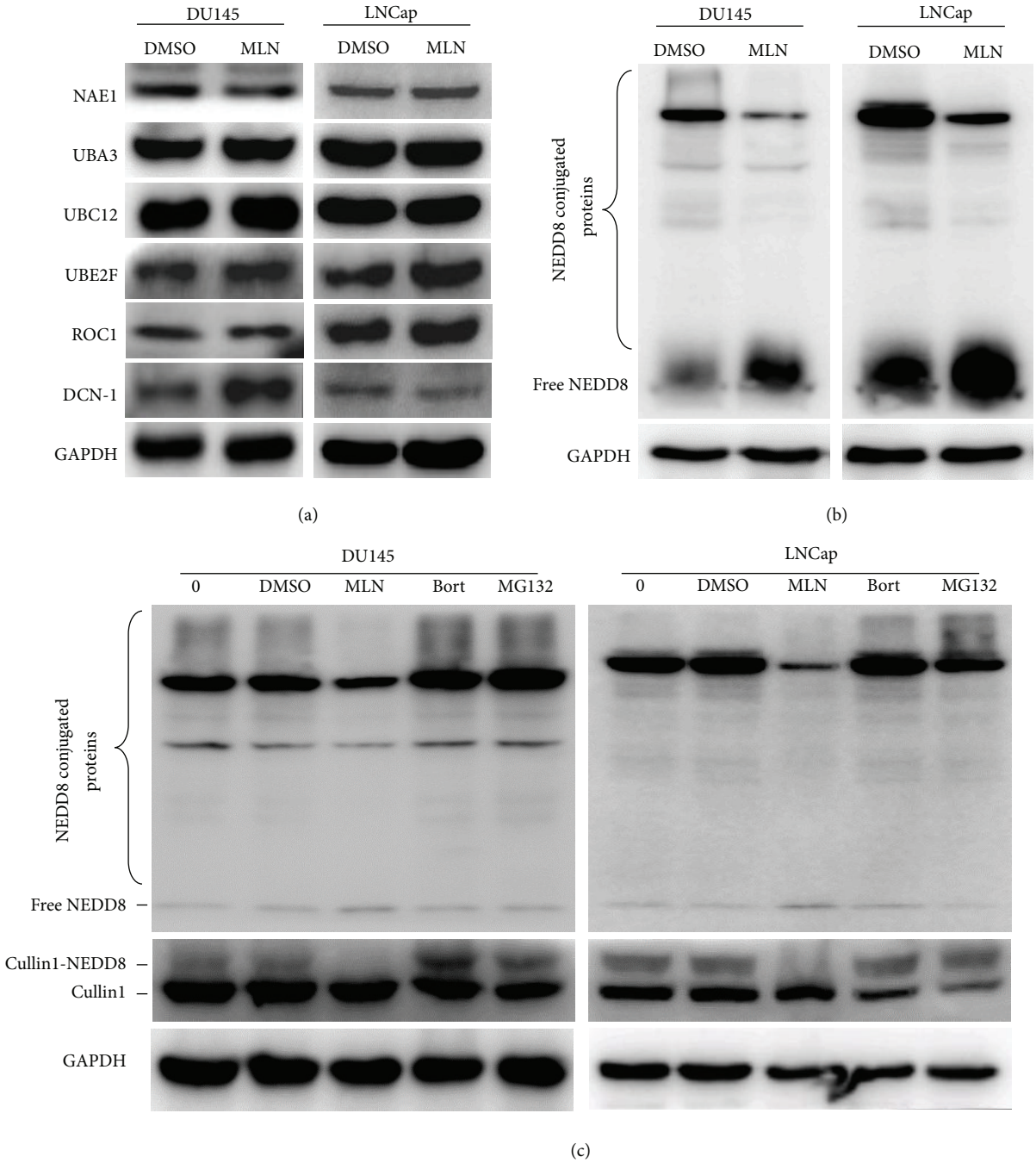


Figure 1: The neddylation pathway was functional and targetable in prostate cancer cells. (a) The components of the neddylation pathway were expressed in prostate cancer cells. Subconfluent cells were subjected to MLN4924 treatment (1 μM) for 12 hours and harvested for immunoblotting (IB) using antibodies against NAE1, UBA3, UBC12, UBE2F, ROC-1, and DCN-1 with GAPDH as a loading control. (b) Neddylation pathway was activated and targetable in prostate cancer cells. Cells were subjected to MLN4924 (1 μM) for 12 hours and harvested for IB using antibodies against NEDD8 with GAPDH as a loading control. (c) MLN4924 specifically inhibited neddylation pathway. Cells were subjected to MLN4924 (1 μM), Bortezomib (1 μM), or MG132 (20 μM) for 4 hours and harvested for IB using antibodies against NEDD8 and cullin1 with GAPDH as a loading control. MLN, MLN4924; Bort, Bortezomib.

3.3. MLN4924 Inhibited Cullin Neddylation and Inactivated CRLs. To address the potential mechanisms underlying the inhibitory effect of MLN4924 on the growth of prostate cancer cells, the expression of a panel of tumor-suppressive CRLs substrates was determined in treated cells. As shown in Figure 3, cullin neddylation was completely blocked by MLN4924, indicating the inactivation of CRLs. As a result, CRLs substrates, including cell cycle inhibitors (p21, p27), NF-κB signaling inhibitor IκBα, and DNA replication licensing proteins (CDT1 and ORC1), were accumulated upon

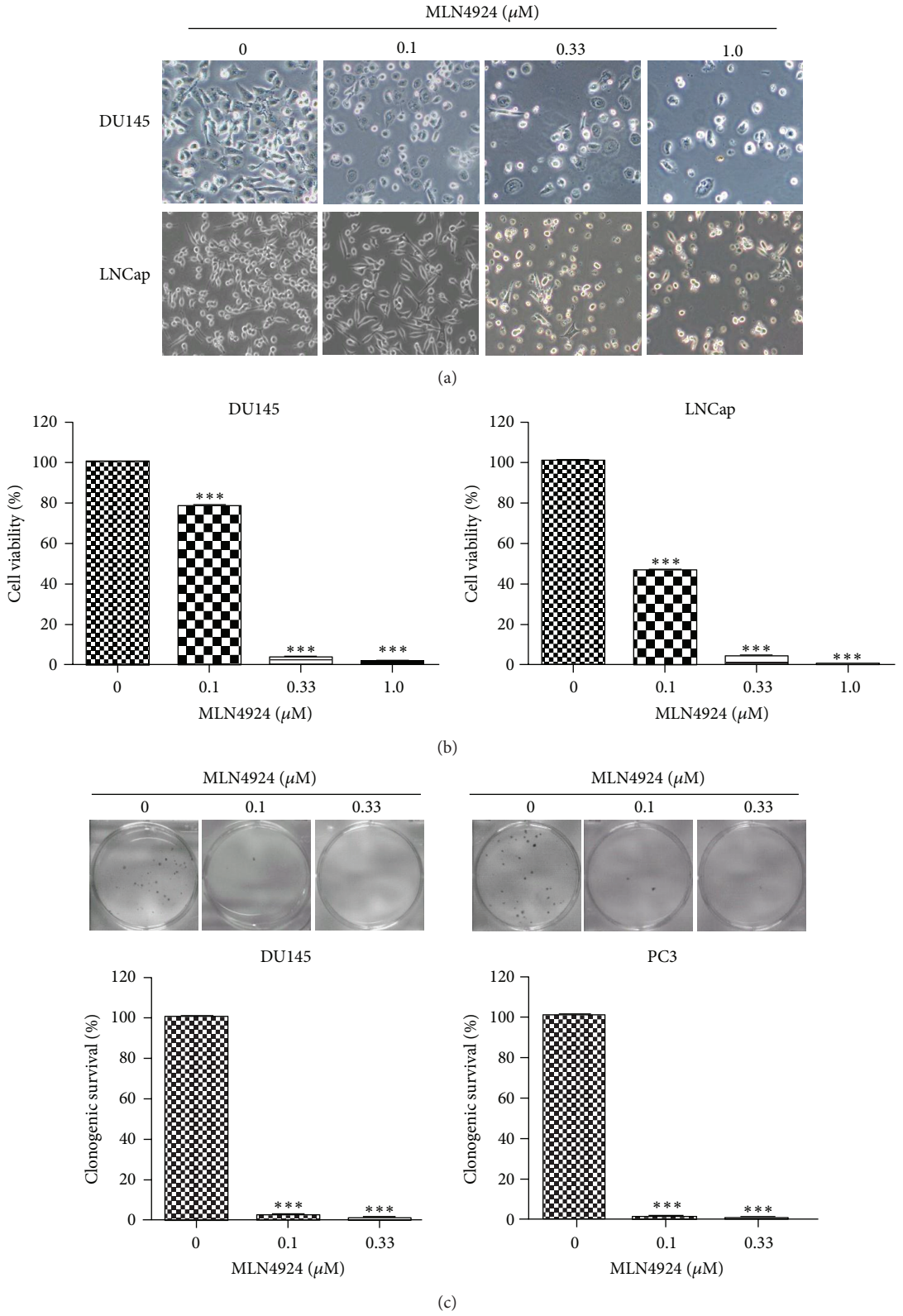


Figure 2: MLN4924 inhibited prostate cancer cell growth. (a) Morphological observations of MLN4924-treated cells. Cells were treated with indicated concentrations of MLN4924 for 48 hours and then photographed. (b) MLN4924 inhibited the proliferation of prostate cancer cells. Cells were seeded in 96-well plates in triplicate and treated with indicated concentrations of MLN4924 for 96 hours; cell viability was determined by ATPlite kit ($^{***}P < 0.0001$, $n = 3$). (c) MLN4924 inhibited clonogenic cell survival of prostate cancer cells. DU145 and PC3 cells were seeded into 60 mm dishes in duplicate and then grown in the presence or absence of MLN4924 for 12 days. The colonies with more than 50 cells were counted, following crystal violet staining ($^{***}P < 0.0001$, $n = 3$).

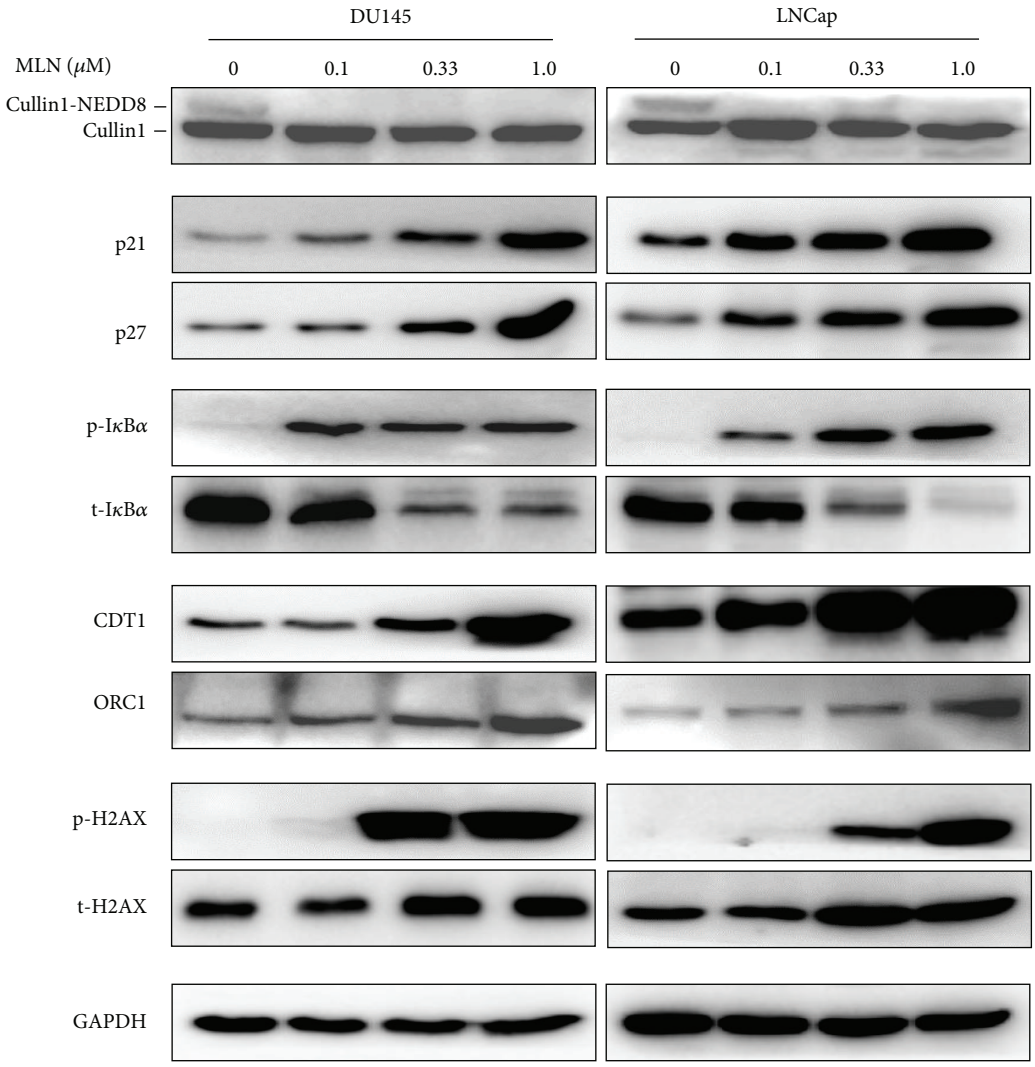


Figure 3: MLN4924 induced accumulation of CRLs substrates and triggered DNA damage in prostate cancer cells. Subconfluent cells were treated with MLN4924 (0, 0.1, 0.33, 1.0 μM) for 48 hours, followed by IB analysis using antibodies against p21, p27, p-IκBα, t-IκBα, CDT1, ORC1, p-H2AX, and t-H2AX with GAPDH as a loading control.

MLN4924 treatment. Also, we found that the expression level of phosphorylation H2AX (Figure 3), a classical DNA damage marker, was elevated in MLN4924-treated cells, which was very likely triggered by the accumulation of CDT1 and ORC1 [23].

3.4. Inactivation of CRLs by MLN4924 Induced Cell Cycle Arrest and Apoptosis. To further investigate the nature of MLN4924-mediated growth suppression of prostate cancer cells, we performed the cell cycle profile analysis and found that MLN4924 notably triggered G2/M cell-cycle arrest in DU145 cell lines (Figure 4(a)). To further determine at which phase cells were arrested, we examined the expression of G2-M phase transition inhibitor WEE1 and the mitotic marker p-histone H3. As shown in Figure 4(b), MLN4924 significantly induced the increase of WEE1 and decrease of p-histone H3, revealing that MLN4924-treated cells were arrested at G2 phase. Moreover, MLN4924 treatment induced the expression of cleaved Caspase-3 and cleaved PARP (the biochemical indicators of apoptotic induction) (Figure 4(c)), which was consistent with previous observation (Figure 2(a)) that MLN4924-treated cells displayed apoptosis-like morphology.

4. Discussion

With the aging population, the incidence of prostate cancer appears to steadily increase in recent years. However, the current systemic therapy is far from satisfaction due to (1) acquired drug resistance, (2) severe treatment-related adverse effects, and (3) low anticancer efficacy, which ultimately results in recurrence and metastasis. Therefore, new systemic therapy strategies are in urgent need to improve the currently available prostate cancer treatment. The neddylation pathway was recently found to be overactivated in a couple of cancer types [18, 26] and has been considered a promising anticancer

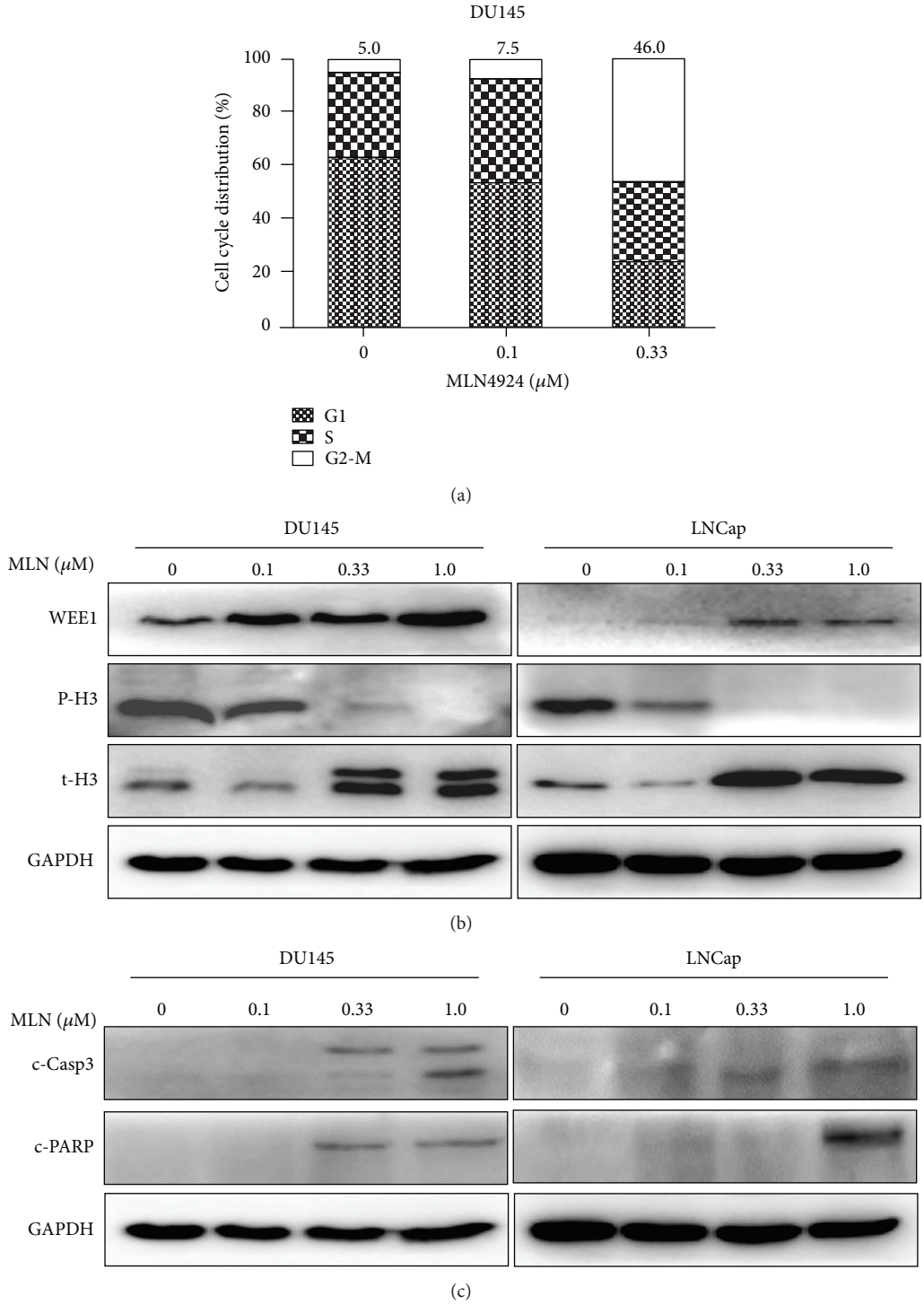


Figure 4: MLN4924 induced G2 phase cell cycle arrest and apoptosis in prostate cancer cells. Subconfluent cells were treated with MLN4924. Forty-eight hours later, one portion of cells was used for cell cycle profile analysis (a), while the other portion was subjected to IB analysis ((b) and (c)). (a) MLN4924 induced G2 phase cell cycle arrest. Cell percentages at G2 phase were 5%, 7.5%, and 46% respectively, when treated with MLN4924 at 0, 0.1 and 0.33 μM. (b) IB analysis to determine the expression of WEE1 and p-histone H3. (c) MLN4924 induced apoptosis in prostate cancer cells. Cells were treated with MLN4924 for 48 hours, followed by IB analysis using antibodies against c-Caspase-3 and c-PARP with GAPDH as a loading control.

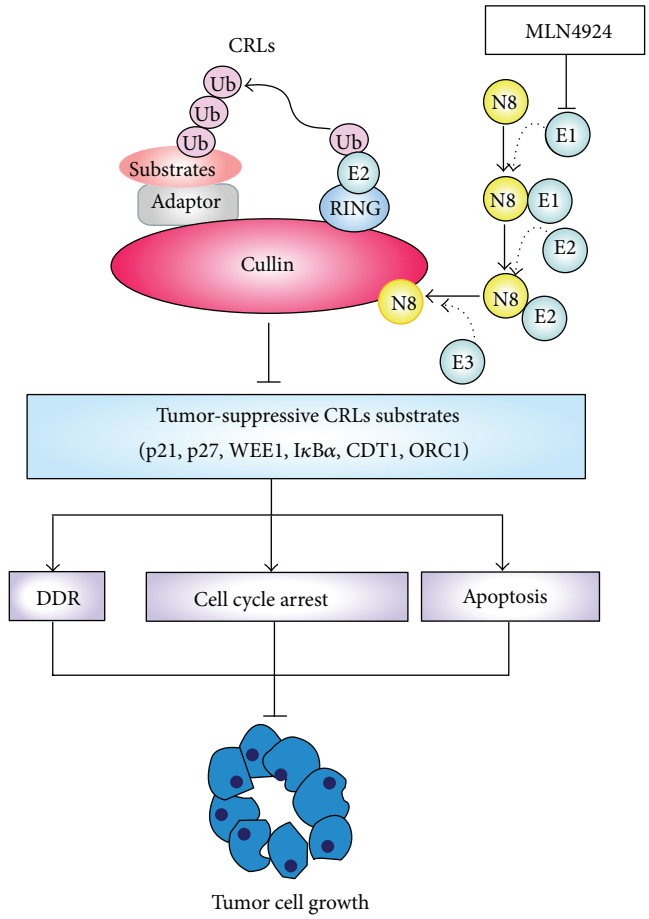


Figure 5: The proposed mechanism underlying the inhibitory effect of MLN4924 in prostate cancer cells. MLN4924 inactivates CRLs by inhibiting cullin neddylation and thus induces accumulation of CRLs substrates. These consequently trigger a series of critical cellular effects including DNA damage response, G2 cycle arrest, and apoptosis, which are responsible for growth suppression of prostate cancer cells. DDR, DNA damage response.

target [18] in a number of malignancies. The efforts to screen for small-molecular inhibitor against neddylation pathway have led to the discovery of MLN4924, an inhibitor of NAE. By blocking neddylation of cullin, the best-characterized target of neddylation pathway thus far, MLN4924 has the ability to inactivate CRLs, leads to accumulation of its substrates, and thus eventually leads to suppression of several solid tumors and hematological malignancies both *in vitro* and *in vivo* [9, 21]. In the present study, we found that the neddylation pathway was activated in prostate cancer cells. Moreover, we found that MLN4924 was potent in inhibiting tumor growth in both hormone-sensitive (LNCap) and hormone-resistant (DU145) human prostate carcinoma cell lines.

Previous studies reported that blockage of cullin neddylation by MLN4924 was enabled to inactivate CRLs and thus induced multiple cellular effects, including G2 phase arrest, DNA damage response, and apoptosis/senescence [14, 18, 25]. Our results demonstrate that similar mechanisms of growth suppression are shared by prostate cancer upon neddylation inhibition. In prostate cancer cells, neddylation inactivation by MLN4924 blocked cullin neddylation, inhibited CRLs activity, and thus triggered DNA damage, cell cycle arrest, and apoptosis by inducing the accumulation of well-known CRLs substrates, including (1) cell cycle inhibitors p21, p27, and WEE1; (2) NF-κB inhibitor IκBα; and (3) DNA replication licensing proteins CDT1 and ORC1 (Figure 5) [14, 23, 27]. These observations suggest that protein neddylation is a conserved signaling pathway essential for the survival of prostate cancer cells. Collectively, targeting neddylation is feasible and reasonable for the treatment of prostate cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by National Basic Research Program of China (973 program, 2012CB910302), National Natural Science Foundation Grant of China (Grant nos. 81172092, 81372196, and 31071204), the Program for Professor

of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning, Shanghai Pujiang Talent Program (12PJ1400600), and a grant from Fudan University Shanghai Cancer Center in China to Lijun Jia.

References

[1] R. Siegel, C. Desantis, K. Virgo et al., "Cancer treatment and survivorship statistics, 2012," *CA Cancer Journal for Clinicians*, vol. 62, no. 4, pp. 220–241, 2012.

[2] J. Ferlay, P. Autier, M. Boniol, M. Heanue, M. Colombet, and P. Boyle, "Estimates of the cancer incidence and mortality in Europe in 2006," *Annals of Oncology*, vol. 18, no. 3, pp. 581–592, 2007.

[3] Z. Zhou, X. Zhu, J. Xia et al., "Short-term versus long-term hormone therapy plus radiotherapy or prostatectomy for prostate cancer: A systematic review and meta-analysis," *Journal of Cancer Research and Clinical Oncology*, vol. 139, no. 5, pp. 783–796, 2013.

[4] B. J. Feldman and D. Feldman, "The development of androgen-independent prostate cancer," *Nature Reviews Cancer*, vol. 1, no. 1, pp. 34–45, 2001.

[5] D. P. Xirodimas, "Novel substrates and functions for the ubiquitin-like molecule NEDD8," *Biochemical Society Transactions*, vol. 36, part 5, pp. 802–806, 2008.

[6] D. P. Xirodimas, M. K. Saville, J. C. Bourdon, R. T. Hay, and D. P. Lane, "Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity," *Cell*, vol. 118, no. 1, pp. 83–97, 2004.

[7] W. Zuo, F. Huang, Y. J. Chiang et al., "c-Cbl-mediated neddylation antagonizes ubiquitination and degradation of the TGF-β type II receptor," *Molecular Cell*, vol. 49, no. 3, pp. 499–510, 2013.

[8] S. J. Loftus, G. Liu, S. M. Carr, S. Munro, and N. B. la Thangue, "NEDDylation regulates E2F-1-dependent transcription," *EMBO Reports*, vol. 13, no. 9, pp. 811–818, 2012.

[9] T. A. Soucy, P. G. Smith, and M. Rolfe, "Targeting NEDD8-activated cullin-RING ligases for the treatment of cancer," *Clinical Cancer Research*, vol. 15, no. 12, pp. 3912–3916, 2009.

[10] E. Sakata, Y. Yamaguchi, Y. Miyauchi et al., "Direct interactions between NEDD8 and ubiquitin E2 conjugating enzymes upregulate cullin-based E3 ligase activity," *Nature Structural & Molecular Biology*, vol. 14, no. 2, pp. 167–168, 2007.

[11] A. Saha and R. J. Deshaies, "Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation," *Molecular Cell*, vol. 32, no. 1, pp. 21–31, 2008.

[12] L. Jia and Y. Sun, "SCF E3 ubiquitin ligases as anticancer targets," *Current Cancer Drug Targets*, vol. 11, no. 3, pp. 347–356, 2011.

[13] L. K. Teixeira and S. I. Reed, "Ubiquitin ligases and cell cycle control," *Annual Review of Biochemistry*, vol. 82, pp. 387–414, 2013.

[14] T. A. Soucy, P. G. Smith, M. A. Milhollen et al., "An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer," *Nature*, vol. 458, no. 7239, pp. 732–736, 2009.

[15] L. Jia, H. Li, and Y. Sun, "Induction of p21-dependent senescence by an NAE inhibitor, MLN4924, as a mechanism of growth suppression," *Neoplasia*, vol. 13, no. 6, pp. 561–569, 2011.

[16] J. L. Blank, X. J. Liu, K. Cosmopoulos et al., "Novel DNA damage checkpoints mediating cell death induced by the NEDD8-activating enzyme inhibitor MLN4924," *Cancer Research*, vol. 73, no. 1, pp. 225–234, 2013.

[17] J. E. Brownell, M. D. Sintchak, J. M. Gavin et al., "Substrate-assisted inhibition of ubiquitin-like protein-activating enzymes: the NEDD8 E1 inhibitor MLN4924 forms a NEDD8-AMP mimetic in situ," *Molecular Cell*, vol. 37, no. 1, pp. 102–111, 2010.

[18] Y. Zhao, X. Xiong, L. Jia, and Y. Sun, "Targeting Cullin-RING ligases by MLN4924 induces autophagy via modulating the HIF1-REDD1-TSC1-mTORC1-DEPTOR axis," *Cell Death & Disease*, vol. 3, no. 9, article e386, 2012.

[19] J. J. Lin, M. A. Milhollen, P. G. Smith, U. Narayanan, and A. Dutta, "NEDD8-targeting drug MLN4924 elicits DNA rereplication by stabilizing Cdt1 in S phase, triggering checkpoint activation, apoptosis, and senescence in cancer cells," *Cancer Research*, vol. 70, no. 24, pp. 10310–10320, 2010.

[20] C. Mackintosh, D. J. García-Domínguez, J. L. Ordóñez et al., "WEE1 accumulation and deregulation of S-phase proteins mediate MLN4924 potent inhibitory effect on Ewing sarcoma cells," *Oncogene*, vol. 32, no. 11, pp. 1441–1451, 2013.

[21] T. A. Soucy, L. R. Dick, P. G. Smith, M. A. Milhollen, and J. E. Brownell, "The NEDD8 conjugation pathway and its relevance in cancer biology and therapy," *Genes & Cancer*, vol. 1, no. 7, pp. 708–716, 2010.

[22] T. Tanaka, T. Nakatani, and T. Kamitani, "Negative regulation of NEDD8 conjugation pathway by novel molecules and agents for anticancer therapy," *Current Pharmaceutical Design*, vol. 19, no. 22, pp. 4131–4139, 2013.

[23] Z. Luo, G. Yu, H. W. Lee et al., "The Nedd8-activating enzyme inhibitor MLN4924 induces autophagy and apoptosis to suppress liver cancer cell growth," *Cancer Research*, vol. 72, no. 13, pp. 3360–3371, 2012.

[24] L. Jia, M. S. Soengas, and Y. Sun, "ROC1/RBX1 E3 ubiquitin ligase silencing suppresses tumor cell growth via sequential induction of G2-M arrest, apoptosis, and senescence," *Cancer Research*, vol. 69, no. 12, pp. 4974–4982, 2009.

[25] L. Li, B. Liu, T. Dong et al., "Neddylation pathway regulates the proliferation and survival of macrophages," *Biochemical and Biophysical Research Communications*, vol. 432, no. 3, pp. 494–498, 2013.

[26] C. Salon, E. Brambilla, C. Brambilla, S. Lantuejoul, S. Gazzeri, and B. Eymin, "Altered pattern of Cul-1 protein expression and neddylation in human lung tumours: relationships with CANDI and cyclin E protein levels," *Journal of Pathology*, vol. 213, no. 3, pp. 303–310, 2007.

[27] L. Jia, J. S. Bickel, J. Wu et al., "RBX1 (RING box protein 1) E3 ubiquitin ligase is required for genomic integrity by modulating DNA replication licensing proteins," *Journal of Biological Chemistry*, vol. 286, no. 5, pp. 3379–3386, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 875305, 11 pages
http://dx.doi.org/10.1155/2014/875305

Review Article

Dissecting the Role of Bone Marrow Stromal Cells on Bone Metastases

Denise Buenrostro,[1,2] Serk In Park,[2,3] and Julie A. Sterling[1,2,3]

[1] *Department of Veterans Affairs, Tennessee Valley Healthcare System, Nashville, TN 37212, USA*
[2] *Center for Bone Biology, Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University, 2215B Garland Avenue, 1235 MRBIV, Nashville, TN 37232, USA*
[3] *Department of Cancer Biology, Vanderbilt University, Nashville, TN 37232, USA*

Correspondence should be addressed to Serk In Park; serkin.park@vanderbilt.edu
and Julie A. Sterling; julie.sterling@vanderbilt.edu

Received 9 May 2014; Accepted 10 June 2014; Published 26 June 2014

Academic Editor: Zhen Chen

Tumor-induced bone disease is a dynamic process that involves interactions with many cell types. Once metastatic cancer cells reach the bone, they are in contact with many different cell types that are present in the cell-rich bone marrow. These cells include the immune cells, myeloid cells, fibroblasts, osteoblasts, osteoclasts, and mesenchymal stem cells. Each of these cell populations can influence the behavior or gene expression of both the tumor cells and the bone microenvironment. Additionally, the tumor itself can alter the behavior of these bone marrow cells which further alters both the microenvironment and the tumor cells. While many groups focus on studying these interactions, much remains unknown. A better understanding of the interactions between the tumor cells and the bone microenvironment will improve our knowledge on how tumors establish in bone and may lead to improvements in diagnosing and treating bone metastases. This review details our current knowledge on the interactions between tumor cells that reside in bone and their microenvironment.

1. Introduction

Despite recent advances in early detection and therapeutic approaches, metastases still remain the major problem for cancer patients. In particular, bone metastases account for decreased quality of life and ultimately death of prostate, breast, and lung cancer patients. However, current therapeutic approaches are insufficient to effectively cure or prevent bone metastasis. Tumor metastasis is a tightly regulated multistep process, in which specific interactions between disseminating tumor cells and the cells constituting the recipient organ microenvironment play important roles. Increasing evidence supports the prometastatic functions of the microenvironment, with many studies indicating the importance of bone marrow cells in the metastatic niche. Many early studies have shown that these bone marrow cells set up a metastatic niche at the secondary site that allows for cells to establish [1, 2]. Subsequent studies have specifically isolated myeloid-derived suppressor cells [3–6], myofibroblast [7–9], and tumor-associated macrophages [10–12]. Each of these has some overlapping roles in metastasis, but each class of cells is a distinct bone marrow cell type with distinct roles in metastasis (summarized in Figure 1). While these classes of cells were isolated and shown to be important in metastases, many groups are still actively trying to clarify their precise molecular role in the metastatic process. Researchers expect that advanced knowledge on how these cells regulate the tumor microenvironment will allow development of novel therapeutic approaches to alter the niche less hospitable to the cells and therefore reducing or preventing tumor growth. It is also possible that understanding the niche will allow clinicians to better predict which patients may develop secondary disease and which organs may be affected.

2. Bone Cells

The importance of interactions between tumor cells and other cells in the bone microenvironment was demonstrated in

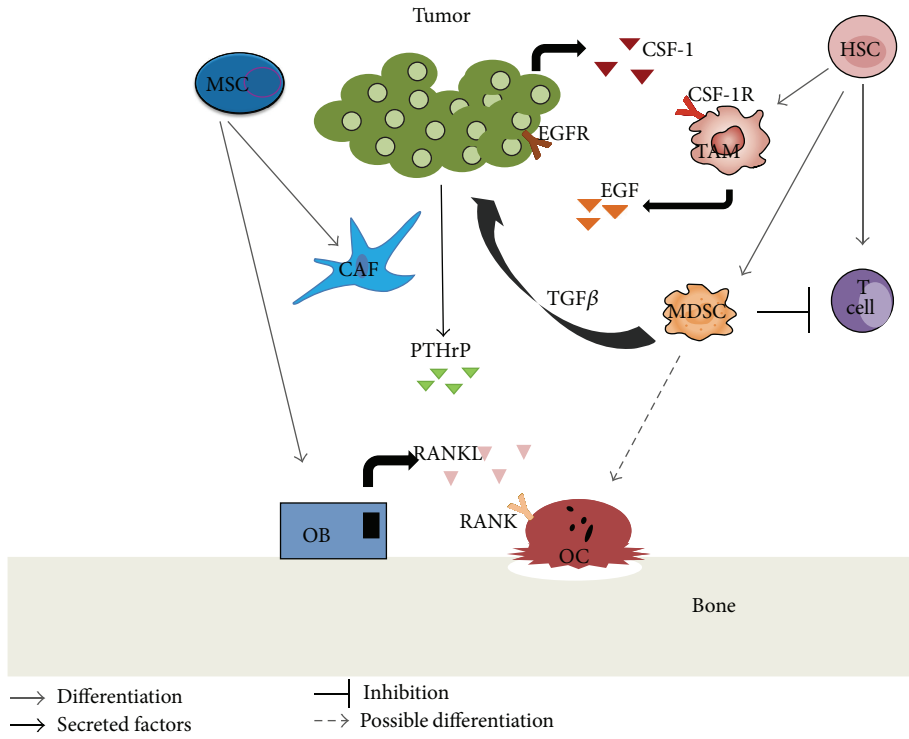


Figure 1: Tumor microenvironment interactions. Tumor cells interact with the cell populations present in the bone marrow. These include cells such as the fibroblasts, osteoblasts, osteoclasts, immune cells, and others as depicted here.

the 1990s by the work of Dr. Greg Mundy and others in the field. Their work strongly showed that there was a vicious cycle between the tumor cells and cells in the bone microenvironment. This work showed that tumor cells secreted factors that stimulated bone destruction, while bone destruction caused the release of growth factors from the bone matrix that further stimulated the tumor cell growth and production of factors that further enhanced bone destruction [13–15].

2.1. Osteoclasts. Osteoclasts are multinucleated cells that are responsible for bone resorption. A functional osteoclast has the ability to resorb mineralized bone matrix as part of normal bone remodeling that occurs during an individual's lifetime [16, 17]. Osteoclasts differentiate from myeloid progenitor cells under the influence of growth factors and cytokines such as macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) [18, 19]. Physiological bone resorption is a tightly regulated process that involves signals from osteoblasts as well as signals from other cells found in the microenvironment. Osteoclast differentiation, maturation, and activation are dependent on RANK/RANKL/osteoprotegerin (OPG) signaling pathway [20, 21]. OPG is a soluble decoy receptor for RANKL, expressed by osteoblasts and negatively regulates osteoclast activation [19, 21, 22]. Deregulation of this process, such as too much resorption or too little, can lead to increased risk of fracture as well as other bone-related diseases [17].

Overactive osteoclasts can be detrimental and play a role in several diseases such as osteoporosis, pycnodysostosis, and Paget's disease, which occur due to increased bone resorption and bone loss [23]. Primary cancers of breast, lung, and prostate cancer have a propensity to metastasis to bone [22, 24–26]. These cancers cells secrete factors such as parathyroid hormone-related protein (PTHrP) which stimulate osteoclast-mediated bone destruction through the RANK/RANKL/OPG signaling pathway [22, 27]. During bone destruction growth factors including transforming growth factor beta (TGF-β), insulin-like growth factors (IGFs), and others are released from the bone matrix, which can stimulate further tumor growth and the production of tumor-derived factors (such as PTHrP) that can stimulate further bone destruction [15, 22, 28].

Even though much is known about the role of osteoclasts within the vicious cycle, many of their functions are yet to be explored. Increased osteoclast activity can be due to several different factors but the end results seem to be the same, which is that over activation of these cells promotes osteolysis and tumor cell growth, because factors released from bone during resorption stimulate tumor cell proliferation. CXCR4 is found on osteoclast precursors and regulates hematopoietic and tumor cell homing to bone. In studies where mice were reconstituted with $Cxcr4^{-/-}$ hematopoietic cells had increased bone resorption and bone loss, specifically $Cxcr4^{-/-}$ osteoclasts had higher resorptive activity and faster differentiation compared to control osteoclasts. The authors concluded that because the reconstituted mice had increased tumor growth in bone compared to control mice that disruption in CXCR4 may increase osteoclastogenesis leading to increased resorption and tumor burden [29]. A recent paper

published by Ell et al. showed that mice injected with pre-miR-141 and pre-miR-219 had reduced osteoclastic activity and osteolytic bone metastasis [30]. $Src^{-/-}$ mice have shown impaired osteoclast functions [31, 32], and Src inhibitors have shown to suppress bone resorption effects [33]. These data suggest that Src may be an ideal therapeutic target to suppress tumor cells (frequently expressing high Src activities) and osteoclasts (requiring Src for function) at the same time [34, 35]. However, recent phase III clinical trial results showed that addition of dasatinib (a Src family kinase inhibitor) to docetaxel did not significantly improve overall survival of castration-resistant prostate cancer patients [36]. Araujo et al., the lead investigator of the failed clinical trial, pointed out that further understanding of Src inhibitors' mode of action could identify a better therapeutic role, because the clinical trial included heterogeneous patient population [36].

Inhibition of osteoclast activity by a variety of different factors has shown to decrease tumor burden in a mouse breast cancer bone metastasis model [37]. The most commonly used class of osteoclast inhibitors includes bisphosphonates, which bind to the bone promoting osteoclast apoptosis and inhibiting osteoclast mediated bone resorption [38]. Bisphosphonates (including zoledronate, alendronate, ibandronate, etc.) have been highly successful for reducing skeletal related events in patients with osteoporosis and with tumor-induced bone destruction [39, 40].

Alternatively, RANKL inhibitory antibodies have been promising both clinically and in preclinical models where they can increase time to skeletal related events (SRE) [41, 42]. Denosumab (Prolia, XGEVA), a monoclonal antibody against RANKL, was recently demonstrated to significantly increase time to SRE compared to a bisphosphonate, zoledronic acid, in breast and prostate cancer patients with bone disease [43, 44]. The debate between clinicians regarding which treatment is more efficacious continues, but both options are clearly effective and have their benefits. One concern regarding both treatments is the serious, yet rare, side-effects such as atypical fractures and osteonecrosis of the jaw [25, 37, 38]. Additionally, neither treatment has been shown to cure bone metastases or significantly increase survival in patients with bone metastases.

2.2. Osteoblasts. Osteoblasts are mesenchymal-origin cells lining the endosteal surface of bone and constitute approximately 4–6% of all bone cells. Osteoblasts produce organic matrix of bone and subsequently deposit inorganic components (e.g., calcium and phosphate), resulting in mineralized hard tissue. In addition to their physiologic functions, osteoblasts are important components of the metastatic bone microenvironment. The best-characterized role of osteoblasts in bone metastasis is described in the "vicious cycle hypothesis" where osteoblasts produce M-CSF and RANKL, two essential factors for osteoclastogenesis [14, 27, 45]. Subsequent studies followed to understand how molecular alterations in osteoblasts contribute to create a congenial microenvironment for metastatic tumor cells. Schneider et al. demonstrated that expansion of osteoblasts by administration of bone-anabolic agents such as parathyroid hormone (PTH) increased prostate tumor cell localization and growth in bone [46], suggesting that higher bone turnover rates (i.e., increased activity and number of osteoblasts) are associated with bone metastasis. Other studies have suggested that osteoblasts can function as a prometastatic population of cells. The first experimental evidence to support this come from the physiological phenomena of hematopoietic stem cell (HSC) homing in bone. HSCs migrate and repopulate the bone marrow immediately after birth, while the liver is the primary site of hematopoiesis during feral development. Taichman et al. demonstrated that CXCL12/SDF-1 (expressed by osteoblasts and endothelial cells) and its receptor (CXCR4, expressed by prostate cancer cells) regulate bone-tropism of prostate cancer cells [47]. In addition to the CXCL12/CXCR4/CXCR7 axis [48], Annexin II, expressed by osteoblasts and endothelium, regulates HSC adhesion, homing, and engraftment [49]. More recently, Jung et al. demonstrated that differential levels of growth arrest specific- (GAS-) 6 protein in the bone stromal cells (dominantly osteoblasts) induce metastatic tumor cell dormancy and determine site-specificity (i.e., increased localization in vertebrae and hind limb long bone compared with fore limb bones) of murine experimental metastasis model of human prostate cancer [50]. Furthermore, the same group provided pivotal evidence that osteoblastic niche for HSC is the direct target of tumor cell localization in bone [51]. The authors demonstrated that increasing the HSC niche size (via administration of PTH to induce osteoblast proliferation) promoted skeletal localization of prostate cancer cells, while decreasing the niche size (via conditional ablation of osteoblasts) reduced tumor cell localization. The author further investigated whether HSC compete with metastatic cancer cells for occupancy in the bone marrow. Administration of AMD3100 (a clinical regimen to mobilize HSC) mobilized metastatic cancer cells in the niche back into the circulation, indicating that HSC compete with bone-tropic cancer cells. These data collectively suggest that adhesion molecules and chemokine/chemokine receptors expressed on osteoblasts contribute to localization and subsequent growth of metastatic tumor cells in bone.

Increasing evidence supports that osteoblastic cells contribute to the metastatic progression by releasing cytokines and growth factors in the microenvironment. We have recently demonstrated that primary prostate tumor cells distantly instigate osteoblasts (via PTHrP in the systemic circulation) to increase vascular endothelial growth factor- (VEGF-) A, interleukin- (IL-) 6, and C-C chemokine ligand- (CCL-) 2 in the bone microenvironment and that VEGF-A and IL-6 in turn stimulate myeloid-derived suppressor cells with increased angiogenic potentials [52]. Indeed, hematopoietic lineage cells are dependent on bone cells (predominantly osteoblastic cells) for proliferation, mobilization, and function. This concept of "osteoimmunology" is now expanding to the role of osteoblasts in regulating other adjacent bone marrow cells (e.g., hematopoietic lineage cells with prometastatic functions, such as myeloid-derived suppressor cells). Interestingly, those prometastatic cytokines (in particular, VEGF-A and IL-6) stimulate osteoblasts to produce more VEGF-A and IL-6, suggesting that osteoblastic cells may function as an amplification mechanism of cytokines in the bone microenvironment.

3. Immune Cells

3.1. Myeloid Derive Suppressor Cells. The role and existence of myeloid derived suppressor cells (MDSCs) have been quite controversial among scientists since their initial discovery in 1978 [53]. Initially they were recognized as natural suppressor cells located in the bone marrow and spleen that were able to suppress cell-mediated immunity [54]. These cells did not contain cell surface markers that resembled T cells, B cells, macrophages, or natural killer cells which made it difficult to phenotypically characterize them [55, 56]. MDSCs are a heterogeneous population of myeloid cells that are at different stages of differentiation. This population includes immature macrophages, granulocytes, and dendritic cells as well as myeloid progenitor cells [5, 57, 58]. In mice these cells can be characterized into two major subtypes, monocytic-MDSCs and granulocytic-MDSCs, through lymphocyte antigens Ly6C and Ly6G [59]. Both subtypes have immune suppressive functions that are regulated through distinct mechanisms. Granulocytic-MDSCs have been found to express higher levels of ROS (reactive oxygen species) and low levels of NO (nitric oxide) verses monocytic-MDSCs expressing higher NO and lower ROS expression [59, 60]. Suppressive MDSCs are not found in healthy hosts; only their nonsuppressive counterpart iMCs (immature myeloid cells) are present. MDSCs need to be activated to express suppressive function and are only present at sites of chronic pathological conditions such as infection and cancer [53].

Recently these cells have been recognized to play an important role in tumor progression in many solid tumors by inhibiting antitumor immune responses and by promoting tolerance [58, 61]. These cells have been deemed protumorigenic due to their suppression of T cells, promotion of angiogenesis, invasion, and metastasis [5, 6, 53, 62]. MDSCs have been directly linked to promoting tumor invasion and metastasis through the production and secretion of factors such as MMPs, IFNγ, IL-10, and TGF-β [6, 61]. They have also been known to suppress the immune system by promoting tolerance by accumulating T regulatory cells [58, 61, 63]. In cancer, MDSCs are activated by tumor-secreted factors such as Toll-like receptors (TLRs), IL-4, IL-13, and TGF-β that activate several different signaling pathways [64]. Specific MDSC expansion in the tumor microenvironment is guided through tumor-derived factors and factors from the microenvironment that is context specific dictating which population (monocytic versus granulocytic) is increased [46].

The presence and accumulation of MDSCs has been well reported in several human cancers as well as different disease types in the last several years. A positive correlation between stage and MDSC peripheral density has been reported in both melanoma and head and neck squamous cell carcinoma (HNSCC) patients [65]. A 15 percent increase in circulating $CD14^+$ $HLA\text{-}DR^{-/lo}$ cells was correlated with advanced stage (III and IV) as compared to early stage (I and II) HNSCC patients [65]. MDSCs containing the phenotype $LIN^-HLA\text{-}DR^-CD33^+CD11b^+$ have been isolated from the blood of patients with glioblastoma, breast, colon, lung, and kidney cancers [58, 62, 66, 67]. MDSCs containing the phenotype $CD11b^+CD14^-HLA\text{-}DR^{-/low}CD33^+CD15^+$ were found in the bone marrow and the peripheral blood of patients with active multiple myeloma compared with healthy donors [68].

The role that MDSCs play in human tumor-induced bone disease is still relatively unknown. With the use of mouse models, several published papers have demonstrated that MDSCs play an important role in bone metastasis. This is consistent with what is known about MDSC's contribution in the primary tumor environment. What is unknown is if MDSCs perform a direct role in promoting tumor establishment or tumor proliferation in bone by assisting the tumor itself or indirectly by secreting protumorigenic factors that prime the bone allowing it to become a hospitable host. Published papers have used mouse models to show that MDSCs can promote tumor growth in bone [52, 57, 69]. In a prostate cancer mouse model, it was demonstrated that tumor-derived PTHrP indirectly increases MDSC's angiogenic potential therefore contributing to tumor growth and angiogenesis [52]. Danilin and colleagues showed that MDSCs contribute to breast cancer osteolysis by inducing expression of Gli2 and PTHrP in tumor-bearing mice. These factors stimulate osteoclast-mediated bone destruction leading to increased bone lesions compared to control mice [57]. This group also showed that MDSCs isolated from tumor-bearing mice had the potential to differentiate into osteoclasts *in vitro* and *in vivo* [57]. Sawant et al. published this as well and explained that the reason MDSCs could differentiate into osteoclasts is because they are novel osteoclast progenitors driving bone metastasis during cancer progression [69].

MDSCs as a potential therapeutic target have been the topic of discussion since their identification. Studies have shown that eliminating MDSCs increases immune-surveillance and decreases tumor growth [63, 70, 71]. There are many different ways to target MDSCs including growth factors (anti-VEGF antibodies), chemokines (anti-CCL2 antibodies), cytotoxic drugs (Gemcitabine), enzyme inhibitors (amino-bisphosphonate), signaling inhibitors (sunitinib), and inducing differentiation (ATRA-All-trans retinoic acid) [72]. Src inhibitors have shown promise in targeting MDSCs by inhibiting their recruitment and MMP-9 gene expression in the tumor microenvironment [73]. Gemcitabine is a nucleoside metabolic inhibitor used to treat several types of cancers and has been shown to decrease MDSC levels in tumor-bearing mice by inhibiting expansion [74, 75]; however, its precise mechanism of MDSC inhibition is not fully understood. Bisphosphonates, which are routinely prescribed for cancer patients with bone metastasis, have also been demonstrated to decrease MDSC expansion in tumor-bearing mice through the reduction of MMP-9 expression [76]. Additionally, STAT3 inhibitors have also been successful at targeting MDSC in preclinical models [65]. While more studies are needed to understand the mechanisms of action, it is clear that targeting MDSCs clinically is both possible and promising therapeutically.

3.2. Tumor-Associated Macrophages. Macrophages are professional phagocytes that are differentiated from the myeloid lineage and are identified by the expression of certain markers as well as by the phenotypic differences among them [77, 78]. They have roles in development, homeostasis, tissue repair,

and immunity and have been linked to many diseases including cancer [77, 78]. These are plastic cells and their phenotype is consistently modulated by the local microenvironment [79]. Macrophages can be classified by their immunological responses such as classically activated macrophages (M1) that are involved in inflammatory responses and alternatively activated macrophages (M2) that are involved in wound healing [77, 78, 80, 81]. M2 macrophages have been implicated in having protumor properties due to the cytokines, chemokines, and growth factors that they release such as VEGF, IL-10, TGF-β, EGF, and MMPs, among many others [77, 82]. These protumor macrophages are referred to as tumor-associated macrophages (TAMs) and are considered to be phenotypically similar to M2 macrophages [81, 83, 84].

Macrophage growth, chemotaxis, and differentiation are controlled by several chemokines including CCL-2 (also known as monocyte chemoattractant protein [MCP]-1) and growth factors such as CSF-1 [77]. CSF-1 is the regulator of the differentiation, proliferation, and survival of macrophages and their precursors [85]. CSF-1 overexpression has been implicated in the poor prognosis of several cancers and is currently being investigated as a possible therapeutic target [85–89]. In an invasive breast cancer mouse model, macrophages have been implicated in assisting tumor cell motility by participating in an epidermal growth factor- (EGF-) CSF-1 paracrine loop where tumor cells secrete CSF-1 and macrophages contain the corresponding receptor and vice versa [66, 67, 69, 75]. CCL2 is a chemokine that has been implicated in assisting cancer metastasis by mediating a crosstalk between cancer cells and the stromal cells that are present in the tumor microenvironment [90]. CCL2 is expressed by many tumor types as well as by the peripheral myeloid population [91]. Roca and colleagues showed that CCL2 stimulation induces peripheral blood monocytes to differentiate to M2 macrophages compared to unstimulated control monocytes [91].

In several papers macrophages have been reported to promote tumor initiation, progression, invasion, and metastasis [77, 79, 84]. Activated macrophages produce inflammatory factors such as reactive oxygen and nitrogen species in response to signals from other immune cells creating a constant inflamed stromal environment [80]. Chronic inflammation generates a stromal environment susceptible to mutations and has been linked to tumor initiation and growth. Progression of a mass from a neoplasia/adenoma to an early carcinoma is prompted through their secretion of VEGF and other angiogenic factors stimulating angiogenic switch [80]. Several groups have shown that an increase in macrophage density correlates with poor patient prognosis and survival in thyroid, lung, breast, and hepatocellular cancers [84, 89, 92, 93]. However, in other cancers such as stomach, colorectal, and pancreatic cancer, a high macrophage density is correlated with a good patient prognosis [80, 94].

The role of macrophages at the primary site is well established but their function at distant metastatic sites is still being highly investigated. Myeloid derived cells have been found to accumulate at distant sites priming the environment for tumor colonization [1, 2]. This notion of a premetastatic niche has been around for several years and has been found to be important in the primary site but has yet to be proven to exist in bone. This theory encompasses that once there is an established primary tumor site, hematopoietic progenitor cells are signaled to migrate from the bone marrow into secondary metastatic sites, such as the lung, and alter the microenvironment leading to activation of integrins and chemokines that promote attachment, survival, and growth of tumor cells [1]. Proving that this process occurs in bone has been challenging because hematopoietic progenitor cells originate in the bone marrow and do not have to migrate to reach the bone microenvironment. It is more likely that in bone microenvironment stromal cells including macrophages are "reeducated" by tumor-derived factors and begin priming the bone before tumor establishment occurs.

Several therapeutic approaches to target macrophages have been explored. One approach includes the inhibition of TGF-β signaling, which was demonstrated through preclinical studies by deleting TGFβ type 2 receptor (RII) in the macrophages. These studies demonstrated that animals with RII deficient macrophages displayed a reduction in tumor growth due to decreased secretion of myeloid factors that assist in tumor progression [82, 95]. Other therapeutic approaches target macrophage factors such as CSF-1 and its receptor [85, 87]. Currently, in clinical trials are small molecules and monoclonal antibodies that inhibit CSF-1 and prevent its binding, or the tyrosine kinase activity [96]. Other therapeutic strategies include preventing the recruitment of macrophages through inhibition of inflammatory monocyte trafficking with anti-CCL2 or CCR2 antibodies [96]. However, a recent phase II clinical trial for carlumab (anti-CCL2 monoclonal antibody) in metastatic prostate cancer patients did not support antitumoral activity as a single agent (PMID 22907596). Since TAMs, macrophages that have been educated by the tumor cells and assist in cancer progression have been implicated in causing resistance to tamoxifen in breast cancer and to androgen receptor antagonists in prostate cancer; a potential future therapeutic strategy could be to reeducate TAMs to express an antitumor phenotype that would work against the tumor instead of with it [79, 80, 83, 84].

3.3. Other Immune Cells. The bone marrow is a rich environment for many different immune cells including the B-cells, T-cells, and NK-cells, all of which are known to be important in cancer progression and soft tissue metastases [97]. Yet despite their proximity and abundance in bone metastases, relatively few studies have been performed to investigate their role in tumor-induced bone disease. This is in part due to the fact that the vast majority of bone metastasis studies utilize human tumors in immune-deficient mice, most commonly these models of T-cell deficient mice, but other models are also lacking B-cells (SCID, *Rag 2*−/−, *Rag 1* −/−). This makes understanding the role of T- and B-cells in bone metastases challenging.

T-cells are well-known to inhibit tumor growth, and in line with this finding it has been shown that stimulating T-cell response in mice reduced tumor burden in bone while reducing it blocks tumor growth in bone [98]. However, a recent study demonstrated that tumor associated T-cells can

induce osteolytic bone disease prior to bone colonization. In this study they show that T-cell produced RANKL can induce osteoclastogenesis and bone destruction [99]. These data suggest that T-cells may have a dual role in bone disease in that they can reduce tumor growth but stimulate bone destruction. Regardless, since the majority of cancer and bone studies utilize T-cell deficient mice, it is clear that tumor cells can grow and metastasize to bone in the absence of T-cells.

Much less information exists describing the interactions between tumors in bone and B-cells or NK cells. A few manuscripts describe interactions between NK cells and tumors in bone. Specifically, they show that inhibiting NK cells increases tumor take in animal models of prostate cancer [100]. Other papers describe that NK cells are reduced in prostate cancer [101] but that forced expression of NK associated ligands can reduce tumor growth [102, 103]. Another immune lineage cell that has been implicated in cancer induced bone disease is the Megakaryocytes. Li et al. demonstrated that megakaryocytes could reduce prostate tumor cell growth and increase apoptosis, while their expansion *in vivo* reduced tumor-induced bone destruction [104].

4. Cancer-Associated Fibroblast (CAF)

Fibroblasts are another cell type that is abundant in the bone marrow microenvironment. CAFs are defined as fibroblast that reside in the tumor mass and are capable of promoting tumor growth. These cells are typically myofibroblast-like cells that express α-Smooth muscle actin (α-SMA), vimentin, and fibroblast specific protein-1 (FSP1) [105]. Some of the early studies showed that these fibroblasts could be recruited from the bone marrow to the tumor [106] and that they could stimulate malignant transformation [7], tumor cell growth, and invasion [107]. These effects on tumor cell growth are thought to be mediated through CXCL12 [108] and TGF-β [109]. Other pathways including Wnt signaling [110], bone morphogenetic proteins [111], and MMPs [112] have also been associated with their invasive potential. Other papers have demonstrated that in addition to factors secreted by fibroblasts that they can induce a more invasive phenotype through physical properties as well. One study showed that the increase in fibroblasts increased the stiffness of the tumor, which can activate pathways within the tumor cells that induces a more invasive phenotype [113]. Interestingly, our previous publications have demonstrated that rigidity influences gene expression in tumor cells [114]. Taken together, this suggests that rigidity may also influence expression in the fibroblast and further contribute to tumor-induced bone disease.

In addition to regulating invasiveness of the primary site, other studies have investigated the role of CAFs in the establishment of secondary sites. For example CAFs have been shown to be recruited to sites of liver metastases in colon cancer [115]. Additionally, CAFs have been associated with bone metastases, in which the loss of TGF-β receptor type II (RII) in the CAFs stimulated prostate cancer cell growth in the bone. More importantly, a recent paper by Joan Massague's group demonstrated that CAF content in triple negative tumors was associated with bone metastases, but not lung, in patient samples [9].

Because of their association with tumor growth, invasion, and metastasis, CAFs make a compelling target for the development of therapeutics. This is also compounded by the fact that CAFs have been associated with chemotherapeutic resistance [116–118]. One group found that CAF-induced resistance to tamoxifen could be reversed using metformin or arsenic trioxide [119]. Other groups tried to target fibroblast activating protein using an anti-FAP antibody (sibrotuzumab) in clinical trials of metastatic colorectal cancer, but these studies showed no significant efficacy [120–122]. However, the use of FAP conjugated therapies has been shown to increase drug efficacy and reduce side-effects associated with chemotherapy [123, 124].

5. Mesenchymal Stem Cell (MSC)

MSCs are a pluripotent population of bone marrow cells that can differentiate with many different cell types, including osteoblasts, adipocytes, chondrocytes, and fibroblasts. Similar to CAFs, MSCs have been shown to associate to sites of tumor in many different tumor types [125] and have been demonstrated to promote proliferation and migration [9, 126, 127]. In Massague's recent paper, they showed that MSCs induced a transcriptional shift in tumors similar to CAFs and that MSCs could recapitulate the CAF phenotype [9]. This suggests that in breast cancer CAFs and MSCs function similarly. However, unlike CAFs, MSC association with tumors has not been completely associated with negative outcomes, and in some cases MSCs may inhibit tumor growth. In fact, in some malignancies, such as multiple myeloma (MM), MSCs are used therapeutically. Some treatments for MM involve cell-based therapies in which patients are given autologous stem cell transplants, under the reasoning that this may recapitulate normal immune cells that may fight the disease [128]. A recent myeloma study suggests that using MSCs with high Fas ligand in multiple myeloma bearing mice increased apoptosis of the myeloma cells [129]. Since MSCs "track" to tumors some groups have developed modified MSCs as cargo for the delivery of therapeutics [125], but due to treatment concerns they have not been tested clinically. Clearly more needs to be understood about MSCs and how to select for more specific populations.

6. Conclusions

The bone microenvironment is a rich milieu of different cell types, with each having a specific role on tumor cells both that metastasize to the bone and to other sites. While the past decade has seen an increase in research devoted to understanding the role of each cell-type in different malignancies, there are still many questions. In reality it is likely that these cells work together to regulate tumor growth, invasion, and metastasis and that new approaches need to be undertaken to study the complex interaction that occur between these multiple cell types. Many groups are beginning to collaborate with systems biologists, engineers, and

computer scientists to investigate these interactions in a more comprehensive manner. Once we better understand these interactions, more possibilities of therapeutic interventions will become possible.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank the Department of Veterans Affairs (1I01BX001957-JS), Cellular, Biochemical, and Molecular Science Training Program (5T32GM008554-DB), the National Cancer Institute (1R01CA163499-JS), and the Vanderbilt Center for Bone Biology (JS, SP) for financial support.

References

[1] R. N. Kaplan, S. Rafii, and D. Lyden, "Preparing the "soil": the premetastatic niche," *Cancer Research*, vol. 66, no. 23, pp. 11089–11093, 2006.

[2] R. N. Kaplan, R. D. Riba, S. Zacharoulis et al., "VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche," *Nature*, vol. 438, no. 7069, pp. 820–827, 2005.

[3] B. Almand, J. I. Clark, E. Nikitina et al., "Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer," *Journal of Immunology*, vol. 166, no. 1, pp. 678–689, 2001.

[4] D. Gabrilovich, T. Ishida, T. Oyama et al., "Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo," *Blood*, vol. 92, no. 11, pp. 4150–4166, 1998.

[5] L. Yang, L. M. DeBusk, K. Fukuda et al., "Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis," *Cancer Cell*, vol. 6, no. 4, pp. 409–421, 2004.

[6] L. Yang, J. Huang, X. Ren et al., "Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis," *Cancer Cell*, vol. 13, no. 1, pp. 23–35, 2008.

[7] G. R. Cunha, S. W. Hayward, and Y. Z. Wang, "Role of stroma in carcinogenesis of the prostate," *Differentiation*, vol. 70, no. 9-10, pp. 473–485, 2002.

[8] A. F. Olumi, G. D. Grossfeld, S. W. Hayward, P. R. Carroll, T. D. Tlsty, and G. R. Cunha, "Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium," *Cancer Research*, vol. 59, no. 19, pp. 5002–5011, 1999.

[9] X. H. Zhang, X. Jin, S. Malladi et al., "Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma," *Cell*, vol. 154, no. 5, pp. 1060–1073, 2013.

[10] R. D. Leek, C. E. Lewis, R. Whitehouse, M. Greenall, J. Clarke, and A. L. Harris, "Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma," *Cancer Research*, vol. 56, no. 20, pp. 4625–4629, 1996.

[11] C. E. Lewis, R. Leek, A. Harris, and J. O. McGee, "Cytokine regulation of angiogenesis in breast cancer: the role of tumor-associated macrophages," *Journal of Leukocyte Biology*, vol. 57, no. 5, pp. 747–751, 1995.

[12] M. Dutsch-Wicherek and W. Kazmierczak, "Creation of a suppressive microenvironment by macrophages and cancer-associated fibroblasts," *Frontiers in Bioscience*, vol. 18, no. 3, pp. 1003–1016, 2013.

[13] T. A. Guise and G. R. Mundy, "Cancer and bone," *Endocrine Reviews*, vol. 19, no. 1, pp. 18–54, 1998.

[14] G. R. Mundy, "Metastasis to bone: causes, consequences and therapeutic opportunities," *Nature Reviews: Cancer*, vol. 2, no. 8, pp. 584–593, 2002.

[15] J. A. Sterling, J. R. Edwards, T. J. Martin, and G. R. Mundy, "Advances in the biology of bone metastasis: how the skeleton affects tumor behavior," *Bone*, vol. 48, no. 1, pp. 6–15, 2011.

[16] T. J. Martin, "Historically significant events in the discovery of RANK/RANKL/OPG," *World Journal of Orthopedics*, vol. 4, no. 4, pp. 186–197, 2013.

[17] J. C. Crockett, M. J. Rogers, F. P. Coxon, L. J. Hocking, and M. H. Helfrich, "Bone remodelling at a glance," *Journal of Cell Science*, vol. 124, no. 7, pp. 991–998, 2011.

[18] B. Boyce, Z. Yao, and L. Xing, "Osteoclasts have multiple roles in bone in addition to bone resorption," *Critical Reviews in Eukaryotic Gene Expression*, vol. 19, no. 3, pp. 171–180, 2009.

[19] B. F. Boyce, E. M. Schwarz, and L. Xing, "Osteoclast precursors: cytokine-stimulated immunomodulators of inflammatory bone disease," *Current Opinion in Rheumatology*, vol. 18, no. 4, pp. 427–432, 2006.

[20] D. A. Glass II, P. Bialek, J. D. Ahn et al., "Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation," *Developmental Cell*, vol. 8, no. 5, pp. 751–764, 2005.

[21] G. D. Roodman, "Mechanisms of bone metastasis," *The New England Journal of Medicine*, vol. 350, no. 16, pp. 1655–1664, 2004.

[22] L. Feller, B. Kramer, and J. Lemmer, "A short account of metastatic bone disease," *Cancer Cell International*, vol. 11, article 24, 2011.

[23] J. R. Edwards, "Osteoclasts: malefactors of disease and targets for treatment," *Discovery Medicine*, vol. 13, no. 70, pp. 201–210, 2012.

[24] J. Zhuang, J. Zhang, S. T. Lwin et al., "Osteoclasts in multiple myeloma are derived from Gr-1+CD11b+myeloid-derived suppressor cells," *PLoS ONE*, vol. 7, no. 11, Article ID e48871, 2012.

[25] L. Mercatali, M. Ricci, E. Scarpi et al., "RANK/RANK-L/OPG in patients with bone metastases treated with anticancer agents and zoledronic acid: a prospective study," *International Journal of Molecular Sciences*, vol. 14, no. 6, pp. 10683–10693, 2013.

[26] R. Jin, J. A. Sterling, J. R. Edwards et al., "Activation of NF-kappa B signaling promotes growth of prostate cancer cells in bone," *PLoS ONE*, vol. 8, no. 4, Article ID e60983, 2013.

[27] K. N. Weilbaecher, T. A. Guise, and L. K. McCauley, "Cancer to bone: a fatal attraction," *Nature Reviews Cancer*, vol. 11, no. 6, pp. 411–425, 2011.

[28] D. R. Clohisy, C. M. Ogilvie, R. J. Carpenter, and M. L. R. Ramnaraine, "Localized, tumor-associated osteolysis involves the recruitment and activation of osteoclasts," *Journal of Orthopaedic Research*, vol. 14, no. 1, pp. 2–6, 1996.

[29] A. C. Hirbe, J. Rubin, Ö. Uluçkan et al., "Disruption of CXCR4 enhances osteoclastogenesis and tumor growth in bone," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 35, pp. 14062–14067, 2007.

[30] B. Ell, L. Mercatali, T. Ibrahim et al., "Tumor-induced osteoclast miRNA changes as regulators and biomarkers of osteolytic bone metastasis," *Cancer Cell*, vol. 24, no. 4, pp. 542–556, 2013.

[31] B. F. Boyce, T. Yoneda, C. Lowe, P. Soriano, and G. R. Mundy, "Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice," *Journal of Clinical Investigation*, vol. 90, no. 4, pp. 1622–1627, 1992.

[32] P. Soriano, C. Montgomery, R. Geske, and A. Bradley, "Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice," *Cell*, vol. 64, no. 4, pp. 693–702, 1991.

[33] A. Garcia-Gomez, E. M. Ocio, E. Crusoe et al., "Dasatinib as a bone-modifying agent: anabolic and anti-resorptive effects," *PLoS ONE*, vol. 7, no. 4, Article ID e34914, 2012.

[34] B. Boyce and L. Xing, "Src inhibitors in the treatment of metastatic bone disease: rationale and clinical data," *Clinical Investigation*, vol. 1, no. 12, pp. 1695–1706, 2011.

[35] G. E. Gallick, P. G. Corn, A. J. Zurita, and S. Lin, "Small-molecule protein tyrosine kinase inhibitors for the treatment of metastatic prostate cancer," *Future Medicinal Chemistry*, vol. 4, no. 1, pp. 107–119, 2012.

[36] J. C. Araujo, G. C. Trudel, F. Saad et al., "Docetaxel and dasatinib or placebo in men with metastatic castration-resistant prostate cancer (READY): a randomised, double-blind phase 3 trial," *The Lancet Oncology*, vol. 14, no. 13, pp. 1307–1316, 2013.

[37] J. Canon, R. Bryant, M. Roudier, D. G. Branstetter, and W. C. Dougall, "RANKL inhibition combined with tamoxifen treatment increases anti-tumor efficacy and prevents tumor-induced bone destruction in an estrogen receptor-positive breast cancer bone metastasis model," *Breast Cancer Research and Treatment*, vol. 135, no. 3, pp. 771–780, 2012.

[38] B. F. Boyce, "Advances in osteoclast biology reveal potential new drug targets and new roles for osteoclasts," *Journal of Bone and Mineral Research*, vol. 28, no. 4, pp. 711–722, 2013.

[39] S. Khosla, J. P. Bilezikian, D. W. Dempster et al., "Benefits and risks of bisphosphonate therapy for osteoporosis," *Journal of Clinical Endocrinology and Metabolism*, vol. 97, no. 7, pp. 2272–2282, 2012.

[40] M. McClung, S. T. Harris, P. D. Miller et al., "Bisphosphonate therapy for osteoporosis: benefits, risks, and drug holiday," *The American Journal of Medicine*, vol. 126, no. 1, pp. 13–20, 2013.

[41] J. Canon, R. Bryant, M. Roudier et al., "Inhibition of RANKL increases the anti-tumor effect of the EGFR inhibitor panitumumab in a murine model of bone metastasis," *Bone*, vol. 46, no. 6, pp. 1613–1619, 2010.

[42] P. M. Holland, R. Miller, J. Jones et al., "Combined therapy with the RANKL inhibitor RANK-Fc and rhApo2L/TRAIL/dulanermin reduces bone lesions and skeletal tumor burden in a model of breast cancer skeletal metastasis," *Cancer Biology & Therapy*, vol. 9, no. 7, pp. 539–550, 2010.

[43] A. T. Stopeck, A. Lipton, J. Body et al., "Denosumab compared with zoledronic acid for the treatment of bone metastases in patients with advanced breast cancer: a randomized, double-blind study," *Journal of Clinical Oncology*, vol. 28, no. 35, pp. 5132–5139, 2010.

[44] K. Fizazi, M. Carducci, M. Smith et al., "Denosumab versus zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study," *The Lancet*, vol. 377, no. 9768, pp. 813–822, 2011.

[45] T. A. Guise, J. J. Yin, S. D. Taylor et al., "Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis," *The Journal of Clinical Investigation*, vol. 98, no. 7, pp. 1544–1549, 1996.

[46] A. Schneider, L. M. Kalikin, A. C. Mattos et al., "Bone turnover mediates preferential localization of prostate cancer in the skeleton," *Endocrinology*, vol. 146, no. 4, pp. 1727–1736, 2005.

[47] R. S. Taichman, C. Cooper, E. T. Keller, K. J. Pienta, N. S. Taichman, and L. K. McCauley, "Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone," *Cancer Research*, vol. 62, no. 6, pp. 1832–1837, 2002.

[48] X. Sun, G. Cheng, M. Hao et al., "CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression," *Cancer and Metastasis Reviews*, vol. 29, no. 4, pp. 709–722, 2010.

[49] Y. Jung, J. Wang, J. Song et al., "Annexin II expressed by osteoblasts and endothelial cells regulates stem cell adhesion, homing, and engraftment following transplantation," *Blood*, vol. 110, no. 1, pp. 82–90, 2007.

[50] Y. Jung, Y. Shiozawa, J. Wang et al., "Prevalence of prostate cancer metastases after intravenous inoculation provides clues into the molecular basis of dormancy in the bone marrow microenvironment," *Neoplasia*, vol. 14, no. 5, pp. 429–439, 2012.

[51] Y. Jung, Y. Shiozawa, J. Wang et al., "Annexin-2 is a regulator of stromal cell-derived factor-1/CXCL12 function in the hematopoietic stem cell endosteal niche," *Experimental Hematology*, vol. 39, no. 2, pp. 151.e1–166.e1, 2011.

[52] S. I. Park, C. Lee, W. D. Sadler et al., "Parathyroid hormone-related protein drives a CD11b+Gr1+ cell-mediated positive feedback loop to support prostate cancer growth," *Cancer Research*, vol. 73, no. 22, pp. 6574–6583, 2013.

[53] J. E. Talmadge and D. I. Gabrilovich, "History of myeloid-derived suppressor cells," *Nature Reviews Cancer*, vol. 13, no. 10, pp. 739–752, 2013.

[54] J. A. Bennett, V. Srinivasa Rao, and M. S. Mitchell, "Systemic bacillus Calmette-Guerin (BCG) activates natural suppressor cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 75, no. 10, pp. 5142–5144, 1978.

[55] S. Slavin and S. Strober, "Induction of allograft tolerance after total lymphoid irradiation (TLI): development of suppressor cells of the mixed leukocyte reaction (MLR)," *The Journal of Immunology*, vol. 123, no. 2, pp. 942–946, 1979.

[56] A. K. Duwe and S. K. Singhal, "The immunoregulatory role of bone marrow. I. Suppression of the induction of antibody responses to T-dependent and T-independent antigens by cells in the bone marrow," *Cellular Immunology*, vol. 43, no. 2, pp. 362–371, 1979.

[57] S. Danilin, A. R. Merkel, J. R. Johnson, R. W. Johnson, J. R. Edwards, and J. A. Sterling, "Myeloid-derived suppressor cells expand during breast cancer progression and promote tumor-induced bone destruction," *Oncoimmunology*, vol. 1, no. 9, pp. 1484–1494, 2012.

[58] B. Hoechst, J. Gamrekelashvili, M. P. Manns, T. F. Greten, and F. Korangy, "Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells," *Blood*, vol. 117, no. 24, pp. 6532–6541, 2011.

[59] J. Youn, S. Nagaraj, M. Collazo, and D. I. Gabrilovich, "Subsets of myeloid-derived suppressor cells in tumor-bearing mice," *Journal of Immunology*, vol. 181, no. 8, pp. 5791–5802, 2008.

[60] K. Movahedi, M. Guilliams, J. Van Den Bossche et al., "Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell suppressive activity," *Blood*, vol. 111, no. 8, pp. 4233–4244, 2008.

[61] P. Serafini, S. Mgebroff, K. Noonan, and I. Borrello, "Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells," *Cancer Research*, vol. 68, no. 13, pp. 5439–5449, 2008.

[62] S. Kusmartsev, Z. Su, A. Heiser et al., "Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal

cell carcinoma," *Clinical Cancer Research*, vol. 14, no. 24, pp. 8270–8278, 2008.

[63] P. Sinha, V. K. Clements, S. K. Bunt, S. M. Albelda, and S. Ostrand-Rosenberg, "Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response," *The Journal of Immunology*, vol. 179, no. 2, pp. 977–983, 2007.

[64] M. J. Delano, P. O. Scumpia, J. S. Weinstein et al., "MyD88-dependent expansion of an immature GR-1 +CD11b+ population induces T cell suppression and Th2 polarization in sepsis," *Journal of Experimental Medicine*, vol. 204, no. 6, pp. 1463–1474, 2007.

[65] D. Vasquez-Dunddel, F. Pan, Q. Zeng et al., "STAT3 regulates arginase-i in myeloid-derived suppressor cells from cancer patients," *The Journal of Clinical Investigation*, vol. 123, no. 4, pp. 1580–1589, 2013.

[66] T. F. Greten, M. P. Manns, and F. Korangy, "Myeloid derived suppressor cells in human diseases," *International Immunopharmacology*, vol. 11, no. 7, pp. 802–807, 2011.

[67] C. M. Diaz-Montero, M. L. Salem, M. I. Nishimura, E. Garrett-Mayer, D. J. Cole, and A. J. Montero, "Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy," *Cancer Immunology, Immunotherapy*, vol. 58, no. 1, pp. 49–59, 2009.

[68] G. T. Görgün, G. Whitehill, J. L. Anderson et al., "Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans," *Blood*, vol. 121, no. 15, pp. 2975–2987, 2013.

[69] A. Sawant, J. Deshane, J. Jules et al., "Myeloid-derived suppressor cells function as novel osteoclast progenitors enhancing bone loss in breast cancer," *Cancer Research*, vol. 73, no. 2, pp. 672–682, 2013.

[70] D. I. Gabrilovich, M. P. Velders, E. M. Sotomayor, and W. M. Kast, "Mechanism of immune dysfunction in cancer mediated by immature Gr-1$^+$ myeloid cells," *The Journal of Immunology*, vol. 166, no. 9, pp. 5398–5406, 2001.

[71] Y. G. Najjar and J. H. Finke, "Clinical perspectives on targeting of myeloid derived suppressor cells in the treatment of cancer," *Frontiers in Oncology*, vol. 3, article 49, 2013.

[72] D. I. Gabrilovich, S. Ostrand-Rosenberg, and V. Bronte, "Coordinated regulation of myeloid cells by tumours," *Nature Reviews Immunology*, vol. 12, no. 4, pp. 253–268, 2012.

[73] W. Liang, M. Kujawski, J. Wu et al., "Antitumor activity of targeting Src kinases in endothelial and myeloid cell compartments of the tumor microenvironment," *Clinical Cancer Research*, vol. 16, no. 3, pp. 924–935, 2010.

[74] H. K. Le, L. Graham, E. Cha, J. K. Morales, M. H. Manjili, and H. D. Bear, "Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice," *International Immunopharmacology*, vol. 9, no. 7-8, pp. 900–909, 2009.

[75] E. Suzuki, V. Kapoor, A. S. Jassar, L. R. Kaiser, and S. M. Albelda, "Gemcitabine selectively eliminates splenic Gr-1$^+$/CD11b$^+$ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity," *Clinical Cancer Research*, vol. 11, no. 18, pp. 6713–6721, 2005.

[76] C. Melani, S. Sangaletti, F. M. Barazzetta, Z. Werb, and M. P. Colombo, "Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma," *Cancer Research*, vol. 67, no. 23, pp. 11438–11446, 2007.

[77] J. W. Pollard, "Trophic macrophages in development and disease," *Nature Reviews Immunology*, vol. 9, no. 4, pp. 259–270, 2009.

[78] T. A. Wynn, A. Chawla, and J. W. Pollard, "Macrophage biology in development, homeostasis and disease," *Nature*, vol. 496, no. 7446, pp. 445–455, 2013.

[79] J. W. Pollard, "Tumour-educated macrophages promote tumour progression and metastasis," *Nature Reviews Cancer*, vol. 4, no. 1, pp. 71–78, 2004.

[80] B. Z. Qian and J. W. Pollard, "Macrophage diversity enhances tumor progression and metastasis," *Cell*, vol. 141, no. 1, pp. 39–51, 2010.

[81] D. Rodríguez, R. Silvera, R. Carrio et al., "Tumor microenvironment profoundly modifies functional status of macrophages: peritoneal and tumor-associated macrophages are two very different subpopulations," *Cellular Immunology*, vol. 283, no. 1-2, pp. 51–60, 2013.

[82] Y. Pang, S. K. Gara, B. R. Achyut et al., "TGF-β Signaling in myeloid cells is required for tumor metastasis," *Cancer Discovery*, vol. 3, no. 8, pp. 936–951, 2013.

[83] M. R. Galdiero, E. Bonavita, I. Barajon, C. Garlanda, A. Mantovani, and S. Jaillon, "Tumor associated macrophages and neutrophils in cancer," *Immunobiology*, vol. 218, no. 11, pp. 1402–1410, 2013.

[84] Q. J. Xuan, J. X. Wang, A. Nanding et al., "Tumor-associated macrophages are correlated with tamoxifen resistance in the postmenopausal breast cancer patients," *Pathology Oncology Research*, vol. 20, no. 3, pp. 619–624, 2014.

[85] E. Y. Lin, A. V. Nguyen, R. G. Russell, and J. W. Pollard, "Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy," *Journal of Experimental Medicine*, vol. 193, no. 6, pp. 727–739, 2001.

[86] M. Groblewska, B. Mroczko, U. Wereszczyńska-Siemiatkowska, P. Myśliwiec, B. Kedra, and M. Szmitkowski, "Serum levels of granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) in pancreatic cancer patients," *Clinical Chemical Laboratory Medicine*, vol. 45, no. 1, pp. 30–34, 2007.

[87] E. Y. Lin, V. Gouon-Evans, A. V. Nguyen, and J. W. Pollard, "The macrophage growth factor CSF-1 in mammary gland development and tumor progression," *Journal of Mammary Gland Biology and Neoplasia*, vol. 7, no. 2, pp. 147–162, 2002.

[88] E. Sapi and B. M. Kacinski, "The role of CSF-1 in normal and neoplastic breast physiology," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 220, no. 1, pp. 1–8, 1999.

[89] X. D. Zhu, J. B. Zhang, P. Y. Zhuang et al., "High expression of macrophage colony-stimulating factor in peritumoral liver tissue is associated with poor survival after curative resection of hepatocellular carcinoma," *Journal of Clinical Oncology*, vol. 26, no. 16, pp. 2707–2716, 2008.

[90] M. Li, D. A. Knight, L. A. Snyder, M. J. Smyth, and T. J. Stewart, "A role for CCL2 in both tumor progression and immunosurveillance," *OncoImmunology*, vol. 2, no. 7, Article ID e25474, 2013.

[91] H. Roca, Z. S. Varcos, S. Sud, M. J. Craig, and K. J. Pienta, "CCL2 and interleukin-6 promote survival of human CD11b$^+$ peripheral blood mononuclear cells and induce M2-type macrophage polarization," *The Journal of Biological Chemistry*, vol. 284, no. 49, pp. 34342–34354, 2009.

[92] M. Ryder, R. A. Ghossein, J. C. M. Ricarte-Filho, J. A. Knauf, and J. A. Fagin, "Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer," *Endocrine-Related Cancer*, vol. 15, no. 4, pp. 1069–1074, 2008.

[93] J. J. W. Chen, Y. Lin, P. Yao et al., "Tumor-associated macrophages: the double-edged sword in cancer progression," *Journal of Clinical Oncology*, vol. 23, no. 5, pp. 953–964, 2005.

[94] C. E. Lewis and J. W. Pollard, "Distinct role of macrophages in different tumor microenvironments," *Cancer Research*, vol. 66, no. 2, pp. 605–612, 2006.

[95] S. V. Novitskiy, M. W. Pickup, A. Chytil, D. Polosukhina, P. Owens, and H. L. Moses, "Deletion of TGF-β signaling in myeloid cells enhances their anti-tumorigenic properties," *Journal of Leukocyte Biology*, vol. 92, no. 3, pp. 641–651, 2012.

[96] J. A. Hamilton and A. Achuthan, "Colony stimulating factors and myeloid cell biology in health and disease," *Trends in Immunology*, vol. 34, no. 2, pp. 81–89, 2013.

[97] S. I. Park, F. N. Soki, and L. K. Mccauley, "Roles of bone marrow cells in skeletal metastases: no longer bystanders," *Cancer Microenvironment*, vol. 4, no. 3, pp. 237–246, 2011.

[98] K. Zhang, S. Kim, V. Cremasco et al., "$CD8^+$ T cells regulate bone tumor burden independent of osteoclast resorption," *Cancer Research*, vol. 71, no. 14, pp. 4799–4808, 2011.

[99] A. C. Monteiro, A. C. Leal, T. Gonçalves-Silva et al., "T cells induce pre-metastatic osteolytic disease and help bone metastases establishment in a mouse model of metastatic breast cancer," *PLoS ONE*, vol. 8, no. 7, Article ID e68171, 2013.

[100] M. J. Smyth, J. Swann, E. Cretney, N. Zerafa, W. M. Yokoyama, and Y. Hayakawa, "NKG2D function protects the host from tumor initiation," *The Journal of Experimental Medicine*, vol. 202, no. 5, pp. 583–588, 2005.

[101] G. Liu, S. Lu, X. Wang et al., "Perturbation of NK cell peripheral homeostasis accelerates prostate carcinoma metastasis," *Journal of Clinical Investigation*, vol. 123, no. 10, pp. 4410–4422, 2013.

[102] A. Cerwenka, J. L. Baron, and L. L. Lanier, "Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 20, pp. 11521–11526, 2001.

[103] A. Diefenbach, E. R. Jensen, A. M. Jamieson, and D. H. Raulet, "Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity," *Nature*, vol. 413, no. 6852, pp. 165–171, 2001.

[104] X. Li, A. J. Koh, Z. Wang et al., "Inhibitory effects of megakaryocytic cells in prostate cancer skeletal metastasis," *Journal of Bone and Mineral Research*, vol. 26, no. 1, pp. 125–134, 2011.

[105] S. Madar, I. Goldstein, and V. Rotter, "'Cancer associated fibroblasts'—more than meets the eye," *Trends in Molecular Medicine*, vol. 19, no. 8, pp. 447–453, 2013.

[106] N. C. Direkze, K. Hodivala-Dilke, R. Jeffery et al., "Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts," *Cancer Research*, vol. 64, no. 23, pp. 8492–8495, 2004.

[107] N. Cheng, N. A. Bhowmick, A. Chytil et al., "Loss of TGf-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-, MSP- and HGF-mediated signaling networks," *Oncogene*, vol. 24, no. 32, pp. 5053–5068, 2005.

[108] A. Orimo, P. B. Gupta, D. C. Sgroi et al., "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion," *Cell*, vol. 121, no. 3, pp. 335–348, 2005.

[109] N. A. Bhowmick, A. Chytil, D. Plieth et al., "TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia," *Science*, vol. 303, no. 5659, pp. 848–851, 2004.

[110] V. R. Placencio, A. Sharif-Afshar, X. Li et al., "Stromal transforming growth factor-β signaling mediates prostatic response to androgen ablation by paracrine Wnt activity," *Cancer Research*, vol. 68, no. 12, pp. 4709–4718, 2008.

[111] P. Owens, H. Polikowsky, M. W. Pickup et al., "Bone morphogenetic proteins stimulate mammary fibroblasts to promote mammary carcinoma cell invasion," *PLoS ONE*, vol. 8, no. 6, Article ID e67533, 2013.

[112] S. M. Eck, A. L. Côté, W. D. Winkelman, and C. E. Brinckerhoff, "CXCR4 and matrix metalloproteinase-1 are elevated in breast carcinoma-associated fibroblasts and in normal mammary fibroblasts exposed to factors secreted by breast cancer cells," *Molecular Cancer Research*, vol. 7, no. 7, pp. 1033–1044, 2009.

[113] S. Kumar and V. M. Weaver, "Mechanics, malignancy, and metastasis: the force journey of a tumor cell," *Cancer and Metastasis Reviews*, vol. 28, no. 1-2, pp. 113–127, 2009.

[114] N. S. Ruppender, A. R. Merkel, T. J. Martin, G. R. Mundy, J. A. Sterling, and S. A. Guelcher, "Matrix rigidity induces osteolytic gene expression of metastatic breast cancer cells," *PLoS ONE*, vol. 5, no. 11, Article ID e15451, 2010.

[115] A. Suetsugu, Y. Osawa, M. Nagaki et al., "Imaging the recruitment of cancer-associated fibroblasts by liver-metastatic colon cancer," *Journal of Cellular Biochemistry*, vol. 112, no. 3, pp. 949–953, 2011.

[116] M. Loeffler, J. A. Krüger, A. G. Niethammer, and R. A. Reisfeld, "Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1955–1962, 2006.

[117] Y. Mao, E. T. Keller, D. H. Garfield, K. Shen, and J. Wang, "Stromal cells in tumor microenvironment and breast cancer," *Cancer and Metastasis Reviews*, vol. 32, no. 1-2, pp. 303–315, 2013.

[118] J. Tchou and J. Conejo-Garcia, "Targeting the tumor stroma as a novel treatment strategy for breast cancer: shifting from the neoplastic cell-centric to a stroma-centric paradigm," *Advances in Pharmacology*, vol. 65, pp. 45–61, 2012.

[119] U. E. Martinez-Outschoorn, A. Goldberg, Z. Lin et al., "Anti-estrogen resistance in breast cancer is induced by the tumor microenvironment and can be overcome by inhibiting mitochondrial function in epithelial cancer cells," *Cancer Biology & Therapy*, vol. 12, no. 10, pp. 924–938, 2011.

[120] A. M. Scott, G. Wiseman, S. Welt et al., "A phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer," *Clinical Cancer Research*, vol. 9, no. 5, pp. 1639–1647, 2003.

[121] R. D. Hofheinz, S. Al-Batran, F. Hartmann et al., "Stromal antigen targeting by a humanised monoclonal antibody: an early phase II trial of sibrotuzumab in patients with metastatic colorectal cancer," *Onkologie*, vol. 26, no. 1, pp. 44–48, 2003.

[122] S. Togo, U. M. Polanska, Y. Horimoto, and A. Orimo, "Carcinoma-associated fibroblasts are a promising therapeutic target," *Cancers*, vol. 5, no. 1, pp. 149–169, 2013.

[123] C. Huang, C. Suen, C. Lin et al., "Cleavage-site specificity of prolyl endopeptidase FAP investigated with a full-length protein substrate," *Journal of Biochemistry*, vol. 149, no. 6, pp. 685–692, 2011.

[124] A. M. LeBeau, W. N. Brennen, S. Aggarwal, and S. R. Denmeade, "Targeting the cancer stroma with a fibroblast activation protein-activated promelittin protoxin," *Molecular Cancer Therapeutics*, vol. 8, no. 5, pp. 1378–1386, 2009.

[125] J. K. Chan and P. Y. Lam, "Human mesenchymal stem cells and their paracrine factors for the treatment of brain tumors," *Cancer Gene Therapy*, vol. 20, no. 10, pp. 539–543, 2013.

[126] B. Liang-Kuan, Z. Nan, L. Cheng et al., "Kidney cancer cells secrete IL-8 to activate Akt and promote migration of mesenchymal stem cells," *Urologic Oncology*, vol. 32, no. 5, pp. 607–612, 2014.

[127] A. E. Karnoub, A. B. Dash, A. P. Vo et al., "Mesenchymal stem cells within tumour stroma promote breast cancer metastasis," *Nature*, vol. 449, no. 7162, pp. 557–563, 2007.

[128] C. Gasparetto, "Stem cell transplantation for multiple myeloma," *Cancer Control*, vol. 11, no. 2, pp. 119–129, 2004.

[129] I. Atsuta, S. Liu, Y. Miura et al., "Mesenchymal stem cells inhibit multiple myeloma cells via the Fas/Fas ligand pathway," *Stem Cell Research Therapy*, vol. 4, no. 5, p. 111, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 123010, 8 pages
http://dx.doi.org/10.1155/2014/123010

Review Article

NTPDase5/PCPH as a New Target in Highly Aggressive Tumors: A Systematic Review

Paula Andreghetto Bracco, Ana Paula Santin Bertoni, and Márcia Rosângela Wink

Laboratório de Biologia Celular, Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rua Sarmento Leite, Rua Sarmento Leite, No. 245, 90050-170 Porto Alegre, RS, Brazil

Correspondence should be addressed to Márcia Rosângela Wink; marciawink@yahoo.com.br

Received 26 February 2014; Accepted 18 May 2014; Published 23 June 2014

Academic Editor: Zhen Chen

The protooncogene *PCPH* was recently identified as being the ectonucleoside triphosphate diphosphohydrolase 5 (*ENTPD5*). This protooncogene is converted into an oncogene by a single base pair deletion, resulting in frame change and producing a premature stop codon, leading to a mutated protein (mt-PCPH) with only 27 kDa, which is much smaller than the original 47 kDa protein. Overexpression of the PCPH as well as the mutated PCPH increases the cellular resistance to stress and apoptosis. This is intriguing considering that the active form, that is, the oncogene, is the mutated PCPH. Several studies analyzed the expression of NTPDase5/mt-PCPH in a wide range of tumor cells and evaluated its role and mechanisms in cancer and other pathogenic processes. The main point of this review is to integrate the findings and proposed theories about the role played by NTPDase5/mt-PCPH in cancer progression, considering that these proteins have been suggested as potential early diagnostic tools and therapy targets.

1. Introduction

The ectonucleoside triphosphate diphosphohydrolase 5 (NTPDase5; EC: 3.6.1.6), also known as CD39L4 (*CD39 antigen-like 4*) [1], is an enzyme that acts mostly on diphosphate nucleosides, rather than triphosphate nucleosides. It can be secreted from mammalian cells through an aminoterminus hydrophobic domain that encodes a signal peptide sequence [2]. The CD39L4 has three potential glycosylation sites and has the capacity to form disulfide dimers; however, none of these characteristics seem to significantly contribute to the ADPase or UDPase activity, which distinguishes the CD39L4 from the other proteins in this family [3]. The NTPDase5 also differs from the other members of E-NTPDase family, as it is the only one characterized as a protooncogene, also known as PCPH [4, 5].

The *PCPH* is a highly conserved gene from yeast to humans, suggesting basic roles in eukaryotic cells function [6]. It is located on chromosomes 12 and 14 in mice and humans, respectively, and it is transformed into an oncogene, called *mt-PCPH,* through a single base pair depletion resulting in frame change and producing a premature stop codon, originating a protein with 246 amino acids (25 kDa) instead of the normal protein with 469 amino acids (47 kDa) [7].

In the first studies, it was not possible to conclude whether the PCPH had nucleotide degrading activity, since it shares the apyrase conserved regions [7]. In 2001, Páez and collaborators showed a degree of genetic similarity of 98% between the protooncogene *PCPH* and *ENTPD5* and also determined that PCPH and NTPDase5 were capable of degrading ADP and ATP (preferentially ADP) [4]. First, it was shown that extracts from stable mt-PCPH-transformed NIH3T3 cells presented GDPase activity, using in-gel activity (performed by native gel electrophoresis of the extracts and staining for inorganic phosphate) and standard GDPase assays [8]. Later, the same group, by a different experimental approach, demonstrated that NTPDase activity of mt-PCPH was undetectable *in vitro* or when tested *in situ* in living cells [9]. The authors stated that this discrepancy could be due to the production of recombinant mt-PCPH in non-human expression systems.

The *PCPH* gene was initially identified as a protooncogene, due to its frequent mutation in the 3-methylcholantrene-induced tumorigenesis process in a Syrian hamster fibroblast cell line [6]. Subsequent studies were mainly based on the hypothesis that the carcinogenic action of PCPH/NTPDase5 could modulate the cellular balance of ATP, potentially interfering with cellular functions such as cell cycle, apoptosis, autophagy, proliferation, and differentiation [5].

The major functional difference between the normal NTPDase5 and the mt-PCPH oncoprotein is that the former provides lower levels of protection against apoptotic agents, including chemotherapeutic drugs, and radiation than the latter. This role of mt-PCPH seems to be mediated by its ability to promote a Ras-independent sustained activation of the ERK pathway [7, 8, 10]. The different expression profiles of these proteins and consequent changes in the cellular resistance observed in the studies reviewed suggest that it may be involved in the survival, growth, or migratory activity of some kinds of tumors. Yet, it is not very clear how this protein, in fact, contributes to the development of the different types of neoplasias. In addition, it was observed that NTPDase5 KO mice presented an increase in cases of liver pathology and neoplasia [11], adding more doubts about how the level of its expression influences cancer development.

The aim of this work is to provide an overview, in a systematic review format, of the expression profile of the NTPDase5 and mt-PCPH in cancer cell lines and tumor samples, in comparison to healthy tissues, and describe the proposed mechanisms by which the mutated and WT proteins act in the neoplastic development.

2. Method

This review describes a "literature overview" about PCPH/NTPDase5 in all types of cancer.

2.1. Search Details. We performed an electronic search on January, 2014, for papers indexed in PubMed and Scopus database. The search strategy comprised only the medical subject heading (MeSH) term "ENTPD5 or NTPDase5." For inclusion in this review, papers had to describe any relation of PCPH/NTPDase5 to cancer. No language restriction was applied. By this search strategy, 55 papers were identified. After reviewing their abstracts, 14 eligible papers were chosen and three citations retrieved from manual search were included, providing 17 papers that examined PCPH/NTPDase5 expression status in cancer (Figure 1).

Data were extracted from each original study about PCPH/NTPDase5 gene or protein expression in normal and pathological state as well as its isoform expression patterns in tissues and tumor cells.

3. Results and Discussion

3.1. Expression Profile of ENTPDase5. *ENTPD5* is expressed at different levels in several types of normal and neoplastic tissues suggesting a tissue-specific regulation [12, 13]. Interestingly, the first studies on this subject revealed the presence of 27 kDa PCPH immune-related polypeptide (believed to be the truncated, i.e., mutated, *PCPH* oncogene) in various cell extracts of healthy epithelial tissues and the expression of 47 kDa polypeptide (believed to be the full length *PCPH* gene), in most of the tumor cell lines studied, hypothesizing that there was a potential relationship between the levels of these proteins and the neoplastic phenotype [12]. However, high levels of the normal NTPDase5 have also been identified in healthy liver and kidney tissues [13]. It is important to mention that these studies were performed with an anti-PCPH polyclonal antiserum raised in rabbits with a purified bacterial recombinant PCPH [8, 12, 13]. Considering that the PCPH/NTPDase5 shares the conserved apyrase regions [1], it is possible that this antiserum also recognizes other members of the E-NTPDase family.

Thus, it is possible to conclude that the expression of ENTPD5/mt-PCPH in tumors studied so far is highly variable, as described in Tables 1 and 2.

Breast tumors induced in mice exhibited increased expression of the normal NTPDase5 and decreased expression of the mt-PCPH when compared with healthy mammary gland, and this difference was more evident in malignant tumors in comparison to benign tumors [13]. A similar expression profile was observed when comparing neoplastic cell lines of the larynx and healthy cells from a primary culture of the larynx. A higher mt-PCPH expression was observed in the healthy cells when compared with carcinoma cells lines: UM-SSC-11, UM-SCC-12, UM-SCC-23, and SCC-81B. Interestingly, a loss of this protein expression was associated with an increase in the expression of the normal NTPDase5 [19]. In human normal and neoplastic breast samples, however, the normal NTPDase5 appeared in all tested samples both benign and malignant. Expression of mt-PCPH correlated positively with the aggressiveness of the breast carcinoma and was not detected in the benign tumors [18].

Testicular germ tumor cell lines NCCIT and NT2/D1 also presented high levels of NTPDase5 and very low levels of mt-PCPH [17]. However, most clinical specimens of germ tumor cell of 54 patients showed an increase in expression of mt-PCPH when compared with healthy adjacent tissue [17]. Additionally, the expression of mt-PCPH is increased in the precursor lesions of germ tumor cell.

Immunohistochemical analysis also identified that, in cases of laryngeal cancer and testicular germ tumor cells, the concentration of NTPDase5 is higher in areas of differentiation and neoplastic transformation, with low proliferation, than in areas with high proliferation rates, raising the hypothesis that this protein acts more in the initial processes of cancer than in well-advanced, malignant neoplastic phenotypes [17, 19].

Studies with clinical specimens of prostate cancer and prostate tumor cell lines RWPE-1, LNCaP, C4-2, and PC-3 demonstrated that NTPDase5 is not significantly expressed in healthy prostate tissue but is present in cases of benign hyperplasia and is more pronouncedly expressed in tumor samples. Also, this protein was not detected in the tumor

Table 1: NTPDase5 profile expression in tumor cell lines.

Author; year	Tumor cell line	Control group	Methods	NTPDase5 expression	mt-PCPH expression
Beckenkamp et al., 2014 [14]	Cervical cancer cell lines SiHa, HeLa, and C33A	Normal immortalized keratinocytes (HaCaT cells)	RT-PCR	Expressed in all cell lines with higher levels in SiHa cells than HeLa, C33A, and HaCaT cells	Not described
Zadran et al., 2012 [15]	Human brain tumor cell lines U87 and U87vIII	Not described	WB	Expressed in U87 and at higher levels in U87vIII	Not described
Villar et al., 2007 [16]	Human prostate tumor cells lines LNCaP, C42, and PC3	Nonneoplastic human prostatic epithelial cells (RWPE-1)	RT-PCR and WB	Not detected in RWPE-1 but it was highly detected in LNCaP and in both C42 and PC3 was expressed at lower levels	Not described
Regadera et al., 2006 [17]	Testicular germ cell tumors NCCIT (mutant Tp53) and NT2/D1 (wild-type Tp53)	Not described	WB	Both cell lines expressed several NTPDase5-immunorelated polypeptides (ranged 20–90 KDa)	Low molecular-size polypeptides were less abundant in NT2/D1 than NCCIT cells
Blánquez et al., 2004 [18]	Human benign and malignant neoplastic breast samples	Normal human breast tissue samples	WB	Both normal tissue and benign and malignant breast tumors samples showed the expression of the NTPDase5 protein	Only the more aggressive breast tumor samples expressed the mt-PCPH
Blánquez et al., 2002 [19]	Cell lines cultured from explants of laryngeal tumors (SCC) at stages II, III, and IV	Primary laryngeal epithelial cells (LECs) from the normal margin of surgical specimens	WB	Expression related directly to the evolution of the three grades of laryngeal dysplasia, characterized by increments of cell proliferation in parallel with changes in epithelial differentiation	LECs expressed more mt-PCPH than normal NTPDase5 and SCCs presented a loss in the mt-NTPDase5
Rouzaut et al., 2001 [12]	20 mammary tumor derived cell lines	Not described	WB	Detectable in 8 of the 20 cells lines	The mt-PCPH, after prolonged exposures, was detectable in all but two cell lines
Rouzaut et al., 2001 [12]	18 tumor cell lines derived from the central or peripheral nervous system	Not described	WB	Absent in 13 cell lines and barely detectable in 4 cell lines	Expressed in 13 cell lines
Rouzaut et al., 2001 [12]	6 colon tumor cell lines	Not described	WB	Expressed highly in all six cell lines	Expressed in 4 cell lines
Rouzaut et al., 2001 [12]	5 lung tumor cell lines	Not described	WB	Detectable at low levels in all five cell lines	Detectable at low levels in 4 cell lines
Rouzaut et al., 2001 [12]	1 pancreas tumor cell line	Not described	WB	Highly expressed	Detectable at low levels

Legend: U87vII: U87 glioblastoma cells transduced to express the epidermal growth factor receptor vIII; WB: Western blot.

Table 2: NTPDase5/mt-PCPH profile expression in clinical samples.

Author; year	Tissue study	Methods	Sampling size	Control group	NTPDase5 expression	mt-PCPH expression
Zadran et al., 2012 [15]	Primary glioblastoma multiforme (GBMs)	Tissue microarrays, IHC, and WB	140 patients	Adjacent normal brain	Elevated levels were observed in GBM cores when compared to adjacent normal tissues	Not described
Mikula et al., 2010 [20]	Adenocarcinomas and colonic adenomas	Mass spectrometry and qRT-PCR	5 adenocarcinomas; 12 colonic adenomas; 4 normal mucosas	Normal mucosa	Continuously downregulated in a progression from normal mucosa to adenocarcinoma	Not described
Villar et al., 2007 [16]	Prostate normal, hyperplasic, and tumor cells	IHC	63 patients	Normal human prostate	Not detected in normal prostate, detected slightly in HPB, and elevated in PIN and prostate carcinoma (samples with a *Gleason* score less *than 7* present lower levels)	Not described
Regadera et al., 2006 [17]	Testicular tumors	IHC	54 patients	Normal testicular tissue	Increased expression in testicular tumors relative to normal tissue; present in well-differentiated squamous epithelia and lost in dedifferentiated squamous cells	Not described
Blánquez et al., 2002 [19]	Laryngeal mild, moderate, and severe stages dysplastic lesions	IHC	59 patients	Normal laryngeal mucosa	Expressed at lower levels in severe than in mild dysplastic cases and at much lower levels than in the normal tissue	Not described
Blánquez et al., 2004 [18]	Human breast tumors	IHC and WB	54 patients	Normal human breast samples	Undetectable in normal and benign samples and increase in carcinoma *in situ* and more strongly in invasive ductal and lobular carcinoma	Absent in benign human breast and low molecular weight polypeptides in ductal and lobular carcinoma
Solanas et al., 2002 [13]	Rat mammary benign and malignant tumors induced	IHC and WB	35 malignant tumors; 19 benign tumors	Normal rat mammary gland	Tumor samples showed higher levels and were more expressed in the malignant tumors	Tumor samples showed decrease in the mt-NTPDase5 expression when compared with the normal tissue

Legend: HPB: hyperplasia prostate benign; PIN: prostatic intraepithelial neoplasia; IHC: immunohistochemistry; WB: Western blot.

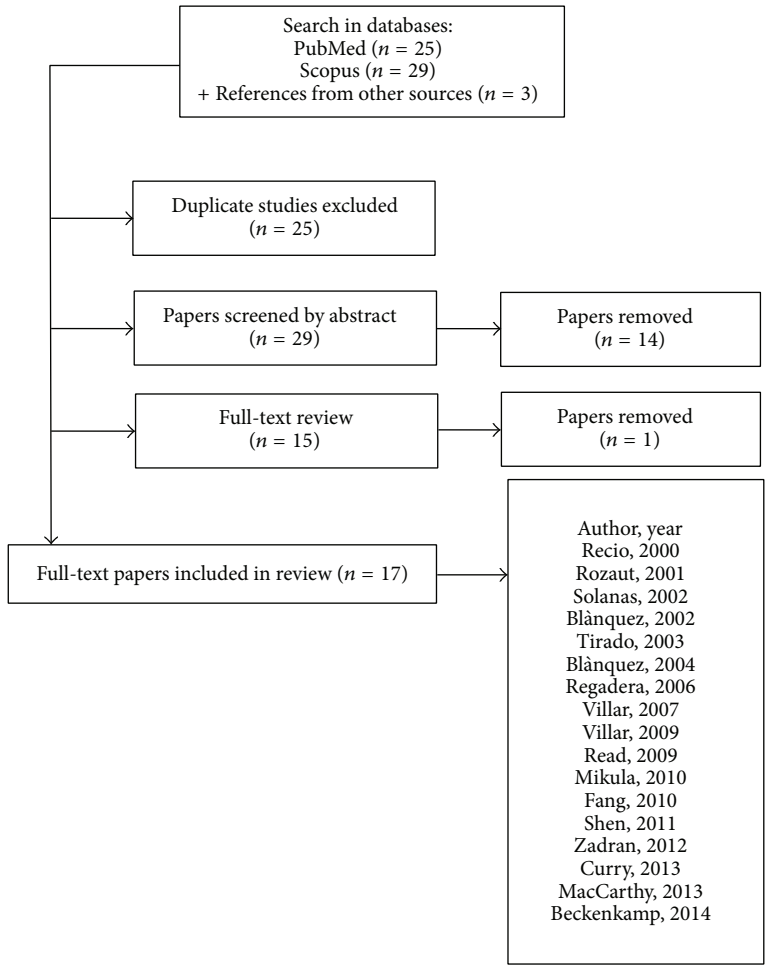


Figure 1: Methodological flow chart of the search strategy in PubMed and Scopus databases.

cell line PC-3 but was expressed in LNCaP cell line. In addition, a positive relationship between the level of NTPDase5 expression, and especially mt-PCPH expression, and the invasiveness of prostate cancer was found, thus associating the expression of the NTPDase5 more with cancer motility than with its proliferation [16].

Clinical samples of glioblastoma multiforme (GBMs) showed a higher level of NTPDase5 expression when compared to adjacent normal tissues [15]. In addition, the samples with higher expression levels of this protein were associated with significantly lower survival rates when compared to samples with lower NTPDase5 expression. However, the *NTPDase5* expression profile in individual samples of normal colon, adenomas, and colon adenocarcinomas presented that the *ENTPD5* gene expression decreased with the increase of lesion malignancy [20].

When an increase in either the NTPDase5 or the mt-PCPH expression was present, this was observed as early as nonneoplastic lesions, suggesting that the deregulation of these proteins is involved in the initial stages of neoplastic development. Thus, it is possible to consider the use of the NTPDase5 as a tool for early identification of various neoplastic cells [13, 16, 17].

Recently, cervical human cancer cells SiHa (HPV 16-positive), HeLa (HPV 18-positive), and C33A (HPV-negative) were shown to present different levels of *ENTPD5* gene expression, and the highest expression observed in SiHa cells suggests a link between *ENTPD5* and oncogenic viral proteins in cervical cancer development [14].

3.2. How NTPDase5 Acts in Cancer Progression. The neoplastic transforming activity is the role of the mt-PCPH that represents the major functional difference between the normal protooncogene and mutated active oncogene. It is supposed that this activity is due to the ability of this protein to cause a Ras-independent sustained activation of ERK1 [8].

Although the normal NTPDase5 lacks the transforming ability, it is suggested that the NTPDase5 and especially the mt-PCPH confer resistance to cells subjected to stress conditions [7]. Such resistance is conferred in part by the diphosphohydrolase activity of this enzyme, which causes a reduction in the intracellular ATP and inactivation of the stress-activated protein kinases, which is reversed by returning the intracellular ATP to physiological levels [21].

One of the cell protection mechanisms against apoptosis afforded by NTPDase5 is through inhibition of mTOR.

After cellular exposure to ionizing radiation, mTOR plays a proapoptotic role and this role is antagonized by the expression of mt-PCPH protein or by the overexpression of the normal protein NTPDase5. They are responsible for blocking the activation of mTOR and its translocation from the cytoplasm to the nucleus, preventing the phosphorylation of p53 at Ser^{18}. Phosphorylation of p53 mediates the release of cytochrome c by mitochondria and the subsequent activation of caspase 9/3, inducing the apoptosis [8]. This signaling pathway is influenced by intracellular ATP concentrations, and an increase in the expression of NTPDase5 and mt-PCPH blocks this pathway and reduces the levels of apoptosis (Figure 2).

It was also observed that the overexpression of the NTPDase5 protein and more significantly the mt-PCPH actually decreases the intracellular concentration of ATP and confers resistance not only to stress-induced apoptosis but also to those induced by chemotherapy. The overexpression of these enzymes increased resistance of prostate tumor cells when in contact with cisplatin and of colorectal carcinoma cells when in contact with oxaliplatin [9, 22].

The proposed mechanism by which NTPDase5/mt-PCPH increases the neoplastic cell resistance to cisplatin is due to the ability of this protein to prevent the dephosphorylation of the kinase PKCα induced by chemotherapy. By keeping the PKCα phosphorylated at Thr638, this protein phosphorylates and stabilizes the antiapoptotic protein Bcl-2 at Ser^{70}, making this enzyme resistant to cisplatin-induced proteasome degradation pathway, as suggested by Villar et al. [22]. Furthermore, it was observed that NTPDase5 interacts functionally with not only the PKCα but also the PKCδ protein, which was recognized as a key mediator in the NTPDase5 functions related to changes in the cell growth and invasive activity of the pancreatic tumor cells (Figure 2) [22].

Fang et al. [23] have demonstrated the involvement of the NTPDase5 also as an important link in the PI3K/PTEN loop-signaling pathway, which promotes cell growth and survival and is frequently found active in tumor cells. In this work, the authors found that PTEN knockout cells had increased expression of NTPDase5, and its overexpression is correlated with the activation of AKT, especially in cell lines of various tumor types and in primary tumor samples (Figure 2). These knockout cells also showed an increased degradation of ATP to AMP; however, as the substrates of NTPDase5 are UDP and ADP, it was found that the ATP degradation pathway occurs in the presence of the enzyme CMPK1, which removes a phosphate of ATP to phosphorylate an UMP molecule, generating UDP and ADP, respectively.

NTPDase5, by activating AKT, also plays a critical role in triggering the Warburg effect, leading to an increase in anaerobic glycolysis even in the presence of oxygen, increasing the levels of lactate and production of important macromolecules for cell proliferation, promoting angiogenesis and metastasis [23, 24]. Cells with activated PI3K/AKT pathway have a higher level of protein translation, which causes an overloading in the endoplasmic reticulum and increases the chances of a deficient folding process and consequent malformed proteins. The NTPDase5 is a protein present in the endoplasmic reticulum, and overexpression of this enzyme increases the degradation of UDP to UMP promoting protein N-glycosylation and folding, reducing the stress in the tumor cells endoplasmic reticulum [23, 24]. This mechanism allows growth factor receptors such as EGFR, IGFR, and HER-2 to be expressed and properly folded, maintaining high levels of these receptors in tumor cells. In fact, *NTPDase5* knockout in cells possessing active AKT impairs the expression and glycosylation of these receptors [23, 24].

In addition to participating in the PI3K/PTEN signaling pathway, which is overactive in approximately 90% of GBMs, NTPDase5 also plays an important role in the development of this cancer. This protein plays a modulatory role in the bioenergetics of this malignancy, increasing the catabolic efficiency of the aerobic glycolysis. In addition, when *NTPDase5* is suppressed, it causes a decrease in fatty acid oxidation and promotes an increase in the ATP influx and of the autophagic vacuoles in the cytoplasm [15].

Tumors of the respiratory system have a heterogeneity in what concerns the activation of Akt and PTEN inhibition. Those with this signaling pathway being active, however, are more resistant to treatments that involve starvation [25]. Indeed, suppression of the *NTPDase5* in lung carcinoma cells results in a decrease in Akt activity, decreased levels of IGF-IR growth factor receptor, and reduced cell proliferation under conditions of starvation, making these cells more susceptible to this type of treatment [25].

Finally, overexpression of *mt-PCPH* in colorectal carcinoma caused an increase in chemotherapy resistance [9]. The authors showed that a crucial event for this observation was a decrease in the intracellular levels of ATP [9]. However, no ATPase activity was observed in the mt-PCPH protein, probably due to a loss of conserved catalytic determinant regions in the truncated form, which may affect its tertiary structure and then the enzymatic function. This suggests that ATP degradation caused by the mutated protein may occur through interaction with other proteins and not through its NTPDase activity *per se*. Such findings can redirect all research involving the action of mt-PCPH in neoplastic processes and need to be further investigated [9].

4. Conclusions

In most of the types of cancers studied, the NTPDase5/mt-PCPH shows a change in its expression levels even in precursors of the malignant and benign lesions, which makes this protein a potential tool for early diagnosis of tumorigenesis. This enzyme has also been identified as a key element in a number of pathways known to be frequently activated in neoplastic processes and which give tumor cells a survival advantage when compared to healthy cells. In most cases, the participation of the NTPDase5/mt-PCPH occurs with a change in the intracellular ATP concentration and with participation of this enzyme in the phosphorylation and activation processes of proteins with antiapoptotic activity, conferring to the tumor cells resistance against apoptosis by stress or by chemotherapy treatments. The two main

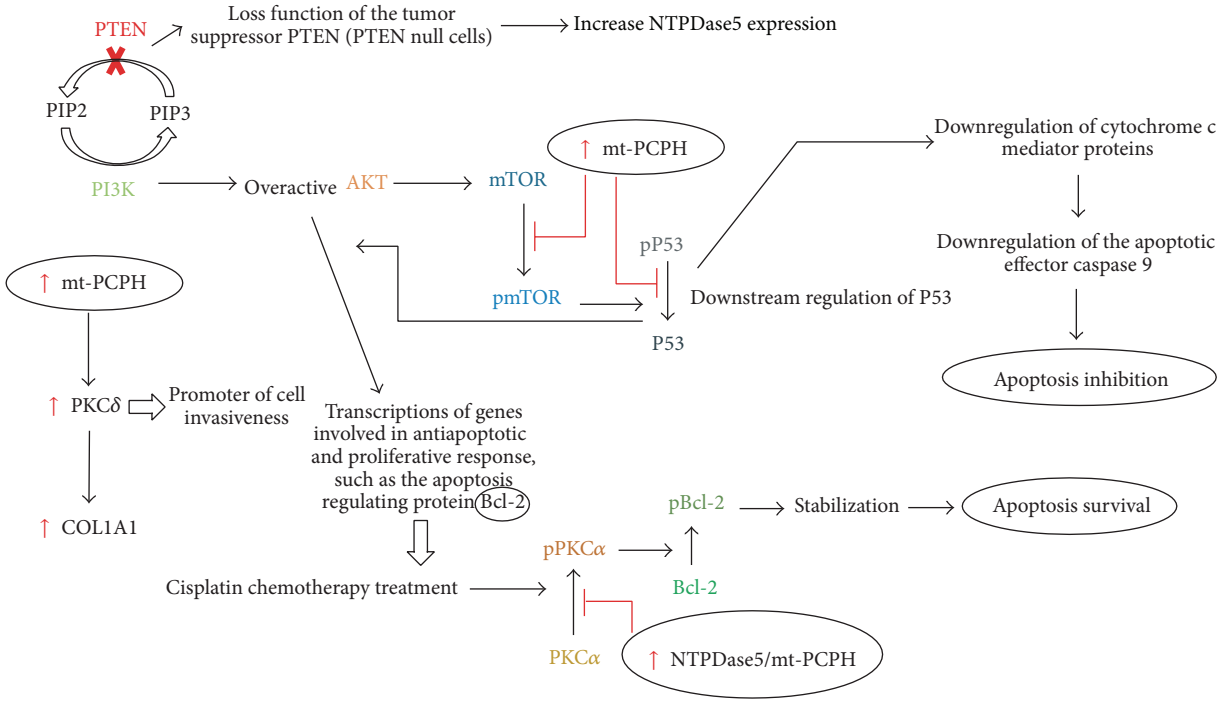


Figure 2: Integration of the proposed pathways by which the NTPDase5/mt-PCPH acts on the neoplastic progression. Due to the lack of information about the role of these proteins in cancer development and progression, this scheme presents all data published so far, not taking into consideration in which cell the proposed mechanisms were studied although it is possible that some of the contradictions presented may be a direct consequence of this fact. The figure demonstrates how the loss of the tumor suppressor PTEN possibly causes an increase in NTPDase5 expression and an overactive PI3K/AKT pathway. AKT and mTOR regulate cell growth and survival, such as Bcl-2 gene leading to an increase in apoptotic resistance. Furthermore it is also suggested that the NTPDase5 interacts with PKCδ, upregulating its levels and inducing cancer cell invasiveness. pP53, pPKCα, and pBcl-2 are the phosphorylated forms of the respective proteins and correspond to the phosphorylation of p53 at Ser^{18}, pPKC at Trh^{638}, and Bcl-2 at Ser^{70}.

pathways related to the NTPDase5/mt-PCPH activity are the mTOR and the PI3K/PTEN signaling pathways, which are directly related, since the inhibition of PTEN results in a PI3K and consequent AKT overactivation, which in turn regulates the growth of tumor cells by different signaling pathways, one being its effect on mTOR (Figure 2).

This review included all the data published so far regarding the role of the proteins NTPDase5/mt-PCPH in cancer development and progression. Due to the scarcity of studies with NTPDase5/mt-PCPH, it is difficult to establish tissue- or cell-type-specific functions. However, it seems relatively well established that *ENTPD5* and mainly *mt-PCPH* expression are related with tumorigenic transformation. The findings presented in the studies reviewed raise the idea of using NTPDase5 as a possible target for cancer treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Dr. Guido Lenz (Laboratório de Sinalização e Plasticidade Celular, UFRGS) for the critical revision of the paper and valuable corrections. Paula Andreghetto Bracco was a recipient of a Master fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior); Márcia Rosângela Wink is recipient of research fellowship from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico); Ana Paula Santin Bertoni is recipient of CAPES-PNPD fellowship. This study was supported by the Pronem-FAPERGS (Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul 11/2072-2), the CNPq (475882/2012-1), and the PROBITEC-CAPES (004/2012).

References

[1] B. P. Chadwick and A.-M. Frischauf, "The CD39-like gene family: identification of three new human members (CD39L2, CD39L3, and CD39L4), their murine homologues, and a member of the gene family from Drosophila melanogaster," *Genomics*, vol. 50, no. 3, pp. 357–367, 1998.

[2] J. J. Mulero, G. Yeung, S. T. Nelken, and J. E. Ford, "CD39-L4 is a secreted human apyrase, specific for the hydrolysis of nucleoside diphosphates," *Journal of Biological Chemistry*, vol. 274, no. 29, pp. 20064–20067, 1999.

[3] J. J. Mulero, G. Yeung, S. T. Nelken, J. M. Bright, D. W. McGowan, and J. E. Ford, "Biochemical characterization of CD39L4," *Biochemistry*, vol. 39, no. 42, pp. 12924–12928, 2000.

[4] J. G. Páez, J. A. Recio, A. Rouzaut, and V. Notario, "Identity between the PCPH proto-oncogene and the CD39L4 (ENTPD5) ectonucleoside triphosphate diphosphohydrolase gene," *International Journal of Oncology*, vol. 19, no. 6, pp. 1249–1254, 2001.

[5] J. A. Recio, N. Zambrano, L. de La Pena et al., "cDNA isolation, expression, and chromosomal localization of the mouse pcph proto-oncogene," *Molecular Carcinogenesis*, vol. 26, no. 2, pp. 130–136, 1999.

[6] J. A. Velasco, R. Castro, M. A. Avila et al., "cph, a novel oncogene which cooperates with H-ras in the transformation of NIH3T3 fibroblasts," *Oncogene*, vol. 9, no. 7, pp. 2065–2069, 1994.

[7] J. A. Velasco, M. A. Avila, and V. Notario, "The product of the cph oncogene is a truncated, nucleotide-binding protein that enhances cellular survival to stress," *Oncogene*, vol. 18, no. 3, pp. 689–701, 1999.

[8] J. A. Recio, J. G. Páez, B. Maskeri, M. Loveland, J. A. Velasco, and V. Notario, "Both normal and transforming PCPH proteins have guanosine diphosphatase activity but only the oncoprotein cooperates with ras in activating extracellular signal-regulated kinase ERK1," *Cancer Research*, vol. 60, no. 6, pp. 1720–1728, 2000.

[9] C. M. MacCarthy and V. Notario, "The ENTPD5/mt-PCPH oncoprotein is a catalytically inactive member of the ectonucleoside triphosphate diphosphohydrolase family," *International Journal of Oncology*, vol. 43, no. 4, pp. 1244–1252, 2013.

[10] O. M. Tirado, S. Mateo-Lozano, S. Sanders, L. E. Dettin, and V. Notario, "The PCPH oncoprotein antagonizes the proapoptotic role of the mammalian target of rapamycin in the response of normal fibroblasts to ionizing radiation," *Cancer Research*, vol. 63, no. 19, pp. 6290–6298, 2003.

[11] R. Read, G. Hansen, J. Kramer, R. Finch, L. Li, and P. Vogel, "Ectonucleoside triphosphate diphosphohydrolase type 5 (Entpd5)-deficient mice develop progressive hepatopathy, hepatocellular tumors, and spermatogenic arrest," *Veterinary Pathology*, vol. 46, no. 3, pp. 491–504, 2009.

[12] A. Rouzaut, J. A. Recio, and V. Notario, "Expression of the protein product of the PCPH proto-oncogene in human tumor cell lines," *Radiation Research*, vol. 155, no. 1, pp. 181–187, 2001.

[13] M. Solanas, E. Escrich, A. Rouzaut, I. Costa, A. Martínez, and V. Notario, "Deregulated expression of the PCPH proto-oncogene in rat mammary tumors induced with 7, 12-dimethylbenz[a]anthracene," *Molecular Carcinogenesis*, vol. 33, no. 4, pp. 219–227, 2002.

[14] A. Beckenkamp, D. B. Santana, A. N. Bruno et al., "Ectonucleotidase expression profile and activity in human cervical cancer cell lines," *Biochemistry and Cell Biology*, vol. 92, no. 2, pp. 95–104, 2014.

[15] S. Zadran, A. Amighi, E. Otiniano, K. Wong, and H. Zadran, "ENTPD5-mediated modulation of ATP results in altered metabolism and decreased survival in gliomablastoma multiforme," *Tumour Biology*, vol. 33, no. 6, pp. 2411–2421, 2012.

[16] J. Villar, M. I. Arenas, C. M. MacCarthy, M. J. Blánquez, O. M. Tirado, and V. Notario, "PCPH/ENTPD5 expression enhances the invasiveness of human prostate cancer cells by a protein kinase Cδ-dependent mechanism," *Cancer Research*, vol. 67, no. 22, pp. 10859–10868, 2007.

[17] J. Regadera, M. J. Blánquez, P. González-Peramato et al., "PCPH expression is an early event in the development of testicular germ cell tumors," *International Journal of Oncology*, vol. 28, no. 3, pp. 595–604, 2006.

[18] M. J. Blánquez, M. I. Arenas, I. Conde, O. M. Tirado, R. Paniagua, and V. Notario, "Deregulated expression of the PCPH proto-oncogene in human breast cancers," *International Journal of Oncology*, vol. 25, no. 4, pp. 821–830, 2004.

[19] M. J. Blánquez, J. Regadera, J. Mariño, R. E. Newman, and V. Notario, "Gradual deregulation and loss of PCPH expression in the progression of human laryngeal neoplasia," *Molecular Carcinogenesis*, vol. 35, no. 4, pp. 186–195, 2002.

[20] M. Mikula, T. Rubel, J. Karczmarski, K. Goryca, M. Dadlez, and J. Ostrowski, "Integrating proteomic and transcriptomic high-throughput surveys for search of new biomarkers of colon tumors," *Functional and Integrative Genomics*, vol. 11, no. 2, pp. 215–224, 2011.

[21] J. A. Recio, J. G. Páez, S. Sanders, T. Kawakami, and V. Notario, "Partial depletion of intracellular ATP mediates the stress-survival function of the PCPH oncoprotein," *Cancer Research*, vol. 62, no. 9, pp. 2690–2694, 2002.

[22] J. Villar, H. S. Quadri, I. Song, Y. Tomita, O. M. Tirado, and V. Notario, "PCPH/ENTPD5 expression confers to prostate cancer cells resistance against cisplatin-induced apoptosis through protein kinase Cα-mediated Bcl-2 stabilization," *Cancer Research*, vol. 69, no. 1, pp. 102–110, 2009.

[23] M. Fang, Z. Shen, S. Huang et al., "The ER UDPase ENTPD5 promotes protein N-glycosylation, the Warburg effect, and proliferation in the PTEN pathway," *Cell*, vol. 143, no. 5, pp. 711–724, 2010.

[24] Z. Shen, S. Huang, M. Fang, and X. Wang, "ENTPD5, an endoplasmic reticulum udpase, alleviates ER stress induced by protein overloading in AKT-activated cancer cells," *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 76, pp. 217–223, 2011.

[25] N. L. Curry, M. Mino-Kenudson, T. G. Oliver et al., "Pten-null tumors cohabiting the same lung display differential AKT activation and sensitivity to dietary restriction," *Cancer Discovery*, vol. 3, no. 8, pp. 908–921, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 934038, 9 pages
http://dx.doi.org/10.1155/2014/934038

Review Article

Tumor and the Microenvironment: A Chance to Reframe the Paradigm of Carcinogenesis?

Mariano Bizzarri[1] and Alessandra Cucina[2]

[1] *Department of Experimental Medicine, "Sapienza" University of Rome, Systems Biology Group, Viale Regina Elena 324, Via A. Scarpa 14, 00161 Rome, Italy*
[2] *Department of Surgery "Pietro Valdoni", "Sapienza" University of Rome, Via A. Scarpa 14, 00161 Rome, Italy*

Correspondence should be addressed to Mariano Bizzarri; mariano.bizzarri@uniroma1.it

Received 7 May 2014; Accepted 27 May 2014; Published 12 June 2014

Academic Editor: Zhen Chen

The somatic mutation theory of carcinogenesis has eventually accumulated an impressive body of shortfalls and paradoxes, as admittedly claimed by its own supporters given that the cell-based approach can hardly explain the emergence of tissue-based processes, like cancer. However, experimental data and alternatives theories developed during the last decades may actually provide a new framework on which cancer research should be reframed. Such issue may be fulfilled embracing new theoretical perspectives, taking the cells-microenvironment interplay as the privileged level of observation and assuming radically different premises as well as new methodological frameworks. Within that perspective, the tumor microenvironment cannot be merely considered akin to new "factor" to be added to an already long list of "signaling factors"; microenvironment represents the physical-biochemical support of the morphogenetic field which drives epithelial cells towards differentiation and phenotype transformation, according to rules understandable only by means of a systems biology approach. That endeavour entails three fundamental aspects: general biological premises, the level of observation (i.e., the systems to which we are looking for), and the principles of biological organization that would help in integrating and understanding experimental data.

1. Introduction: From the "Cancer Hallmarks" to the "Shortfalls" of the Somatic Mutation Theory of Carcinogenesis

Cancer is commonly thought as the result of progressive accumulation of random mutations and increased deregulation of key molecular pathways (somatic mutation theory of carcinogenesis, SMT) [1]. This statement utterly relies on a tacit premise that assumes that the pathogenetic process depends on alterations in a discrete number of signalling pathways, thought to carry "instructive" biological "information." That paradigm prompted the pharmaceutical industry to focus on development of drugs targeting specific molecular components, funding basic research, and technological applications in line with those premises, thus strengthening a bottom-up reductionist approach. Among others, Hanahan and Weinberg have been of the most prominent standard-bearers of the mainstream and they have substantially contributed in popularizing the SMT among scientists [2]. However, recently Weinberg called into question the entire theoretical construct of SMT. Quoting him, "half a century of cancer research had generated an enormous body of observations [⋯] but there were essentially *no insights into how the disease begins and progresses*" [3]. Despite the expectations raised by "the Ame's axiom ("substances act as carcinogens because they have mutagenic activity"), it shortly turned out that most powerful carcinogens are actually not mutagen"; "but fortunately—as Weinberg candidly admits—*I and others were not derailed by discrepant facts.*" Indeed, a whole series of "discrepant facts" and many other paradoxes were ignored or marginalized, while acknowledging that their realistic appraisal would have flawed the dominant paradigm [4]. Nonetheless, the search for mutated oncogenes and/or tumor suppressor continued unabated up to the present. "But even this was an *illusion*, as only became apparent years later [⋯] the identities of mutant cancer-causing genes

varied dramatically from one type of tumor to the next [· · ·]. Each tumor seemed to represent a unique experiment of nature." Rather unexpectedly, however, Weinberg concludes that, at the beginning of 2014, "we cannot really assimilate and interpret most of the data that we accumulate. How will all this play out? I wouldn't pretend to know. It's a job [· · ·] for the next generation. Passing the buck like this is an enormously liberating experience."

2. Thinking of Cancer as a Tissue-Based Disease

The core message of Weinberg's admissions is that current SMT-based cancer research provided no meaningful results to solve the cancer puzzle because it lacks a well-founded and robust theory of biological phenomena. According to Weinberg, "we lack the conceptual paradigms and computational strategies for dealing with this complexity. And equally painful, we do not know how to integrate individual data sets, such as those deriving from cancer genome analyses, with other equally important data sets, such as proteomics." Moreover, that unfortunate situation must still be investigated even more thoroughly, and the "abscess" should be incised widely if we want to get the healing. Indeed, the confusion and contradictions featuring the current state of cancer research can be ascribed to several factors, among which the absence of a true biological theory, inappropriate level on which experiments and observations are made, and associated methodological biases [5]. Biology is indeed facing a dilemma the physics has solved a century ago, that is, how to cope with an overwhelming body of data without having a general theoretical framework into which experimental results could be embedded [6]. In other words, we need to recognize general organizing principles that could enable us to frame a reliable theoretical structure to which the experimental work will be inextricably intertwined [7]. This does not imply that in biology there is no theory at all. No science can be built up in the absence of theoretical constructs. As an example, evolutionary biology offers a general theoretical structure. However, evolution is no more than a general framework within which biological processes are interpreted, for evolution does not allow specific predictions in the way quantum mechanics does in physics. Moreover, the theory of evolution itself is ongoing reinterpretations in the light of some "unexpected" and apparently contradictory data (i.e., the inheritance of acquired characters) [8, 9]. Also, during the last four decades, biology has been grounded on a reductionistic framework [10], based on the central dogma of the biology, which states that information flows unidirectionally (from DNA to proteins, from genotype to phenotype). Implicitly, form and functions in organism would depend solely on "genetic information." Such assumptions have never been presented as a theory, even worse, that model was presented as an unquestionable fact. However, this model is currently encompassing several drawbacks, grounded on conflicts with the gene-centric paradigm [11]. The overall picture is by far more complex than previously thought; biological interactions that take place at lower molecular levels are characterized by unpredictable nonlinear dynamics and become tightly influenced by higher-level organizational constraints [12]. Thereby, as we are actually unable to grasp such overwhelming complexity, we are also unable to set a reliable theory of biological organization [13].

This is especially true in the field of carcinogenesis, where both experimental modelling and theoretical framework have been for a long time dominated by the SMT [14, 15]. SMT explains cancer as a process taking place at the cellular/subcellular level of biological organization and claims that cancer is a problem of regulatory control of cell proliferation and of invasiveness, due to mutations and/or overexpression of a specific class of genes. Yet, current therapeutic approaches based on that paradigm have been proven to be ineffective in clinical cancer management, and the effect of new treatments for cancer on mortality has been largely disappointing [16], to the point that an increasing number of voices are asking to revise current treatment strategies [17, 18]. Undoubtedly, SMT has fostered a significant development of molecule-based technologies, providing so far a huge body of "raw" data on gene and proteomic networks; but, on the other hand, SMT has also generated an *impasse* in cancer studies, given that an increasing number of experimental results contradict its premises [19]. In turn, a number of ad hoc variants have been proposed [20], striving to include the tumor-microenvironment paradigm within SMT, while retaining unaltered the notion that cancer is a cell-based disease [21]. However, as "the current dominant paradigm wherein multiple genetic lesions provide both the impetus for and the Achilles heel of cancer might be inadequate to understand cancer as a disease process" [22], some radical alternatives, grounded on clearly different premises and epistemological settings, have been proposed [23, 24]. Yet, it is rather unpleasant to notice that no mention of those "alternatives" is reported in the Weinberg's paper.

An alternative to SMT is tissue organization field theory (TOFT), which posits that cancer arises from the deregulated interplay among cells (cells/stroma) and their microenvironment [25]. According to TOFT, the microenvironment represents the physical-biochemical support of the morphogenetic field which drives epithelial cells towards differentiation and phenotype transformation, according to rules understandable only by means of a systems approach [26]. Not only microenvironment-cells interplay is a matter of "signalling interaction" but also it involves biophysical factors and field-based effects, usually overlooked by the current scientific mainstream [27]. The structure of determination in TOFT includes both bottom-up and top-down as well as reciprocal causation. Moreover, a profound rewiring of the theoretical assumptions on which SMT has been based is required if one would consider the tumor microenvironment as a basis for making a new paradigm in cancer research. Such rewiring entails three fundamental aspects: (a) general biological premises, (b) the level of observation (i.e., the systems to which we are looking for), and (c) the principles of biological organization that should help in integrating and understanding experimental data. Overall, these features contribute to shape a novel biological theory that is still waiting for a cogent formulation [28]. Yet, in this respect,

SMT and TOFT differ significantly, because they rely on radically different theoretical assumptions [29].

3. Carcinogenesis Theory May Be Rebuild upon the Tumor Microenvironment Paradigm

Studies on tumor microenvironment are dating back even from 1940, when microenvironment was shown to suppress skin carcinogenesis induced by chemical carcinogens [30]. Later, even experiments done to demonstrate that a single or few mutated genes are needed to induce carcinogenesis acknowledged that microenvironmental factors were mandatorily required to promote oncogenesis at the tissue level [31, 32]. As case in point, experimental tumors obtained by inoculating cells with oncogenic virus demonstrated that the context plays a pivotal role in driving the neoplastic transformation. The tumorigenicity of polyoma virus-transformed BALB/C 3T3 cells in syngeneic mice depends on the microenvironment in which these cells were grown rather than on the content of the polyoma middle T oncogene [33]. Moreover, given that no specific genetic traits have been associated so far to the metastatic process, despite aggressive efforts to find a correlation among genome profile and cancer malignancy [34], increased attention has recently been given to the microenvironment thought to "confer" a "metastatic phenotype" [35]. This is closely linked to the "seed and soil" hypothesis, first proposed by Stephen Paget [36].

Indeed, those preliminary investigations highlighted that potent carcinogenic cues could be overridden by embryonic microenvironment [37], a finding that was recently confirmed. Namely, cancer cells cultured in an embryonic environment [38–40] or cocultured in 3D-matrices with normal human cells showed apoptosis and differentiation and eventually were reprogrammed into normal phenotypes [41, 42]. Such effects have been ascribed to undefined "signalling molecules," to morphogens, or to soluble factors provided by the morphogenetic embryonic field. Comparable results have been obtained by culturing cancer cells in 3D-matrices with normal human cells. Indeed, a matrix containing both type I collagen and reconstituted basement membrane and the presence of normal breast fibroblasts constituted the minimal permissive microenvironment to induce near-complete tumor phenotype reversion [43].

For a while, interest on immunologic [44] and angiogenic [45] aspects of tumor microenvironment overshadowed the contribution of the microenvironment on cancer initiation [46]. Yet, it is now firmly established that the microenvironment actively contributes to initiation of carcinogenesis, given that it profoundly influences tissue organization (i.e., the very shape and structure of epithelia) as well as intracellular processes in the cells within epithelial structures, like proliferation, differentiation, and apoptosis [47, 48]. Even subtle differences, in ECM composition and stiffness, selectively foster or inhibit proliferation by modulating cell cycle regulatory molecules and early response genes [49–51]. The microenvironment regulates the transcription of genes associated with differentiating pathways [52, 53] and participates in shaping cells phenotypes by modulating cell-stroma interactions and cytoskeleton architecture [54, 55]. Moreover, cell shape and microenvironmental cues trigger programmed cell death signals, hence driving cells towards apoptosis [56, 57]. Changes in the microenvironment structure or composition frequently lead to tissue fibrosis, augmented collagen crosslink, and tissue stiffening, all of which have been associated to an increased risk of developing cancer [58, 59]. It is not trivial to recall that aging is associated to increase in both tissue stiffness and cancer incidence [60]. In turn, tissue fibrosis and modification of physicochemical properties of ECM may likely influence tumor onset and progression by regulating soluble factors involved in inflammation [61] and angiogenesis [62].

Participation of the microenvironment in carcinogenesis is further supported by phenotypic changes, observed changes in stromal cells residing within the tumor microenvironment. Both cancer-associated fibroblasts [63] and endothelial cells [64] showed indeed altered architecture [65] and paracrine signals expression that ultimately lead to malignancy [66, 67] and genetic instability of the epithelial cell layer [68]. Indeed, in a seminal paper, Maffini et al. [69] demonstrated that, by treating stroma with the chemical carcinogen N-nitroso-methylurea, stromal cells may drive malignant transformation of epithelial cells by disrupting the normal stromal-epithelial interactions, whereas treating directly the epithelial cells does not lead to any cancerous transformation. Conversely, "normalizing" the behavior of "altered" stromal components of the tumor microenvironment may reduce the malignancy phenotype of tumor cells [70]. Similar results have been confirmed by others [71, 72], thereby outlining that the main factor in driving the carcinogenic process lies on the cross-talk in the microenvironment among stroma and epithelial cells.

Such results are in overt contradiction with SMT and its "oncogene" paradigm. Moreover, phenotypic traits cannot be considered a simple, linear output coded by "genomic information." Instead, phenotypic features result from the complex cross-talk among three-dimensional participants within the microenvironment. Additionally, the regulatory control exerted by the microenvironment on the emergence of tumor clones contributes to explain why "occult" tumors are prevented from progressing into overt, clinical cancer [73].

4. Physical Cues Drive Cells Differentiation and Fate

In the last decades it became clear that cell behavior is far from being "controlled" by linear (digital) "commands" but rather by complex networks of molecular interactions and biophysical cues, spanning across different levels of structural and functional organization. Not only those interactions are context-dependent and thus cannot be understood by keeping cells in an inappropriate milieu, like that provided by 2D-cultures, but also they follow nonlinear dynamics which make impractical modeling processes with more than two

variables. The switching between different stable states (representing differentiated or pathological phenotypes) requires that the activity/expression of several "signaling" molecules change in concert. Indeed, phenotype reversions are linked to the simultaneous coexpression of hundreds of different transcription factors and multiple downstream genes [74, 75]. To achieve a state-transition, no single point mutation is sufficient, and a cumulative effect linked to mutations will occur only if a critical state of the system as a whole is reached. It is worth of noting that the transition beyond that critical point "may be prevented or reversed by simultaneously manipulating a number of factors in the extracellular medium" [76]. Indeed, If multiple molecular elements must be tuned simultaneously to change cell phenotype, then it should be hypothesized that only a stimulus, outfitted with pleiotropic property, would perform that task, mainly based on stochastic fluctuations that enable transition from one attractor (phenotype) to another; that model may explain the genome-wide adaptability to environmental changes without requiring specific molecular signaling transducers [77] and why switching in between different cell fates can be triggered by changes in extracellular matrix structure, by inducing cell shape modification, and by adding aspecific chemical substances, electrical ion flows, and magnetic or gravitational fields [78–80]. Overall, those factors shared a meaningful property, given that they are able to modify the morphogenetic field and the biomechanical features of the systems [81].

Cells sense and respond to external physical forces and changes in matrix mechanics by modulating their endogenous cytoskeletal contractility. For instance, the mechanosensitivity of cells lies on the delicate force balance between the endogenous cytoskeletal contractility and external mechanical forces transmitted across the cell-ECM adhesions [82]. The force balance is transmitted across the mechanical continuum of ECM-integrin-CSK, which regulates integrin-mediated adhesion sites (such as FAK and Src signaling), providing the mechanical linkage between the ECM and the actin CSK. Exposure of cells to mechanical strain, fluid shear stress, or plating cells on substrates with varying elastic moduli activates integrins, which promote recruitment of scaffold and signaling proteins to strengthen adhesions and to transmit biochemical signals into the cell. These mechanotransduction pathways establish positive feedback loops in which integrin engagement activates actomyosin CSK contractility, which in turn reinforces adhesions. Thus, the level of CSK contractility generated inside the cell is directly proportional to the adhesion strength and the matrix elastic modulus and dictates their cellular responses [83]. Moreover, the way a cell senses and responds to a biochemical input mainly depends on the *physical state* of both cells and their microenvironment. For instance, TGFβ-1 exerts a "dual" role on cancer cells, and that paradoxical behavior is well recognized as a challenging enigma that, still now, classic molecular biology has not been able to elucidate [84–87]. To the contrary, by referring data to a higher level of observation, that is, when the cell-microenvironment interaction or the tissue levels are kept in consideration, conflicting results end up as such, and paradoxes may likely find a compelling explanation. Indeed, soluble factors like TGFβ-1 may trigger opposite outputs depending on the tissue stiffness; under mechanically unloaded conditions (floating matrices), TGFβ-1 stimulated contraction directly as an agonist and indirectly by preactivating cells to express the myofibroblast phenotype, whereas, under mechanically loaded conditions (stressed matrices), TGFβ-1 had no direct agonist effect on contraction [88].

Physical and biochemical changes occurring within the microenvironment are transmitted from the cytoskeleton to the nucleoskeleton, thus enabling the selective unfolding of chromatin [89]. The DNA is enveloped in histone proteins to form strand, further wrapped and folded. Gene switching (on/off) can proceed properly only if the appropriate section of chromatin is unpacked and exposed to the enzyme machinery. This physical rearrangement of the chromatin is mainly dependent on the tensional forces perceived by the cell-microenvironment system and further transmitted across the focal adhesion along the cytonucleoskeleton to the cell biochemical/genetic machinery. Therefore, different cytoskeleton arrangements end up in activating different gene sequences, leading to triggering different biochemical pathways [90]. The balance between tensional forces and the cytoskeleton architecture modulates thereupon several complex cell functions like apoptosis, differentiation, proliferation, and ECM remodeling among others. That model can help in understanding the "dual" role displayed by a lot of "signaling molecules," selective sensitivity to drugs [91], and why cancer cell behavior may proceed regardless of their "mutated" genes [92]. That is precisely what means "to put the gene in a context," given that cell responses to molecular "signals" tightly lie on the response of individual cells to mechanical tension and to the specific microenvironment in which cells are embedded. To date, an overwhelming body of data has revealed that mechanical tension generated through molecular interactions within the cytoskeleton is indeed critical for modulating molecular activity [93, 94] and to dramatically influence cell form and function [95]. In turn, interactions between epithelial cells and microenvironmental components (namely, stromal cells) change ECM composition as well as its biochemical-biophysical features [96].

Experimental results have provided compelling evidence of the key role played by the microenvironment in cancer initiation. Despite the presence of "growth factor," normal cells cannot grow when they are free of adhesion to ECM [97], or if they are compressed into specific geometric space (i.e., only along a thin epithelial monolayer) [98]. Similarly, stimulated breast cancer cells cease to grow when are detached to their substrate in a microgravity field [99]. Therefore, an increase in "signaling molecules" alone cannot explain cell growth induction, given that physical interaction with the microenvironment enables cells to respond to soluble factors or genetic inputs. Even in autosomal dominant tumor predisposition syndromes, like neurofibromatosis-1 (NF-1), NF-1 inactivation results in increased astrocyte growth, but the augmented proliferation rate is actually unable to induce glioma formation [100]. To observe tumor formation in vivo, brain microglia carrying NF-1 heterozygosity are needed. In that model, microenvironmental components drive the epithelial transformation, mainly by providing

disruption of ECM integrity (through the enhanced release of hyaluronidase) and subsequent activation of the MAPK-pathway. As expected, inhibition of hyaluronidase release or microglia activation dramatically reduces mouse optical glioma proliferation in vivo [101].

Overall, those results highlight how the microenvironment, mainly through its physical components, participates in promoting and shaping the carcinogenic process that can be considered as a "development gone awry" [102]. As recently recognized, "the physical laws and principles that define the behaviour of matter are essential for developing an understanding of the initiation and progression of cancer," thus providing "opportunities for new insights into long-lasting problems in cancer research" [103]. This premise, well-grounded on experimental basis, represents another discontinuity point with respect to SMT which posits that "biological-information" carried out by genes constitutes the only (or the main) causative factor in driving cellular fate and behavior.

5. Microenvironment and Cancer: Methodological Issues

The term "microenvironment" encompasses discrete, interacting elements, such as extracellular matrix (ECM), stromal cells, molecular diffusible factors, configuration of the cell-stroma architecture [104], nonlocal control through field's forces [105], and topologic geometry of the emerging tissue [106]. In order to grasp such overwhelming complexity of interactions, we adopted a radical new perspective, which considers the interplay of both biochemical (proteomic, genetic, and metabolic) and biophysical (stiffness and surface tension) factors operating at different levels [107]. In other words, to understand tissue level phenomena, it is necessary to study the tissue and not single pathway in cells isolated from their tissue environment. The radical change in theoretical perspective requires a shift from the gene-centric paradigm to the cell-microenvironment system [108], a concept introduced as late as 1962 by Smithers [109], claiming a tissue-based "quality," challenging the Boveri's prevailing view of cancer as a cell-based disease. This means that we must change the "level" of observation, by positioning ourselves where the process we are looking for really happens. Thus, "to understand the whole, one must study the whole" [110]; and if one wishes to study a tissue-based disease, like cancer, one must study the tissue.

This paradigmatic switch has important theoretical and methodological implications. First, cell function and behavior cannot longer be studied in isolation, that is, without taking into consideration their three-dimensional microenvironment. Two-dimensional cultures can be viewed for many aspects as true "artifacts," which often makes them unreliable predictors of gene expression, tissue structure, cellular functions, and behaviour [111]. Moreover, also the actual response to many drugs is remarkably flawed by 2D-environment-based experiments [112]; instead, 3D cellular models have the potential to become a fundamental research tool in biology [113], given that they allow the integration of data generated by investigations carried out at different levels. This will result in models of tissues and organisms with enhanced predictive power [114].

Second, tissue and cytoskeleton/nucleoskeleton architecture, as well as mechanical forces (stiffness, shear stress [115], and surface tension), must be adequately weighted and investigated, a rather unusual request for a "traditional" biologist [116].

Third, molecular and genetic changes, involving both the epithelial and the stromal cells, should therefore be investigated in association and linked to the observed modification of the context.

Although much has been learned about molecular components and subcellular processes, the integration of data and models across a wide range of spatial and temporal scales, taking us from observations at the cellular or subcellular level to understand tissue level phenomena, remains an unchartered territory. Moreover, biophysical influences on cell behavior and differentiation can be adequately appreciated only by studying cells in their three-dimensional context and are therefore disregarded by current experimental methodologies almost fully based on 2D cultures. Overall, these considerations highlight another fundamental bias of modern biology, that is, the lack of a general theory for understanding biological organization. In order to cope with the increasingly appreciated complexity of living organism, implicitly, biologists have adopted a reductive approach, mainly based on a gene-centric paradigm, where causative processes are modelled according to a simplified, linear dynamics. However, reality is far more complex than the biochemical diagrams we are asked to trust. Biological complexity entails nonlinear dynamics, stochastic gene expression, interactions between biochemical and biophysical factors, and events acting simultaneously at different levels. From molecules to organs, levels are interrelated and interdependent, so that the organism is able to conserve and adapt the integrity of its structural and functional organization against a back-drop of continuous changes within the organism and its environment. That feature represents the updated interpretation of homeostasis, a concept formulated a century ago by W. Cannon and currently reinterpreted as autoconservation [117], functional stability [118], evolvability, or robustness [119]. Given that homeostasis is dramatically threatened or even disrupted in the course of several diseases, to understand such processes we are obligatory required to apply methodologies that explore nonlinear spatiotemporal systems with multiple levels of structural and functional organization. As pointedly discussed by Noble [120], one cannot understand the physiology or the pathology of cardiac rhythm by only referring to the gene expression and to the features of a single cardiomyocite. Similarly one cannot understand pathologic processes emerging at the cell-microenvironment level by only referring to "abstract" gene-regulatory circuits in the isolated cell.

6. Conclusions

The interaction between cells and their microenvironment, by involving both biochemical and biophysical cues, drives

differentiating processes and contributes in a determinant fashion to cancer emergence. Considering cancer as a tissue-based phenomenon implies a profound rewiring of our experimental methodology, by requiring to move from cells and subcellular structures toward higher levels of organization. Namely, experiments should be undertaken in order to verify how to modify microenvironment biophysical features by means of chemical/pharmacological means in order to prevent or eventually to induce cancer reprogramming in 3D settings or on animal models.

Choosing between competing premises and testing alternative theoretical hypothesis have been the core component of the experimental science since the Renaissance. However, as Kuhn [121] has taught us, a widely accepted paradigm will hardly be dropped before a considerable amount of paradoxes and contradictions has been resolved. Such moment seems to have come. Indeed, the somatic mutation theory has eventually accumulated an impressive body of shortfalls and paradoxes, as admittedly claimed by its own supporters [2], as a cell-based approach can hardly explain the emergence of tissue-based processes, like cancer. However, as Niels Bohr once said "How wonderful that we have met with a paradox. Now we have some hope of making progress" [122]. Nowadays, that progress may be disclosed embracing new theoretical perspectives, taking the cells-microenvironment interplay as the privileged level of observation and assuming radically different premises as well as methodological frameworks [19, 123]. This may probably not be enough, and even new theories could prove to be incomplete. Yet, as once stated by Tzu thousands years ago, a new path, however long it may be, always begins with the first leap [124].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] F. Michor, Y. Iwasa, and M. A. Nowak, "Dynamics of cancer progression," *Nature Reviews Cancer*, vol. 4, no. 3, pp. 197–205, 2004.

[2] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell*, vol. 144, no. 5, pp. 646–674, 2011.

[3] R. A. Weinberg, "Coming full circle-from endless complexity to simplicity and back again," *Cell*, vol. 157, no. 1, pp. 267–271, 2014.

[4] S. G. Baker and B. S. Kramer, "Paradoxes in carcinogenesis: new opportunities for research directions," *BMC Cancer*, vol. 7, article 151, 2007.

[5] O. Wolkenhauer and S. Green, "The search for organizing principles as a cure against reductionism in systems medicine," *The FEBS Journal*, vol. 280, no. 23, pp. 5938–5948, 2013.

[6] D. Noble, "Foreword," in *Perspectives on Organisms. Biological Time, Symmetries, Singularities*, G. Longo and M. Montévil, Eds., p. 7, Springer, New York, NY, USA, 2013.

[7] D. Noble, "Biophysics and systems biology," *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, vol. 368, no. 1914, pp. 1125–1139, 2010.

[8] E. Jablonka and M. J. Lamb, *Evolution in Four Dimensions: Genetic, Epigenetic, Behavioral, and Symbolic Variation in the History of Life*, The MIT Press, Cambridge, Mass, USA, 2005.

[9] T. E. Dickins and Q. Rahman, "The extended evolutionary synthesis and the role of soft inheritance in evolution," *Proceedings of the Royal Society B: Biological Sciences*, vol. 279, no. 1740, pp. 2913–2921, 2012.

[10] A. Cornish-Bowden, "Systems biology—how far has it come?" *Biochemist*, vol. 33, pp. 16–18, 2011.

[11] A. M. Soto and C. Sonnenschein, "One hundred years of somatic mutation theory of carcinogenesis: is it time to switch?" *BioEssays*, vol. 36, no. 1, pp. 118–120, 2014.

[12] S. Dinicola, F. D'Anselmi, A. Pasqualato et al., "A systems biology approach to cancer: fractals, attractors, and nonlinear dynamics," *OMICS*, vol. 15, no. 3, pp. 93–104, 2011.

[13] G. Longo and M. Montévil, *Perspectives on Organisms. Biological Time, Symmetries, Singularities*, Edited by: G. Longo and M. Montévil, Springer, New York, NY, USA, 2013.

[14] M. Bizzarri, A. Cucina, F. Conti, and F. D'Anselmi, "Beyond the oncogene paradigm: understanding complexity in cancerogenesis," *Acta Biotheoretica*, vol. 56, no. 3, pp. 173–196, 2008.

[15] K. Saetzler, C. Sonnenschein, and A. M. Soto, "Systems biology beyond networks: generating order from disorder through self-organization," *Seminars in Cancer Biology*, vol. 21, no. 3, pp. 165–174, 2011.

[16] J. C. Bailar III and H. L. Gornik, "Cancer undefeated," *The New England Journal of Medicine*, vol. 336, no. 22, pp. 1569–1574, 1997.

[17] R. B. Ness, "Fear of failure: why American science is not winning the war on cancer," *Annals of Epidemiology*, vol. 20, no. 2, pp. 89–91, 2010.

[18] D. Hanahan, "Rethinking the war on cancer," *The Lancet*, vol. 383, no. 9916, pp. 558–563, 2014.

[19] S. G. Backer, "Recognizing paradigm instability in theories of carcinogenesis," *British Journal of Medicine & Medical Research*, vol. 4, no. 5, pp. 1149–1163, 2014.

[20] R. Sager, "Expression genetics in cancer: shifting the focus from DNA to RNA," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 3, pp. 952–955, 1997.

[21] C. Sonnenschein, A. M. Soto, A. Rangarajan, and P. Kulkarni, "Competing views on cancer," *Journal of Biosciences*, vol. 39, no. 2, pp. 281–302, 2014.

[22] C. C. Park, M. J. Bissell, and M. H. Barcellos-Hoff, "The influence of the microenvironment on the malignant phenotype," *Molecular Medicine Today*, vol. 6, no. 8, pp. 324–329, 2000.

[23] C. Sonnenschein and A. M. Soto, *The Society of Cells: Cancer and Control of Cell Proliferation*, Springer, New York, NY, USA, 1999.

[24] G. L. G. Miklos, "The human cancer genome project—one more misstep in the war on cancer," *Nature Biotechnology*, vol. 23, no. 5, pp. 535–537, 2005.

[25] C. Sonnenschein and A. M. Soto, "The death of the cancer cell," *Cancer Research*, vol. 71, no. 13, pp. 4334–4337, 2011.

[26] A. M. Soto and C. Sonnenschein, "Is systems biology a promising approach to resolve controversies in cancer research?" *Cancer Cell International*, vol. 12, no. 1, article 12, 2012.

[27] M. Bizzarri, A. Pasqualato, A. Cucina, and V. Pasta, "Physical forces and non linear dynamics mould fractal cell shape: quantitative morphological parameters and cell phenotype," *Histology and Histopathology*, vol. 28, no. 2, pp. 155–174, 2013.

[28] J. Loscalzo and A.-L. Barabasi, "Systems biology and the future of medicine," *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, vol. 3, no. 6, pp. 619–627, 2011.

[29] A. M. Soto and C. Sonnenschein, "The tissue organization field theory of cancer: a testable replacement for the somatic mutation theory," *BioEssays*, vol. 33, no. 5, pp. 332–340, 2011.

[30] I. Berenblum and P. Shubik, "An experimental study of the initiating state of carcinogenesis and a re-examination of the somatic cell mutation theory of cancer," *British Journal of Cancer*, vol. 3, no. 1, pp. 109–118, 1949.

[31] M. J. Bissell, C. Hatie, and M. Calvin, "Is the product of the src gene a promoter?" *Proceedings of the National Academy of Sciences of the United States of America*, vol. 76, no. 1, pp. 348–352, 1979.

[32] M. H. Barcellos-Hoff, "Stromal mediation of radiation carcinogenesis," *Journal of Mammary Gland Biology and Neoplasia*, vol. 15, no. 4, pp. 381–387, 2010.

[33] E. Halachmi and I. P. Witz, "Differential tumorigenicity of 3T3 cells transformed *in vitro* with polyoma virus and *in vivo* selection for high tumorigenicity," *Cancer Research*, vol. 49, no. 9, pp. 2383–2389, 1989.

[34] B. Vogelstein and K. W. Kinzler, "Cancer genes and the pathways they control," *Nature Medicine*, vol. 10, no. 8, pp. 789–799, 2004.

[35] G. P. Gupta and J. Massague, "Cancer metastasis: building a framework," *Cell*, vol. 127, no. 4, pp. 679–695, 2006.

[36] S. Paget, "The distribution of secondary growths in cancer of the breast," *The Lancet*, vol. 133, no. 3421, pp. 571–573, 1889.

[37] G. B. Pierce and C. Wallace, "Differentiation of malignant to benign cells," *Cancer Research*, vol. 31, no. 2, pp. 127–134, 1971.

[38] F. D'Anselmi, M. G. Masiello, A. Cucina et al., "Microenvironment promotes tumor cell reprogramming in human breast cancer cell lines," *PLoS ONE*, vol. 8, no. 12, Article ID e83770, 2013.

[39] M. J. Hendrix, E. A. Seftor, R. E. Seftor, J. Kasemeier-Kulesa, P. M. Kulesa, and L. M. Postovit, "Reprogramming metastatic tumour cells with embryonic microenvironments," *Nature Reviews Cancer*, vol. 7, no. 4, pp. 246–255, 2007.

[40] M. Bizzarri, A. Cucina, P. M. Biava et al., "Embryonic morphogenetic field induces phenotypic reversion in cancer cells. Review article," *Current Pharmaceutical Biotechnology*, vol. 12, no. 2, pp. 243–253, 2011.

[41] M. J. Bissell, P. A. Kenny, and D. C. Radisky, "Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes," *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 70, pp. 343–356, 2005.

[42] P. A. Kenny and M. J. Bissell, "Tumor reversion: correction of malignant behavior by microenvironmental cues," *International Journal of Cancer*, vol. 107, no. 5, pp. 688–695, 2003.

[43] S. Krause, M. V. Maffini, A. M. Soto, and C. Sonnenschein, "The microenvironment determines the breast cancer cells'phenotype: organization of MCF7 cells in 3D cultures," *BMC Cancer*, vol. 10, article 263, 2010.

[44] I. P. Witz and M. G. Hanna Jr., *In Situ Expression of Tumor Immunity*, vol. 10 of *Contemporary Topics in Immunobiology*, Plenum Press, New York, NY, USA, 1980.

[45] J. Folkman, E. Merler, C. Abernathy, and G. Williams, "Isolation of a tumor factor responsible or angiogenesis," *The Journal of Experimental Medicine*, vol. 133, no. 2, pp. 275–288, 1971.

[46] I. P. Witz, "The tumor microenvironment: the making of a paradigm," *Cancer Microenvironment*, vol. 2, no. 1, supplement, pp. S9–S17, 2009.

[47] M. J. Bissell and M. H. Barcellos-Hoff, "The influence of extracellular matrix on gene expression: is structure the message?" *Journal of Cell Science. Supplement*, vol. 8, pp. 327–343, 1987.

[48] A. van den Hooff, "Stromal involvement in malignant growth," *Advances in Cancer Research*, vol. 50, pp. 159–196, 1988.

[49] B. Rana, D. Mischoulon, Y. Xie, N. L. Bucher, and S. R. Farmer, "Cell-extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes: reciprocal expression of C/EBP alpha and immediate-early growth response transcription factors," *Molecular and Cellular Biology*, vol. 14, no. 9, pp. 5858–5869, 1994.

[50] S. Huang, C. S. Chen, and D. E. Ingber, "Control of cyclin D1, $p27^{Kip1}$, and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension," *Molecular Biology of the Cell*, vol. 9, no. 11, pp. 3179–3193, 1998.

[51] L. E. Dike and D. E. Ingber, "Integrin-dependent induction of early growth response genes in capillary endothelial cells," *Journal of Cell Science*, vol. 109, part 12, pp. 2855–2863, 1996.

[52] C. D. Roskelley, P. Y. Desprez, and M. J. Bissell, "Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 26, pp. 12378–12382, 1994.

[53] N. Boudreau, C. Myers, and M. J. Bissell, "From laminin to lamin: regulation of tissue-specific gene expression by the ECM," *Trends in Cell Biology*, vol. 5, no. 1, pp. 1–4, 1995.

[54] C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides, and D. E. Ingber, "Geometric control of cell life and death," *Science*, vol. 276, no. 5317, pp. 1425–1428, 1997.

[55] M. J. Paszek, N. Zahir, K. R. Johnson et al., "Tensional homeostasis and the malignant phenotype," *Cancer Cell*, vol. 8, no. 3, pp. 241–254, 2005.

[56] J. E. Meredith Jr., B. Fazeli, and M. A. Schwartz, "The extracellular matrix as a cell survival factor," *Molecular Biology of the Cell*, vol. 4, no. 9, pp. 953–961, 1993.

[57] N. Boudreau, C. J. Sympson, Z. Werb, and M. J. Bissell, "Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix," *Science*, vol. 267, no. 5199, pp. 891–893, 1995.

[58] C. G. Colpaert, P. B. Vermeulen, S. B. Fox, A. L. Harris, L. Y. Dirix, and E. A. van Marck, "The presence of a fibrotic focus in invasive breast carcinoma correlates with the expression of carbonic anhydrase IX and is a marker of hypoxia and poor prognosis," *Breast Cancer Research and Treatment*, vol. 81, no. 2, pp. 137–147, 2003.

[59] L. J. Martin and N. F. Boyd, "Mammographic density. Potential mechanisms of breast cancer risk associated with mammographic density: hypotheses based on epidemiological evidence," *Breast Cancer Research*, vol. 10, no. 1, article 201, 2008.

[60] K. M. Szauter, T. Cao, C. D. Boyd, and K. Csiszar, "Lysyl oxidase in development, aging and pathologies of the skin," *Pathologie Biologie*, vol. 53, no. 7, pp. 448–456, 2005.

[61] F. Balkwill, K. A. Charles, and A. Mantovani, "Smoldering and polarized inflammation in the initiation and promotion of malignant disease," *Cancer Cell*, vol. 7, no. 3, pp. 211–217, 2005.

[62] L. M. Coussens, W. W. Raymond, G. Bergers et al., "Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis," *Genes & Development*, vol. 13, no. 11, pp. 1382–1397, 1999.

[63] M. Hu, J. Yao, L. Cai et al., "Distinct epigenetic changes in the stromal cells of breast cancers," *Nature Genetics*, vol. 37, no. 8, pp. 899–905, 2005.

[64] B. S. Croix, C. Rago, V. Velculescu et al., "Genes expressed in human tumor endothelium," *Science*, vol. 289, no. 5482, pp. 1197–1202, 2000.

[65] G. Giannelli, L. Milillo, F. Marinosci, L. L. Muzio, R. Serpico, and S. Antonaci, "Altered expression of integrins and basement membrane proteins in malignant and pre-malignant lesions of oralmucosa," *Journal of Biological Regulators and Homeostatic Agents*, vol. 15, no. 4, pp. 375–380, 2001.

[66] L. W. Chung, A. Baseman, V. Assikis, and H. E. Zhau, "Molecular insights into prostate cancer progression: the missing link of tumor microenvironment," *The Journal of Urology*, vol. 173, no. 1, pp. 10–20, 2005.

[67] L. W. Chung, S.-M. Chang, C. Bell, H. Zhau, J. Y. Ro, and A. C. von Eschenbach, "Prostatic carcinogenesis evoked by cellular interaction," *Environmental Health Perspectives*, vol. 77, pp. 23–28, 1988.

[68] R. S. Bindra and P. M. Glazer, "Genetic instability and the tumor microenvironment: towards the concept of microenvironment-induced mutagenesis," *Mutation Research*, vol. 569, no. 1-2, pp. 75–85, 2005.

[69] M. V. Maffini, A. M. Soto, J. M. Calabro, A. A. Ucci, and C. Sonnenschein, "The stroma as a crucial target in rat mammary gland carcinogenesis," *Journal of Cell Science*, vol. 117, part 8, pp. 1495–1502, 2004.

[70] S. Vosseler, N. Mirancea, P. Bohlen, M. M. Mueller, and N. E. Fusenig, "Angiogenesis inhibition by vascular endothelial growth factor receptor-2 blockade reduces stromal matrix metalloproteinase expression, normalizes stromal tissue, and reverts epithelial tumor phenotype in surface heterotransplants," *Cancer Research*, vol. 65, no. 4, pp. 1294–1305, 2005.

[71] M. H. Barcellos-Hoff and S. A. Ravani, "Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells," *Cancer Research*, vol. 60, no. 5, pp. 1254–1260, 2000.

[72] D. H. Nguyen, H. A. Oketch-Rabah, I. Illa-Bochaca et al., "Radiation acts on the microenvironment to affect breast carcinogenesis by distinct mechanisms that decrease cancer latency and affect tumor type," *Cancer Cell*, vol. 19, no. 5, pp. 640–651, 2011.

[73] M. J. Bissell and W. C. Hines, "Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression," *Nature Medicine*, vol. 17, no. 3, pp. 320–329, 2011.

[74] D. Fambrough, K. McClure, A. Kazlauskas, and E. S. Lander, "Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes," *Cell*, vol. 97, no. 6, pp. 727–741, 1999.

[75] M. Wernig, A. Meissner, R. Foreman et al., "*In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state," *Nature*, vol. 448, no. 7151, pp. 318–324, 2007.

[76] K. Qu, A. Abi Haidar, J. Fan et al., "Cancer onset and progression: a genome-wide, nonlinear dynamical systems perspective on onconetworks," *Journal of Theoretical Biology*, vol. 246, no. 2, pp. 234–244, 2007.

[77] A. Kashiwagi, I. Urabe, K. Kaneko, and T. Yomo, "Adaptive response of a gene network to environmental changes by fitness-induced attractor selection," *PLoS ONE*, vol. 1, no. 1, article e49, 2006.

[78] M. Levin and C. G. Stevenson, "Regulation of cell behavior and tissue patterning by bioelectrical signals: challenges and opportunities for biomedical engineering," *Annual Review of Biomedical Engineering*, vol. 14, pp. 295–323, 2012.

[79] M. Bizzarri, A. Cucina, A. Palombo, and M. G. Masiello, "Gravity sensing by cells: mechanisms and theoretical grounds," *Rendiconti Lincei: Scienze Fisiche e Naturali*, vol. 25, no. 1, supplement, pp. S29–S38, 2014.

[80] M. Levin, "Reprogramming cells and tissue patterning via bioelectrical pathways: molecular mechanisms and biomedical opportunities," *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, vol. 5, no. 6, pp. 657–676, 2013.

[81] M. Levin, "Morphogenetic fields in embryogenesis, regeneration, and cancer: non-local control of complex patterning," *Biosystems*, vol. 109, no. 3, pp. 243–261, 2012.

[82] S.-Y. Tee, J. Fu, C. S. Chen, and P. A. Janmey, "Cell shape and substrate rigidity both regulate cell stiffness," *Biophysical Journal*, vol. 100, no. 5, pp. L25–L27, 2011.

[83] M. E. Chicurel, C. S. Chen, and D. E. Ingber, "Cellular control lies in the balance of forces," *Current Opinion in Cell Biology*, vol. 10, no. 2, pp. 232–239, 1998.

[84] R. Derynck, R. J. Akhurst, and A. Balmain, "TGF-β signaling in tumor suppression and cancer progression," *Nature Genetics*, vol. 29, no. 2, pp. 117–129, 2001.

[85] H. L. Moses, E. Y. Yang, and J. A. Pietenpol, "TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights," *Cell*, vol. 63, no. 2, pp. 245–247, 1990.

[86] M. Deckers, M. van Dinther, J. Buijs et al., "The tumor suppressor Smad4 is required for transforming growth factor β-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells," *Cancer Research*, vol. 66, no. 4, pp. 2202–2209, 2006.

[87] B. Bierie and H. L. Moses, "Tumour microenvironment: TGFβ: the molecular Jekyll and Hyde of cancer," *Nature Reviews Cancer*, vol. 6, no. 7, pp. 506–520, 2006.

[88] F. Grinnell and C. H. Ho, "Transforming growth factor β stimulates fibroblast-collagen matrix contraction by different mechanisms in mechanically loaded and unloaded matrices," *Experimental Cell Research*, vol. 273, no. 2, pp. 248–255, 2002.

[89] E. Fedorova and D. Zink, "Nuclear architecture and gene regulation," *Biochimica et Biophysica Acta*, vol. 1783, no. 11, pp. 2174–2184, 2008.

[90] A. Mammoto and D. E. Ingber, "Cytoskeletal control of growth and cell fate switching," *Current Opinion in Cell Biology*, vol. 21, no. 6, pp. 864–870, 2009.

[91] J. Feng, Y. Tang, Y. Xu, Q. Sun, F. Liao, and D. Han, "Substrate stiffness influences the outcome of antitumor drug screening *in vitro*," *Clinical Hemorheology and Microcirculation*, vol. 55, no. 1, pp. 121–131, 2013.

[92] B. Mintz and K. Illmensee, "Normal genetically mosaic mice produced from malignant teratocarcinoma cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 72, no. 9, pp. 3585–3589, 1975.

[93] D. J. Tschumperlin, G. Dal, I. V. Maly et al., "Mechanotransduction through growth-factor shedding into the extracellular space," *Nature*, vol. 429, no. 6987, pp. 83–86, 2004.

[94] P. J. Wipff, D. B. Rifkin, J. J. Meister, and B. Hinz, "Myofibroblast contraction activates latent TGF-β1 from the extracellular matrix," *The Journal of Cell Biology*, vol. 179, no. 6, pp. 1311–1323, 2007.

[95] S. A. Newman, G. Forgacs, and G. B. Müller, "Before programs: the physical origination of multicellular forms," *The International Journal of Developmental Biology*, vol. 50, no. 2-3, pp. 289–299, 2006.

[96] A. Budhu and X. W. Wang, "Transforming the microenvironment: a trick of the metastatic cancer cell," *Cancer Cell*, vol. 22, no. 3, pp. 279–280, 2012.

[97] T. M. Guadagno and R. K. Assoian, "G1/S control of anchorage-independent growth in the fibroblast cell cycle," *The Journal of Cell Biology*, vol. 115, no. 5, pp. 1419–1425, 1991.

[98] M. G. Stoker and H. Rubin, "Density dependent inhibition of cell growth in culture," *Nature*, vol. 215, no. 5097, pp. 171–172, 1967.

[99] F. Testa, A. Palombo, S. Dinicola et al., "Fractal analysis of shape changes in murine osteoblasts cultured under simulated microgravity," *Rendiconti Lincei: Scienze Fisiche e Naturali*, vol. 25, no. 1, supplement, pp. S39–S47, 2014.

[100] M. L. Bajenaru, Y. Zhu, N. M. Hedrick, J. Donahoe, L. F. Parada, and D. H. Gutmann, "Astrocyte-specific inactivation of the neurofibromatosis 1 gene (*NF1*) is insufficient for astrocytoma formation," *Molecular and Cellular Biology*, vol. 22, no. 14, pp. 5100–5113, 2002.

[101] G. C. Daginakatte and D. H. Gutmann, "Neurofibromatosis-1 (*Nf1*) heterozygous brain microglia elaborate paracrine factors that promote *Nf1*-deficient astrocyte and glioma growth," *Human Molecular Genetics*, vol. 16, no. 9, pp. 1098–1112, 2007.

[102] A. M. Soto, M. V. Maffini, and C. Sonnenschein, "Neoplasia as development gone awry: the role of endocrine disruptors," *International Journal of Andrology*, vol. 31, no. 2, pp. 288–293, 2008.

[103] F. Michor, J. Liphardt, M. Ferrari, and J. Widom, "What does physics have to do with cancer?" *Nature Reviews Cancer*, vol. 11, no. 9, pp. 657–670, 2011.

[104] C. M. Nelson and M. J. Bissell, "Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer," *Annual Review of Cell and Developmental Biology*, vol. 22, pp. 287–309, 2006.

[105] M. Levin, "Bioelectric mechanisms in regeneration: unique aspects and future perspectives," *Seminars in Cell & Developmental Biology*, vol. 20, no. 5, pp. 543–556, 2009.

[106] C. M. Nelson, "Geometric control of tissue morphogenesis," *Biochimica et Biophysica Acta*, vol. 1793, no. 5, pp. 903–910, 2009.

[107] J. Fu, Y. K. Wang, M. T. Yang et al., "Mechanical regulation of cell function with geometrically modulated elastomeric substrates," *Nature Methods*, vol. 7, no. 9, pp. 733–736, 2010.

[108] M. Bizzarri, A. Palombo, and A. Cucina, "Theoretical aspects of systems biology," *Progress in Biophysics and Molecular Biology*, vol. 112, no. 1-2, pp. 33–43, 2013.

[109] D. W. Smithers, "An attack on cytologism," *The Lancet*, vol. 279, no. 7228, pp. 493–499, 1962.

[110] H. Kacser, "On parts and wholes in metabolism," in *The Organization of Cell Metabolism*, G. R. Welch and J. S. Clegg, Eds., pp. 327–337, Plenum Press, New York, NY, USA, 1986.

[111] J. W. Haycock, "3D cell culture: a review of current approaches and techniques," *Methods in Molecular Biology*, vol. 695, pp. 1–15, 2011.

[112] S. J. Fey and K. Wrzesinski, "Determination of drug toxicity using 3D spheroids constructed from an immortal human hepatocyte cell line," *Toxicological Sciences*, vol. 127, no. 2, pp. 403–411, 2012.

[113] P. A. Vidi, M. J. Bissell, and S. A. Lelièvre, "Three-dimensional culture of human breast epithelial cells: the how and the why," *Methods in Molecular Biology*, vol. 945, pp. 193–219, 2013.

[114] M. D. Mesarovic, S. N. Sreenath, and J. D. Keene, "Search for organising principles: understanding in systems biology," *Systems biology*, vol. 1, no. 1, pp. 19–27, 2004.

[115] P. F. Davies, "Flow-mediated endothelial mechanotransduction," *Physiological Reviews*, vol. 75, no. 3, pp. 519–560, 1995.

[116] D. B. Agus, J. F. Alexander, W. Arap et al., "A physical sciences network characterization of non-tumorigenic and metastatic cells," *Scientific Reports*, vol. 3, article 1449, 2013.

[117] A. Cornish-Bowden and M. L. Cárdenas, "Self-organization at the origin of life," *Journal of Theoretical Biology*, vol. 252, no. 3, pp. 411–418, 2008.

[118] A. Strand and G. Oftedal, "Functional stability and systems level causation," *Philosophy of Science*, vol. 76, no. 5, pp. 809–820, 2009.

[119] A. Wagner, *Robustness and Evolvability in Living Systems*, Princeton University Press, Princeton, NJ, USA, 2005.

[120] D. Noble, "Modeling the heart—from genes to cells to the whole organ," *Science*, vol. 295, no. 5560, pp. 1678–1682, 2002.

[121] T. S. Kuhn, *The Structure of Scientific Revolutions*, University of Chicago Press, Chicago, Ill, USA, 1962.

[122] R. Moore, *Niels Bohr: The Man, His Science, and the World They Changed*, Edited by: A. Knopf, The MIT Press, New York, NY, USA, 1966.

[123] S. Green and O. Wolkenhauer, "Tracing organizing principles: learning from the history of systems biology," *History and Philosophy of the Life Sciences*, vol. 35, no. 4, pp. 553–576, 2013.

[124] L. Tzu, *Tao-Te-Ching*, Tarcher Cornerstone Editions Series, Jeremy P. Tarcher, New York, NY, USA, 2008.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 198539, 10 pages
http://dx.doi.org/10.1155/2014/198539

Review Article

Immunological Dysregulation in Multiple Myeloma Microenvironment

Alessandra Romano,[1,2,3] Concetta Conticello,[3] Maide Cavalli,[3] Calogero Vetro,[1,3] Alessia La Fauci,[3] Nunziatina Laura Parrinello,[1,3] and Francesco Di Raimondo[1,3]

[1] *Department of Clinical and Molecular Biomedicine, Haematology Section, University of Catania, Catania, Italy*
[2] *Fondazione Veronesi, Roma, Italy*
[3] *Division of Hematology, AOU "Policlinico-Vittorio Emanuele", Catania, Italy*

Correspondence should be addressed to Francesco Di Raimondo; diraimon@unict.it

Received 11 April 2014; Accepted 20 May 2014; Published 11 June 2014

Academic Editor: Lijun Jia

Multiple Myeloma (MM) is a systemic hematologic disease due to uncontrolled proliferation of monoclonal plasma cells (PC) in bone marrow (BM). Emerging in other solid and liquid cancers, the host immune system and the microenvironment have a pivotal role for PC growth, proliferation, survival, migration, and resistance to drugs and are responsible for some clinical manifestations of MM. In MM, microenvironment is represented by the cellular component of a normal bone marrow together with extracellular matrix proteins, adhesion molecules, cytokines, and growth factors produced by both stromal cells and PC themselves. All these components are able to protect PC from cytotoxic effect of chemo- and radiotherapy. This review is focused on the role of immunome to sustain MM progression, the emerging role of myeloid derived suppressor cells, and their potential clinical implications as novel therapeutic target.

1. Introduction

Multiple myeloma (MM) is a systemic hematologic disease due to uncontrolled proliferation of monoclonal plasma cells (PC) in bone marrow (BM). MM symptoms depend on organ damage and include renal failure, anemia due to extensive BM infiltration, hypercalcemia, and pain due to osteolytic bone lesions [1]. A monoclonal proliferation of PC is also present in other conditions that can be considered as preclinical phases of MM, including monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic or smoldering myeloma (SMM). Recent studies have documented that virtually all cases of MM pass through a MGUS phase, although it is often not recognized [2, 3]. The rate of evolution from these preclinical conditions to an overt myeloma is very low and it has been calculated to be 1% per year for MGUS and 10% per year for SMM.

However, while both MGUS and SMM lack the clinical features of MM, they harbor the same genetic alterations of symptomatic myeloma [4, 5]. Therefore, the transformation of MGUS to MM seems to be a multistep process where several factors may play a role. Besides additional, acquired genetic and epigenetic changes of PC, it is likely that a "permissive" microenvironment plays a significant role in the evolution from MGUS to symptomatic myeloma [6].

In fact, a well-recognized feature of MM is the presence of an intimate relationship between PC and bone marrow microenvironment where PC are hosted in special niches and receive multiple signals that maintain their long survival and exert a protective effect on drug-induced apoptosis. Therefore, as emerging in other solid and liquid cancers, the host immune system and the microenvironment have a pivotal role for the PC growth, proliferation, survival, migration, and resistance to drugs and are responsible for some clinical manifestations of MM.

In addition to the cellular component of the microenvironment (represented by stromal cells, osteoblasts, osteoclasts, adipocytes, endothelial cells, and T and natural killer cells), PC interact with extracellular matrix (such as laminin and fibronectin), adhesion molecules (including syndecan1,

VCAM1, and VLA4), cytokines (the most studied being IL-6, TNFα, HGF, and IGF), and growth factors, produced by both stromal cells and PC themselves. All these components are able to protect PC from cytotoxic effect of chemo- and radiotherapy [7, 8]. Indeed, MM PC are not able to grow in a medium without a stromal support and even PC from patients affected by refractory myeloma show drug sensitivity when cultured in vitro [9].

This review is focused on the role of microenvironment and immunome to sustain MM progression and resistance to chemotherapy.

2. MM Microenvironment: In Vivo Models

MM microenvironment has been evaluated in vitro and in several murine models [10], all of them limited for reproducible, convenient, and sensitive monitoring of cellular immunological changes [11]. Currently available murine models for MM include immunocompetent mice, such as the 5TMM series [12, 13] and genetic models of MM [14, 15], or immunocompromised mice, namely, NOD/SCID [16], SCID-hu [17, 18], and NOG [19, 20].

The 5TMM and the genetic models of MM have the advantage of affording preclinical studies in immunocompetent hosts, but molecular and biological differences exist between murine and human MM cells [10]. The number of available murine genetic models of MM and of 5TMM cell lines is extremely restricted and does not represent the heterogeneity of the human disease. Immunological changes can be monitored but are limited to the mouse background and cannot be translated *tout-court* in MM patients.

Moreover, MM is different when developed in mouse or human background [21]. Thus, an emerging need is the development of murine models with both cancer and microenvironment in human background. Available xenograft models of human myeloma into mice to study MM expansion in immune human system include NOD/SCID mice, SCID-Hu model using human fetal bone [21], and SCID-Rab using rabbit bones implanted subcutaneously in unconditioned SCID mice [22].

SCID models have been further modified using synthetic scaffolds instead of fetal human bone to study the expansion of human primary MM cells [23] or using a silicomodel that allows visualizing in a three-dimensional space the dynamics of BM microenvironment and the relevant role of SDF1/CXCR4 axis [24].

Immunodeficient RAG2−/−γc−/− mice (that completely lack B, T, and NK cells) have been used to investigate the graft versus myeloma effect and T-cells modulation from human peripheral blood mononucleated cells PBMC, showing that human PBMC can be safely inoculated in mice to investigate microenvironment. Human mesenchymal stromal cells and bone particles can be also implanted in mice to create a humanized environment for MM cells and bioluminescent imaging is used to follow in a noninvasive way engraft, growth dynamics, and response to therapy of patients PC [25].

However, in these models, immunological impairment present in MM patients is still hard to monitor since the whole immunome cannot be modelled, due to excess of inflammation in models using synthetic scaffolds or the presence fetal immunome in those using fetal bone scaffolds.

However, thanks to these models we addressed many details about the interaction of PC in BM, thus to define the MM "niche" and their relative likely or known relation to myeloma genesis.

3. The MM Niche

The main components of MM niche are [26, 27]

(1) extracellular matrix (ECM): fibrous proteins, proteoglycans, glycosaminoglycans, and small integrin-binding ligand N-linked glycoproteins,

(2) soluble component: cytokines, growth factors, and adhesion molecules,

(3) hematopoietic and nonhematopoietic cells: bone structural cells (stromal cells, adipocytes, osteoclasts, and osteoblasts), immune cells (T lymphocytes, dendritic cells), and the vasculature.

ECM is the dynamic substrate that supports cells anchorage to BM niche and regulates growth factors distribution. Fibrous proteins (such as collagen, laminin, fibronectin, and elastin), proteoglycans (such as heparan sulfate-containing proteoglycans and small leucine-rich repeat proteoglycans (SLRPs): decorin, biglycan, fibromodulin, and lumican), glycosaminoglycans (particularly hyaluronan), and SIBLING proteins (such as osteopontin, bone sialoprotein (BSP), and dentin matrix protein-1 (DMP-1)) constitute ECM. In MM patients ECM composition is altered and variably disorganized [28].

Autocrine and paracrine loops and cell-cell adhesion mechanisms regulate PC production of cytokines and growth factors within the BM microenvironment. These different components are able to induce signaling pathways responsible for PC survival, growth, and migration among which the most important are Ras/Raf/MEK/MAPK pathway, PI3 K/Akt pathway, the JAK/Stat3 pathway, the NFκB pathway, and the wingless-type (Wnt) pathway [29–34].

It has been widely described that NF-κB transcription factor plays a key role in the pathogenesis of multiple myeloma within BM microenvironment where there is an increased MM expression and activation of molecules involved in both the canonical and noncanonical NF-κB pathway [29, 35]. NF-κB signaling pathways play an important role not only for MM cells but also for many other types of stromal cells by inducing the production of prosurvival cytokines such as IL-6, BAFF, or APRIL [36].

Our group has recently described that the majority of MM cells from BM specimens at different stages of disease almost exclusively express the cytoplasmic (inactive) form of NF-κB while, in mesenchymal cells from MM-patients, NF-κB is present in the nuclear active form, further underlining the relevance of BM mesenchymal cells [37]. In addition, the proteasome inhibitor bortezomib, which was described in the past as a NF-κB antagonist, had a consistent antitumor activity against both chemoresistant and chemosensitive MM

cells, regardless of the NF-κB localization, thus suggesting the existence of other molecular targets of proteasome inhibitors in MM [29, 35, 37]. The overexpression and activation of several molecules involved in NF-κB signaling give rise to promising targets for novel anti-MM therapy [38].

Two types of cells primarily compose the cellular compartment of the BM niche: hematopoietic and nonhematopoietic cells, including the vasculature. Among the nonhematopoietic cells (stromal cells, including pericytes, marrow adipocytes, fibroblasts, osteoblasts, osteoclasts, and endothelial cells), we will focus on osteoblast/osteoclast ratio and on the vasculature.

Bone homeostasis is normally maintained by the opposite activity of osteoblasts and osteoclasts. BM malignant PC induce osteoclastogenesis and inhibit bone building by osteoblasts, thus altering bone homeostasis and leading to bone damage. The general view is that MM PC adhere to BM stroma and induce the secretion of different proosteoclasts and antiosteoblasts cytokines. The adhesion is mediated by integrins such as CTLA4-1 integrin and VLA-4 expressed by MM cells and VCAM-1 expressed by stromal cells. This interaction induces osteoclasts resorbing activity and osteolysis [39]. After adhesion, increased bone resorbing activity by osteoclasts and MM survival is mediated by a variety of osteoclast-activating factors, such as macrophage inflammatory protein-1α (MIP-1α), receptor of NF-κB ligand (RANKL), VEGF, TNF-α, IL-1β, HGF, and IL-6, produced by both tumor as well as stromal cells. In particular, RANKL is a member of TNF family whose antagonist is osteoprotegerin (OPG) ligand. When MM cells adhere to stromal cells, they induced expression of RANKL by BM stromal cells, thus promoting osteoclast activity and differentiation. Conversely, decreased number and bone formation activity of osteoblasts are associated with dysregulation of several signaling molecules, among which are dickkoppf 1 (DKK1), IL-3, and IL-7. DKK1 is overexpressed in MM patients with bone lesions. It is able to inhibit Wnt signaling pathway, critical for osteoblast differentiation, and to abolish Wnt-related OPG production. The cytokines IL-3 and IL-7 negatively affect osteoblast survival. Importantly, the main source of IL-3 is the BM CD3+T lymphocytes, suggesting an additional role for T lymphocytes, which also overproduce RANKL in MM BM with bone damage [39, 40]. This complex network of cytokines and signaling pathway induce osteoclasts resorbing activity and osteolysis.

As for bone homeostasis, the balance between proangiogenic and antiangiogenic factors is lost in MM BM niche in favor of neoangiogenesis. Angiogenesis (evaluated in MM BM specimens by microvessel density, MVD) is increased in patients with active myeloma in comparison with MGUS or smoldering MM patients. An "angiogenic switch" is a feature of active myeloma, as a "vascular phase" of disease, while MGUS and smoldering myeloma are arrested in an "avascular phase" [41, 42]. Increased MVD is a poor prognostic factor at diagnosis for patients who undergo high dose chemotherapy with autologous transplant [43].

Angiogenesis in MM is the result of physical factors such as hypoxia and chemical substances such as hypoxia-inducing factors (HIFs), VEGF, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), angiopoietin, platelet derived growth factors (PDGF), and endothelial growth factor (EGF), whose concentration is much higher in BM than in peripheral blood of MM patients [44]. In particular, microenvironment VEGF, produced by both MM PC and BM stromal cells, is able to induce MM growth and survival and egress from BM via its VEGFR-1 receptor on MM cells and to induce angiogenesis through VEGFR-2 on endothelial cells.

In many MM mice models, hypoxia is a specific feature of MM microenvironment. Moreover, the pathways of HIFs, VEGFs, and VEGF receptors are upregulated in the majority of MM cases and are associated with angiogenesis. Both VEGF and HIF could be a therapeutic target of anti-MM therapy with specific small-inhibitor molecules even if many common anti-MM drugs such as bortezomib and lenalidomide are able to inhibit HIF-1α activity and thalidomide-derived immunomodulatory drugs (IMiDs) including lenalidomide and pomalidomide which are *per se* antiangiogenetic drugs [45].

4. Immunome: An Emerging Role in MM Microenvironment

Among the hematopoietic cells, we can consider a variety of cells from hematopoietic stem cells (HSCs) and mesenchymal cells (MSC), to mature erythrocytes, megakaryocytes, platelets, immune cells, such as B and T lymphocytes, natural killer (NK) cells, macrophages, and dendritic cells (DCs).

Macrophages support survival and stimulate proliferation of MM cell lines in vitro and protect PC cells from spontaneous and drug-induced apoptosis, thanks to secretion of IL-6 and vascular endothelial growth factors [46]. Eosinophils contribute to MM cells proliferation in a largely contact-independent manner, though not by IL-6 or APRIL, usually produced by many other kinds of BM cell types to support normal and malignant PC survival and proliferation [47].

An impairment in the function of immune cells has been widely described in MM patients [48]. MM patients have a greater susceptibility to infection and secondary malignancies and this is the clinical counterpart of an intricate cellular interaction involving the PC clone and the BM microenvironment [49]. The MM-related immunological dysfunction is also able to model PC activity and damage capacity [49]. Indeed, the PC-induced modulation of the surrounding microenvironment involves also host immune effectors.

The levels of B cells, NK cells, and CD4+ T cells are inferior when compared to normal control as well as the immunoglobulin reduction which indicate a typical clinical feature of symptomatic MM [49].

The main dysregulated immunological elements include: Treg, TH17, MDSC, and DC. However, the regulation of the immune-effector cells is the result of a complicated cross talk between PC, DC, and CD4+ cells, through indoleamine 2,3 dioxygenase, the programmed death 1-ligand, and the B7H3 action [50, 51].

4.1. Dendritic Cells. The functions of the antigen presenting cells DCs are defective. The direct interaction between DCs and PC resulted in PC survival and spontaneous cell-cell fusion with formation of giant bone resorbing cells. In a recent model of DCs-MM and T lymphocytes interactions in BM microenvironment, the expression of the costimulatory molecule CD28 on MM plasma cells and of ligands CD80/CD86 (that normally activate T cells) on BM DCs induced DCs to produce both IL-6 and the immunosuppressive enzyme indoleamine 2,3 dioxygenase (IDO). IL-6 is one of the most important MM prosurvival factors while IDO is able to deplete an essential amino acid whose absence induces anergy of activated T cells and differentiation of T cells in suppressive $CD25^{high}/FOXP3^{+}/CD4^{+}$ Treg cells. Blockage of CD28 inhibits IL-6 and IDO production by DCs thus abrogating the protective effect of DCs on MM cells [52].

4.2. Treg. Development of CD4 T cells is a plastic process influenced by cytokines of the microenvironment milieu. A paradigm of the adaptive immunity describes in detail the balance between Th1/Th2 CD4 subpopulations, due to specific soluble factors.

Th1/Th2 balance in MM is altered with a reduced production of Th1-like cytokines, such as IL-2 or IFN-γ, with an overexpression of Th2 cytokines, IL-10 and IL-4 [53]. Similarly, a plastic balance between Th1, Treg, and Th17 is emerging, driven by TGF-β. The forkhead/winged helix transcription factor forkhead box P3 (Foxp3) expression identifies that Treg and retinoid-related orphan receptor (ROR-γ) are a marker of Th17. Naïve T cells overexpress ROR-γ and Foxp3, and in presence of TGF-β Foxp3 inhibits ROR-γ leading to Treg expansion. However, in presence of proinflammatory stimuli, including IL-6, IL17-α, and IL17-β this inhibition does not occur thus switching to Th17 phenotype [54–59], promoting Th17 expansion, and inhibiting Treg differentiation.

MM-derived Tregs are unable to regulate T-cell expansion and function [60, 61]. Tregs behavior remains a critical debate in MM [62], due their source (peripheral blood versus bone marrow), the quantification method (absolute count versus percentage), and the immunophenotype used. Some studies showed increased $CD4^{+}CD25^{high}Foxp3^{+}$ Treg with inhibitory functions [63, 64], while others, including data from our group (Parrinello, manuscript in preparation) show lower absolute numbers in MM patients compared with healthy volunteers [65, 66]. In some series MM Tregs were dysfunctional, because of being unable to inhibit anti-CD3 mediated T-cell proliferation [66]. However, FOXP3 expression is not a definitive and unique marker for Tregs, these cells being also characterized as $CD3^{+}CD4^{+}CD25^{+}CD127^{low}$ cells [67, 68].

4.3. Th17. Th17 produce IL-17 and IL-22 cytokines and, as above described, they are strongly related to the effect of IL-21, IL-22, IL-23, and IL-27, whose levels are augmented in MM [69]. PC express the IL-17R (receptor of IL-17) on the cell surface, thus being sensitive to the survival stimuli given by IL-17 cells, as shown in vitro, in murine model and in patients [69]. Several studies indicate an increase of Th17 in the PB and BM of MM patients [69–72], as consequence of increased proinflammatory cytokines leading to Th17 polarization in MM milieu. Th17 expansion along with increased IL-17 suppress immune responses, protect PC from CTL attack, and promote their survival and growth.

Additionally, IL-17 plays a pivotal role in the development of bone disease, since the higher the levels of IL-17 are, the more advanced the bone disease is. IL-17 is also able to determine an upregulation of RANKL on stromal cells thus determining a stimulation of osteoclasts and so the generation of bone lesions [70]. The amount of Th17 in BM is positively correlated to lytic lesions [70], clinical tumor stage, serum lactate dehydrogenase concentration, and serum creatinine concentration [72].

Th17 are a potential predictive factor of therapy response [72]. Curiously, the enrichment of BM with Th1 cells is able to revert this phenomenon [70]. However, a multivariate analysis of these factors is missing.

The relationship between Th17 and Th1 cells [57] could depend on the microenvironment signature, in particular on the function of DC, through IL-6 secretion [73]. A balance between Th17/ and Treg in favor of Th17 has been observed in long-term MM survivors compared to short-term MM survivors [71]. However, the real Treg/Th17 interaction has not completely been defined since some reports indicate that these constitute a cell subset essential for disease progression [69, 70], while other reports indicate that these cells could have an antitumor activity, regulating cell-to-cell cross talk and leading to a long-term control of the disease [71].

It is likely that the study of the whole microenvironment, taking together the myeloid and lymphoid axis, would be useful in the definition of disease pathogenesis and its prognostic implication.

An indirect marker of T-cell dysfunction could be the evaluation of CD200+T cells. CD200 is a transmembrane glycoprotein belonging to the immunoglobulin superfamily, physiologically expressed on lymphocytes to induce immune tolerance, for example, during pregnancy to avoid conflicts between mother and newborn's immune system. Since CD200 is expressed by different tumor cell types [74–77], it has recently described the relevance of the CD200-CD200R axis in cancer immune evasion. In fact, the expression of the cognate ligand of CD200, CD200R, is restricted to certain populations of T cells and mainly to myeloid-derived Ag presenting cells, among which tumor-associated myeloid cells whose suppression, that is, in melanoma model, is able to abrogate tumor formation [78]. The first data on a likely anti-CD200 treatment in neoplastic diseases come from hematologic malignancies where it has been demonstrated that CD200 blockade may represent a novel approach to clinical treatment of CLL [79].

Increased expression of immune-regulatory molecule CD200 has been described also in MM where it is accepted as a negative prognostic factor [80, 81], but it has never been investigated on T-cell subsets in MM.

Our group has recently confirmed that almost the 70% of MM cells in BM specimens express CD200 and, as in melanoma, the dependence of CD200 expression on RAS/RAF/MEK/ERK pathway. Furthermore, on the basis of a reduced immunogenicity in vitro of CD200-positive cells

depending on ERK pathway, explored by mixed lymphocytes cultures, we have supposed that CD200 expression in MM could suppress antitumor response in bone marrow microenvironment suggesting that an anti-CD200 treatment could be therapeutic in MM [82].

Our data do not allow speculating any role for myeloid involvement in this pathway, although CD200R is an inhibitory immune receptor initially described mainly on myeloid cells [83].

4.4. Myeloid Derived Suppressor Cells. Accumulation of myeloid derived suppressor cells (MDSC) has been described in the peripheral blood of patients affected by solid tumors [84–86], and only a few reports are focused on MM, including 11 newly diagnosed patients [87], 13 relapsed/refractory patients [88], and 11 MGUS [89–91].

MDSC favor the tumor escape from immune-surveillance. MDSC exert an immunosuppressive activity mainly on T lymphocytes because of high levels of arginase, which is able to deplete the microenvironment of arginine, an essential amino acid for T-cell activity. MDSC induce also inhibition of T-cell receptor, by nitrosylation, and ROS release and in this way create conditions for cancer progression as well [92].

In tumour-bearing mice, MDSC can be identified in tumor infiltration and in the spleen as myeloid cells at various maturation stage CD11b+Gr1+ and based on the expression of Ly6G can be distinguished in granulocytic and monocytic fractions.

In humans, several MDSC subpopulations have described termed monocytic (mo-MDSC, $CD14^+HLA\text{-}DR^{low/-}$) and granulocytic MDSC (G-MDSC, $CD33^+CD14^+HLA\text{-}DR^{low/-}$), respectively, in absence of a human marker equivalent to Ly6C in mice.

The role of MDSC in MM progression is currently under investigation. Most of our current knowledge on MDSC is obtained by studying solid tumors that expand MDSC in the lymphoid organs, but information on whether cancer cells residing in the bone marrow (BM) are able to directly influence MDSC generation in situ in MM has been recently evaluated by three groups [87, 88, 93].

Using a 5T-2MM murine model [93], in which the MM cells grow in the BM in fully immunocompetent mice, no dramatic changes in the relative abundance of MDSC subsets were noted, suggesting that MDSC expansion is an early event in MM. The same happens in MM patient's bone marrow, where MDSC infiltration is hard to define because of neoplastic plasma cell expansion. However, when the amount of MDSC was calculated as percentage of the nonneoplastic cells (identified as CD138 negative cells), MDSC accumulation in BM of MM patients was evident when compared with BM healthy donors (41.1 (range, 13.3–75.9%) versus 22.9 (range, 7.7–33.3%)) [87]. Data from Dr. Gorgun confirmed an increase of MDSC in the bone marrow, but it was compared to peripheral blood and not bone marrow in healthy subjects [88].

In the murine model, T-cell suppressive capacity of 5T2MM MDSC subsets could already be observed after 3 weeks—a time point at which the tumor load is very low—and was maintained throughout weeks 6, 9, and 12, confirming that MDSC immunosuppression is an early event in MM disease [93]. Using another immunocompetent mouse model, established by intravenous inoculation of BCM, DP42, or ATLN MM cells into syngeneic mice, MDSC accumulated in BM as early as 1 week after tumor inoculation. When these mice were engineered to lose their ability to accumulate MDSC in tumor-bearing hosts (S100A9 knockout), growth of the immunogenic MM cells was significantly reduced showing again that the accumulation of MDSC at early stages of MM plays a critical role in MM progression [87]. In the ATLN model, a significant increase in the proportion and absolute number of MDSC in BM was observed as early as 1 week after tumor cell inoculation, followed in weeks 2-3 by a reduction due to MM expansion in BM and a progressive increase in spleen and lymphonodes. MDSC continued to grow during week two posttumor injection, reflecting the fact that MM cells accumulated in spleen at later time points and to a lesser extent than in BM. In fact, only at the end of week 3 (late MM stage in mouse model), the presence of MDSC in spleens declined, without any difference in the kinetics for G-MDSC or mo-MDSC. However, mo-MDSC were the main subset in MDSC [87]. In another model, mo-MDSC, defined as $CD11b^{high}Ly6G^{low}$, exhibited a larger immunosuppressive activity than $CD11b^{high}Ly6G^{high}$G-MDSC counterpart [93].

In humans, data are still under investigation. Both mo- and G-MDSC subsets sorted from MM bone marrow are immunosuppressive, when cultured in ratio 1 : 1 with T cells stimulated by allogeneic dendritic cells [87]. Both mo- and G-MDSC subsets sorted from MM bone marrow or peripheral blood are immunosuppressive, cultured with autologous T cells for 4 days in the presence of T-cell stimulator factors, because G-MDSC are more immunosuppressive than mo-MDSC [88]. G-MDSC are increased in PB of MM patients and are able to induce the generation of Treg. G-CSF administered to induce stem cell mobilization caused an increase in the number of MDSC in the peripheral blood of patients with MM and a concentration of these immune-suppressive cells in peripheral blood stem cell collections [94, 95].

In our series, including 45 newly diagnosed MM and 60 MGUS, both G-MDSC and mo-MDSC were increased in PB of MM patients, while MGUS exhibited intermediate values between healthy subjects and MM patients. Myeloid compartment (identified as CD66 positive cells) and mature granulocytes exhibited immunosuppressive properties against allogeneic T cells stimulated with phytohemagglutinin (at increasing lymphoid : myeloid ratio) in both MGUS and MM patients, despite te fact that the effect was more evident in MM patients (Parrinello and Romano, manuscript in preparation). Granulocytes obtained from MM patients had greater amount of arginase-1 (a key mediator of G-MDSC immunosuppression) than MGUS or healthy subjects evaluated by RT-PCR (Parrinello and Romano, manuscript in preparation).

Emerging interest of MDSC in MM includes their involvement in other crucial processes for active disease, such as angiogenesis, since MDSC are able to release metalloproteinase-9 [96], and osteoclastogenesis, since MDSC can

work as osteoclast progenitors [97, 98], thereby contributing to osteolytic bone disease in MM [99].

Several strategies are currently under investigation in human cancer to target MDSC in order to improve immune therapies:

(1) deactivation of MDSC (using phosphodiesterase inhibitors, nitroaspirins, synthetic triterpenoids, COX2 inhibitors, ARG1 inhibitors, antiglycan antibodies, CSF-1R, IL-17 inhibitors, and histamine based approaches),

(2) differentiation of MDSC into mature cells (with ATRA, vitamins A or D3, or IL-12),

(3) inhibition of myeloid cell development into MDSC (with N-bisphosphonates, modulators of tyrosine kinases, and STAT3 inhibitors),

(4) depletion of MDSC (using gemcitabine, HSP90 inhibitors, and paclitaxel) as recently reviewed [100].

Phosphodiesterase-5 (PDE-5) inhibitors, including sildenafil and tadalafil, inhibit the degradation of cyclic guanosine monophosphate (cGMP) leading to reduction in ARG1 and NOS2 expression, thus turning off the immunosuppressive property of MDSC [101]. In an in vitro model, sildenafil was able to restore expansion of T cells within the peripheral blood mononuclear cell fraction isolated from MM patients [101] thus leading to PDE5-inhibitors as novel immunotherapy in MM.

A phase II study has been presented at 2013 ASH annual meeting to test whether tadalafil could improve the response to lenalidomide and dexamethasone (NCT01374217 on http://www.clinicaltrials.gov/), in 13 patients who were refractory to lenalidomide-based regimens. However, the study was early stopped for lack of response and potentially of target, since MDSC were not detected in any of the patients at baseline in both blood and marrow and this correlated with the lack of clinical response [102]. However, the same group recently published the clinical benefit in an end-stage MM patient in whom responsiveness to lenalidomide-based therapy was restored upon the addition of tadalafil [103].

Novel agents active against MM, such as lenalidomide, and bortezomib inhibit NF-κB activity as part of their diverse actions contributing to modulating proteins involved in antigen presentation [104]. Recently, modulation by lenalidomide of regulatory cells, with an immunophenotype overlapping with mo-MDSC, defined as $CD33^{+}CD11b^{+}CD14^{-}HLADR^{-}$, has been reported [89], while in vitro studies exclude a direct effect of lenalidomide or bortezomib in MDSC expansion [88]. On the other hand, in lymphoma-bearing mice, lenalidomide can reduce MDSC numbers and reverts cancer-induced immunosuppression [105].

Some chemotherapeutic agents used also in MM, such as cyclophosphamide or anthracyclines, have immunological side effects [106] that include MDSC expansion [107, 108] or inhibition [109], associated to T-cell function modulation, but this aspect has been poorly evaluated in MM.

Nitrogen-containing bisphosphonates, such as zoledronic acid, have a predominant role in the supportive therapy of MM patients [110] with benefit in survival [111–113]. Nitrogen-containing bisphosphonates inhibit bone resorbing osteoclasts, delay tumor growth rate as a consequence of MDSC depletion, and increased recruitment of T cells in murine models of solid tumors [99, 114]. However, no data are currently available about MDSC amount in MM patients treated with bisphosphonates.

5. Conclusions

MM microenvironment is a complex network, which progressively leads to functional impairment of host immune system. In the early stage of the disease, PC proliferation and migration are under the control of an active immune system, which fails to eradicate malignant cells. As long as the balance between immune effectors and PC is preserved, the disease remains completely indolent; this scenario seems to be consistent with the clinical course of MGUS patients. In a minority of subjects, several changes arise in terms of PC number and phenotype, expression of microenvironment-associated cytokines, and impairment in immune response and infiltration. MDSC increase, specific T-cell response is abolished, and finally PC become independent of microenvironment signaling, thus to confer an aggressive clinical course. Some drugs, including IMiDs, bisphosphonate, and cyclophosphamide, can interplay at this level, but use of drugs able of triggering this signaling is an urgent need to improve the efficacy of current treatments.

Conflict of Interests

Francesco Di Raimondo has received honoraria from Janssen-Cilag and Celgene. All other authors declare that they have no conflict of interests regarding the publication of this paper.

References

[1] S. V. Rajkumar and F. Buadi, "Multiple myeloma: new staging systems for diagnosis, prognosis and response evaluation," *Best Practice and Research: Clinical Haematology*, vol. 20, no. 4, pp. 665–680, 2007.

[2] O. Landgren and N. Korde, "Multiple myeloma precursor disease: current clinical and epidemiological insights and future opportunities," *Oncology*, vol. 25, no. 7, pp. 589–590, 2011.

[3] O. Landgren, R. A. Kyle, R. M. Pfeiffer et al., "Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study," *Blood*, vol. 113, no. 22, pp. 5412–5417, 2009.

[4] M. Brousseau, X. Leleu, J. Gerard et al., "Hyperdiploidy is a common finding in monoclonal gammopathy of undetermined significance and monosomy 13 is restricted to these hyperdiploid patients," *Clinical Cancer Research*, vol. 13, no. 20, pp. 6026–6031, 2007.

[5] L. Chiecchio, G. P. Dagrada, A. H. Ibrahim et al., "Timing of acquisition of deletion 13 in plasma cell dyscrasias is dependent on genetic context," *Haematologica*, vol. 94, no. 12, pp. 1708–1713, 2009.

[6] A. Agarwal and I. M. Ghobrial, "Monoclonal gammopathy of undetermined significance and smoldering multiple myeloma:

a review of the current understanding of epidemiology, biology, risk stratification, and management of myeloma precursor disease," *Clinical Cancer Research*, vol. 19, no. 5, pp. 985–994, 2013.

[7] J. A. Fowler, G. R. Mundy, S. T. Lwin, and C. M. Edwards, "Bone marrow stromal cells create a permissive microenvironment for myeloma development: a new stromal role for Wnt inhibitor Dkk1," *Cancer Research*, vol. 72, no. 9, pp. 2183–2189, 2012.

[8] Y. Nefedova, T. H. Landowski, and W. S. Dalton, "Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms," *Leukemia*, vol. 17, no. 6, pp. 1175–1182, 2003.

[9] V. Hughes, "Microenvironment: neighbourhood watch," *Nature*, vol. 480, no. 7377, pp. S48–S49, 2011.

[10] C. S. Mitsiades, K. C. Anderson, and D. R. Carrasco, "Mouse models of human myeloma," *Hematology/Oncology Clinics of North America*, vol. 21, no. 6, pp. 1051–1069, 2007.

[11] Z.-W. Li and W. S. Dalton, "Tumor microenvironment and drug resistance in hematologic malignancies," *Blood Reviews*, vol. 20, no. 6, pp. 333–342, 2006.

[12] J. W. Croese, C. M. vas Nunes, J. Radl, M. H. M. Van Den Enden-Vieveen, R. J. Brondijk, and W. J. A. Boersma, "The 5T2 mouse multiple myeloma model: characterization of 5T2 cells within the bone marrow," *British Journal of Cancer*, vol. 56, no. 5, pp. 555–560, 1987.

[13] K. Vanderkerken, H. De Raeve, E. Goes et al., "Organ involvement and phenotypic adhesion profile of 5T2 and 5T33 myeloma cells in the C57BL/KaLwRij mouse," *British Journal of Cancer*, vol. 76, no. 4, pp. 451–460, 1997.

[14] R. A. Campbell, S. J. Manyak, H. H. Yang et al., "LAGλ1: a clinically relevant drug resistant human multiple myeloma tumor murine model that enables rapid evaluation of treatments for multiple myeloma," *International Journal of Oncology*, vol. 28, no. 6, pp. 1409–1417, 2006.

[15] A. L. Kovalchuk, J. S. Kim, S. S. Park et al., "IL-6 transgenic mouse model for extraosseous plasmacytoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 3, pp. 1509–1514, 2002.

[16] R. LeBlanc, L. P. Catley, T. Hideshima et al., "Proteasome inhibitor PS-341 inhibits human myeloma cell growth in vivo and prolongs survival in a murine model," *Cancer Research*, vol. 62, no. 17, pp. 4996–5000, 2002.

[17] S. Yaccoby, B. Barlogie, and J. Epstein, "Primary myeloma cells growing in SCID-hu mice: a model for studying the biology and treatment of myeloma and its manifestations," *Blood*, vol. 92, no. 8, pp. 2908–2913, 1998.

[18] S. Yaccoby and J. Epstein, "The proliferative potential of myeloma plasma cells manifest in the SCID-hu host," *Blood*, vol. 94, no. 10, pp. 3576–3582, 1999.

[19] M. Z. Dewan, M. Watanabe, K. Terashima et al., "Prompt tumor formation and maintenance of constitutive NF-κB activity of multiple myeloma cells in NOD/SCID/γcnull mice," *Cancer Science*, vol. 95, no. 7, pp. 564–568, 2004.

[20] Y. Miyakawa, Y. Ohnishi, M. Tomisawa et al., "Establishment of a new model of human multiple myeloma using NOD/SCID/γcnull (NOG) mice," *Biochemical and Biophysical Research Communications*, vol. 313, no. 2, pp. 258–262, 2004.

[21] P. Tassone, P. Neri, D. R. Carrasco et al., "A clinically relevant SCID-hu in vivo model of human multiple myeloma," *Blood*, vol. 106, no. 2, pp. 713–716, 2005.

[22] K. Yata and S. Yaccoby, "The SCID-rab model: a novel in vivo system for primary human myeloma demonstrating growth of CD138-expressing malignant cells," *Leukemia*, vol. 18, no. 11, pp. 1891–1897, 2004.

[23] T. Calimeri, E. Battista, F. Conforti et al., "A unique three-dimensional SCID-polymeric scaffold (SCID-synth-hu) model for in vivo expansion of human primary multiple myeloma cells," *Leukemia*, vol. 25, no. 4, pp. 707–711, 2011.

[24] J. Su, L. Zhang, W. Zhang et al., "Targeting the biophysical properties of the myeloma initiating cell niches: a pharmaceutical synergism analysis using multi-scale agent-based modeling," *PloS ONE*, vol. 9, no. 1, Article ID e85059, 2014.

[25] R. W. J. Groen, W. A. Noort, R. A. Raymakers et al., "Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma," *Blood*, vol. 120, no. 3, 2012.

[26] G. W. Basak, A. S. Srivastava, R. Malhotra, and E. Carrier, "Multiple myeloma bone marrow niche," *Current Pharmaceutical Biotechnology*, vol. 10, no. 3, pp. 335–346, 2009.

[27] S. Manier, A. Sacco, X. Leleu, I. M. Ghobrial, and A. M. Roccaro, "Bone marrow microenvironment in multiple myeloma progression," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 157496, 5 pages, 2012.

[28] H. Iriuchishima, K. Takubo, Y. Miyakawa et al., "Neovascular niche for human myeloma cells in immunodeficient mouse bone," *PloS ONE*, vol. 7, no. 2, Article ID e30557, 2012.

[29] T. Hideshima, H. Ikeda, D. Chauhan et al., "Bortezomib induces canonical nuclear factor-κB activation in multiple myeloma cells," *Blood*, vol. 114, no. 5, pp. 1046–1052, 2009.

[30] T. Hideshima, K. Podar, D. Chauhan, and K. C. Anderson, "Cytokines and signal transduction," *Best Practice and Research: Clinical Haematology*, vol. 18, no. 4, pp. 509–524, 2005.

[31] K. Kim, S.-Y. Kong, M. Fulciniti et al., "Blockade of the MEK/ERK signalling cascade by AS703026, a novel selective MEK1/2 inhibitor, induces pleiotropic anti-myeloma activity in vitro and in vivo," *British Journal of Haematology*, vol. 149, no. 4, pp. 537–549, 2010.

[32] K. Podar, D. Chauhan, and K. C. Anderson, "Bone marrow microenvironment and the identification of new targets for myeloma therapy," *Leukemia*, vol. 23, no. 1, pp. 10–24, 2009.

[33] K. Podar, P. G. Richardson, T. Hideshima, D. Chauhan, and K. C. Anderson, "The malignant clone and the bone-marrow environment," *Best Practice and Research in Clinical Haematology*, vol. 20, no. 4, pp. 597–612, 2007.

[34] Y.-T. Tai, M. Fulciniti, T. Hideshima et al., "Targeting MEK induces myeloma-cell cytotoxicity and inhibits osteoclastogenesis," *Blood*, vol. 110, no. 5, pp. 1656–1663, 2007.

[35] T. Hideshima, D. Chauhan, P. Richardson et al., "NF-κB as a therapeutic target in multiple myeloma," *Journal of Biological Chemistry*, vol. 277, no. 19, pp. 16639–16647, 2002.

[36] J. Moreaux, E. Legouffe, E. Jourdan et al., "BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone," *Blood*, vol. 103, no. 8, pp. 3148–3157, 2004.

[37] C. Conticello, R. Giuffrida, L. Adamo et al., "NF-κB localization in multiple myeloma plasma cells and mesenchymal cells," *Leukemia Research*, vol. 35, no. 1, pp. 52–60, 2011.

[38] K. Podar, "Novel targets and derived small molecule inhibitors in multiple Myeloma," *Current Cancer Drug Targets*, vol. 12, no. 7, pp. 797–813, 2012.

[39] D. Zipori, "The hemopoietic stem cell niche versus the microenvironment of the multiple myeloma-tumor initiating cell," *Cancer Microenvironment*, vol. 3, no. 1, pp. 15–28, 2010.

[40] A. Balakumaran, P. G. Robey, N. Fedarko, and O. Landgren, "Bone marrow microenvironment in myelomagenesis: its potential role in early diagnosis," *Expert Review of Molecular Diagnostics*, vol. 10, no. 4, pp. 465–480, 2010.

[41] K. Podar and K. C. Anderson, "Emerging therapies targeting tumor vasculature in multiple myeloma and other hematologic and solid malignancies," *Current Cancer Drug Targets*, vol. 11, no. 9, pp. 1005–1024, 2011.

[42] F. Fan, A. Schimming, D. Jaeger, and K. Podar, "Targeting the tumor microenvironment: focus on angiogenesis," *Journal of Oncology*, vol. 2012, Article ID 281261, 16 pages, 2012.

[43] S. Kumar, M. A. Gertz, A. Dispenzieri et al., "Prognostic value of bone marrow angiogenesis in patients with multiple myeloma undergoing high-dose therapy," *Bone Marrow Transplantation*, vol. 34, no. 3, pp. 235–239, 2004.

[44] F. di Raimondo, M. P. Azzaro, G. A. Palumbo et al., "Angiogenic factors in multiple myeloma: higher levels in bone marrow than in peripheral blood," *Haematologica*, vol. 85, no. 8, pp. 800–805, 2000.

[45] J. Hu, E. van Valckenborgh, E. Menu, E. de Bruyne, and K. Vanderkerken, "Understanding the hypoxic niche of multiple myeloma: therapeutic implications and contributions of mouse models," *Disease Models and Mechanisms*, vol. 5, no. 6, pp. 763–771, 2012.

[46] J. Kim, R. A. Denu, B. A. Dollar et al., "Macrophages and mesenchymal stromal cells support survival and proliferation of multiple myeloma cells," *British Journal of Haematology*, vol. 158, no. 3, pp. 336–346, 2012.

[47] T. W. Wong, H. Kita, C. A. Hanson, D. K. Walters, B. K. Arendt, and D. F. Jelinek, "Induction of malignant plasma cell proliferation by eosinophils," *PloS ONE*, vol. 8, no. 7, Article ID e70554, 2013.

[48] G. Pratt, O. Goodyear, and P. Moss, "Immunodeficiency and immunotherapy in multiple myeloma," *British Journal of Haematology*, vol. 138, no. 5, pp. 563–579, 2007.

[49] M. Rossi, C. Botta, P. Correale, P. Tassone, and P. Tagliaferri, "Immunologic microenvironment and personalized treatment in multiple myeloma," *Expert Opinion on Biological Therapy*, vol. 13, no. 1, supplement, pp. S83–S93, 2013.

[50] D. J. Chung, M. Rossi, E. Romano et al., "Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells," *Blood*, vol. 114, no. 3, pp. 555–563, 2009.

[51] K. Mahnke, S. Ring, T. S. Johnson et al., "Induction of immunosuppressive functions of dendritic cells in vivo by $CD4^+CD25^+$ regulatory T cells: role of B7-H3 expression and antigen presentation," *European Journal of Immunology*, vol. 37, no. 8, pp. 2117–2126, 2007.

[52] J. R. Nair, C. H. Rozanski, and K. P. Lee, "Under one roof: the bone marrow survival niche for multiple myeloma and normal plasma cells," *Oncoimmunology*, vol. 1, no. 3, pp. 388–389, 2012.

[53] A. Sharma, R. Khan, S. Joshi, L. Kumar, and M. Sharma, "Dysregulation in T helper 1/T helper 2 cytokine ratios in patients with multiple myeloma," *Leukemia & Lymphoma*, vol. 51, no. 5, pp. 920–927, 2010.

[54] Z. Chen, F. Lin, Y. Gao et al., "FOXP3 and RORγt: transcriptional regulation of Treg and Th17," *International Immunopharmacology*, vol. 11, no. 5, pp. 536–542, 2011.

[55] S. Q. Crome, A. Y. Wang, and M. K. Levings, "Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease," *Clinical and Experimental Immunology*, vol. 159, no. 2, pp. 109–119, 2010.

[56] B. Hoechst, J. Gamrekelashvili, M. P. Manns, T. F. Greten, and F. Korangy, "Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells," *Blood*, vol. 117, no. 24, pp. 6532–6541, 2011.

[57] I. Kryczek, M. Banerjee, P. Cheng et al., "Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments," *Blood*, vol. 114, no. 6, pp. 1141–1149, 2009.

[58] Y. K. Lee, R. Mukasa, R. D. Hatton, and C. T. Weaver, "Developmental plasticity of Th17 and Treg cells," *Current Opinion in Immunology*, vol. 21, no. 3, pp. 274–280, 2009.

[59] C. Schmidl, L. Hansmann, R. Andreesen, M. Edinger, P. Hoffmann, and M. Rehli, "Epigenetic reprogramming of the RORC locus during in vitro expansion is a distinctive feature of human memory but not naïve Treg," *European Journal of Immunology*, vol. 41, no. 5, pp. 1491–1498, 2011.

[60] B. Maecker, K. S. Anderson, M. S. von Bergwelt-Baildon et al., "Viral antigen-specific CD8+ T-cell responses are impaired in multiple myeloma," *British Journal of Haematology*, vol. 121, no. 6, pp. 842–848, 2003.

[61] G. Görgün, E. Calabrese, E. Soydan et al., "Immunomodulatory effects of lenalidomide and pomalidomide on interaction of tumor and bone marrow accessory cells in multiple myeloma," *Blood*, vol. 116, no. 17, pp. 3227–3237, 2010.

[62] D. E. Joshua, R. D. Brown, P. J. Ho, and J. Gibson, "Regulatory T cells and multiple myeloma," *Clinical Lymphoma and Myeloma*, vol. 8, no. 5, pp. 283–286, 2008.

[63] A. Laronne-Bar-On, D. Zipori, and N. Haran-Ghera, "Increased regulatory versus effector T cell development is associated with thymus atrophy in mouse models of multiple myeloma," *Journal of Immunology*, vol. 181, no. 5, pp. 3714–3724, 2008.

[64] S. Feyler, M. Von Lilienfeld-Toal, S. Jarmin et al., "$CD4^+CD25^+FoxP3^+$ regulatory T cells are increased whilst $CD3^+CD4^-CD8^-\alpha\beta TCR^+$ Double Negative T cells are decreased in the peripheral blood of patients with multiple myeloma which correlates with disease burden," *British Journal of Haematology*, vol. 144, no. 5, pp. 686–695, 2009.

[65] R. Gupta, P. Ganeshan, M. Hakim, R. Verma, A. Sharma, and L. Kumar, "Significantly reduced regulatory T cell population in patients with untreated multiple myeloma," *Leukemia Research*, vol. 35, no. 7, pp. 874–878, 2011.

[66] R. H. Prabhala, P. Neri, J. E. Bae et al., "Dysfunctional T regulatory cells in multiple myeloma," *Blood*, vol. 107, no. 1, pp. 301–304, 2006.

[67] M. Beyer, S. Classen, E. Endl et al., "Comparative approach to define increased regulatory T cells in different cancer subtypes by combined assessment of CD127 and FOXP3," *Clinical and Developmental Immunology*, vol. 2011, Article ID 734036, 12 pages, 2011.

[68] J. Favaloro, R. Brown, E. Aklilu et al., "Myeloma skews regulatory T and pro-inflammatory T helper 17 cell balance in favor of a suppressive state," *Leukemia & Lymphoma*, 2013.

[69] R. H. Prabhala, D. Pelluru, M. Fulciniti et al., "Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma," *Blood*, vol. 115, no. 26, pp. 5385–5392, 2010.

[70] K. Noonan, L. Marchionni, J. Anderson, D. Pardoll, G. D. Roodman, and I. Borrello, "A novel role of IL-17-producing lymphocytes in mediating lytic bone disease in multiple myeloma," *Blood*, vol. 116, no. 18, pp. 3554–3563, 2010.

[71] C. Bryant, H. Suen, R. Brown et al., "Long-term survival in multiple myeloma is associated with a distinct immunological profile, which includes proliferative cytotoxic T-cell clones and a favourable Treg/Th17 balance," *Blood Cancer Journal*, vol. 3, article e148, 2013.

[72] C.-J. Shen, Z.-H. Yuan, Y.-X. Liu, and G.-Y. Hu, "Increased numbers of T helper 17 cells and the correlation with clinicopathological characteristics in multiple myeloma," *Journal of International Medical Research*, vol. 40, no. 2, pp. 556–564, 2012.

[73] K. M. Dhodapkar, S. Barbuto, P. Matthews et al., "Dendritic cells mediate the induction of polyfunctional human IL17-producing cells (Th17-1 cells) enriched in the bone marrow of patients with myeloma," *Blood*, vol. 112, no. 7, pp. 2878–2885, 2008.

[74] G. Adhikary, D. Grun, C. Kerr et al., "Identification of a population of epidermal squamous cell carcinoma cells with enhanced potential for tumor formation," *PloS ONE*, vol. 8, no. 12, Article ID e84324, 2013.

[75] S. J. Coles, E. C. Y. Wang, S. Man et al., "CD200 expression suppresses natural killer cell function and directly inhibits patient anti-tumor response in acute myeloid leukemia," *Leukemia*, vol. 25, no. 5, pp. 792–799, 2011.

[76] H. Olteanu, A. M. Harrington, P. Hari, and S. H. Kroft, "CD200 expression in plasma cell myeloma," *British Journal of Haematology*, vol. 153, no. 3, pp. 408–411, 2011.

[77] G. A. Palumbo, N. Parrinello, G. Fargione et al., "CD200 expression may help in differential diagnosis between mantle cell lymphoma and B-cell chronic lymphocytic leukemia," *Leukemia Research*, vol. 33, no. 9, pp. 1212–1216, 2009.

[78] F. Talebian and X. F. Bai, "The role of tumor expression of CD200 in tumor formation, metastasis and susceptibility to T lymphocyte adoptive transfer therapy," *Oncoimmunology*, vol. 1, no. 6, pp. 971–973, 2012.

[79] R. M. Gorczynski, Z. Chen, S. Shivagnahnam et al., "Potent immunosuppression by a bivalent molecule binding to CD200R and TGF-βR," *Transplantation*, vol. 90, no. 2, pp. 150–159, 2010.

[80] D. Alapat, J. Coviello-Malle, R. Owens et al., "Diagnostic usefulness and prognostic impact of CD200 expression in lymphoid malignancies and plasma cell myeloma," *American Journal of Clinical Pathology*, vol. 137, no. 1, pp. 93–100, 2012.

[81] J. Moreaux, D. Hose, T. Reme et al., "CD200 is a new prognostic factor in multiple myeloma," *Blood*, vol. 108, no. 13, pp. 4194–4197, 2006.

[82] C. Conticello, R. Giuffrida, N. Parrinello et al., "CD200 expression in patients with Multiple Myeloma: another piece of the puzzle," *Leukemia Research*, vol. 37, no. 12, pp. 1616–1621, 2013.

[83] S. Preston, G. J. Wright, K. Starr, A. N. Barclay, and M. H. Brown, "The leukocyte/neuron cell surface antigen OX2 binds to a ligand on macrophages," *European Journal of Immunology*, vol. 27, no. 8, pp. 1911–1918, 1997.

[84] S. Nagaraj, M. Collazo, C. A. Corzo et al., "Regulatory myeloid suppressor cells in health and disease," *Cancer Research*, vol. 69, no. 19, pp. 7503–7506, 2009.

[85] S. Ostrand-Rosenberg, "Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity," *Cancer Immunology, Immunotherapy*, vol. 59, no. 10, pp. 1593–1600, 2010.

[86] S. Ostrand-Rosenberg and P. Sinha, "Myeloid-derived suppressor cells: linking inflammation and cancer," *Journal of Immunology*, vol. 182, no. 8, pp. 4499–4506, 2009.

[87] I. R. Ramachandran, A. Martner, A. Pisklakova et al., "Myeloid-derived suppressor cells regulate growth of multiple myeloma by inhibiting T cells in bone marrow," *Journal of Immunology*, vol. 190, no. 7, pp. 3815–3823, 2013.

[88] G. T. Görgün, G. Whitehill, J. L. Anderson et al., "Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans," *Blood*, vol. 121, no. 15, pp. 2975–2987, 2013.

[89] K. R. Muthu Raja, L. Kovarova, and R. Hajek, "Induction by lenalidomide and dexamethasone combination increases regulatory cells of patients with previously untreated multiple myeloma," *Leukemia & Lymphoma*, vol. 53, no. 7, pp. 1406–1408, 2012.

[90] M. K. Brimnes, A. J. Vangsted, L. M. Knudsen et al., "Increased level of both $CD4^+FOXP3^+$ Regulatory t Cells and $CD14^+HLA\text{-}DR^-$/low myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma," *Scandinavian Journal of Immunology*, vol. 72, no. 6, pp. 540–547, 2010.

[91] Y. Lin, M. P. Gustafson, P. A. Bulur, D. A. Gastineau, T. E. Witzig, and A. B. Dietz, "Immunosuppressive $CD14^+$HLA-DRlow/-monocytes in B-cell non-Hodgkin lymphoma," *Blood*, vol. 117, no. 3, pp. 872–881, 2011.

[92] P. Serafini, I. Borrello, and V. Bronte, "Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression," *Seminars in Cancer Biology*, vol. 16, no. 1, pp. 53–65, 2006.

[93] E. van Valckenborgh, E. Schouppe, K. Movahedi et al., "Multiple myeloma induces the immunosuppressive capacity of distinct myeloid-Derived suppressor cell subpopulations in the bone marrow," *Leukemia*, vol. 26, no. 11, pp. 2424–2428, 2012.

[94] L. Dolcetti, E. Peranzoni, S. Ugel et al., "Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF," *European Journal of Immunology*, vol. 40, no. 1, pp. 22–35, 2010.

[95] J. Favaloro, T. Liyadipitiya, R. Brown et al., "Myeloid derived suppressor cells are numerically, functionally and phenotypically different in patients with multiple myeloma," *Leukemia & Lymphoma*, 2014.

[96] L. Yang, L. M. DeBusk, K. Fukuda et al., "Expansion of myeloid immune suppressor Gr^+CD11b^+ cells in tumor-bearing host directly promotes tumor angiogenesis," *Cancer Cell*, vol. 6, no. 4, pp. 409–421, 2004.

[97] L. Yang, C. M. Edwards, and G. R. Mundy, "$Gr\text{-}1^+CD11b^+$ myeloid-derived suppressor cells: formidable partners in tumor metastasis," *Journal of Bone and Mineral Research*, vol. 25, no. 8, pp. 1701–1706, 2010.

[98] A. Sawant, J. Deshane, J. Jules et al., "Myeloid-derived suppressor cells function as novel osteoclast progenitors enhancing bone loss in breast cancer," *Cancer Research*, vol. 73, no. 2, pp. 672–682, 2013.

[99] C. Melani, S. Sangaletti, F. M. Barazzetta, Z. Werb, and M. P. Colombo, "Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration

in tumor stroma," *Cancer Research*, vol. 67, no. 23, pp. 11438–11446, 2007.

[100] R. Wesolowski, J. Markowitz, and W. Carson, "Myeloid derived suppressor cells—a new therapeutic target in the treatment of cancer," *Journal For ImmunoTherapy of Cancer*, vol. 1, no. 1, article 10, 2013.

[101] P. Serafini, K. Meckel, M. Kelso et al., "Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function," *Journal of Experimental Medicine*, vol. 203, no. 12, pp. 2691–2702, 2006.

[102] L. Rudraraju, X. Ye, K. Noonan, C. A. Huff, and I. M. Borrello, "Administration of an oral PDE5 inhibitor, tadalafil In conjunction with a lenalidomide containing regimen in patients with multiple myeloma," *Blood*, vol. 122, no. 21, article 1959, 2013.

[103] K. A. Noonan, N. Ghosh, L. Rudraraju, M. Bui, and I. Borrello, "Targeting immune suppression with PDE-5 inhibition in end stage multiple myeloma: a case study," *Cancer Immunology Research*, 2014.

[104] N. Mitsiades, C. S. Mitsiades, V. Poulaki et al., "Biologic sequelae of nuclear factor-κB blockade in multiple myeloma: therapeutic applications," *Blood*, vol. 99, no. 11, pp. 4079–4086, 2002.

[105] I. Sakamaki, L. W. Kwak, S.-C. Cha et al., "Lenalidomide enhances the protective effect of a therapeutic vaccine and reverses immune suppression in mice bearing established lymphomas," *Leukemia*, 2013.

[106] L. Zitvogel, L. Galluzzi, M. Smyth, and G. Kroemer, "Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance," *Immunity*, vol. 39, no. 1, pp. 74–88, 2013.

[107] P. Liu, J. Jaffar, I. Hellstrom, and K. E. Hellstrom, "Administration of cyclophosphamide changes the immune profile of tumor-bearing mice," *Journal of Immunotherapy*, vol. 33, no. 1, pp. 53–59, 2010.

[108] A. Sistigu, S. Viaud, N. Chaput, L. Bracci, E. Proietti, and L. Zitvogel, "Immunomodulatory effects of cyclophosphamide and implementations for vaccine design," *Seminars in immunopathology*, vol. 33, no. 4, pp. 369–383, 2011.

[109] D. Alizadeh, M. Trad, N. T. Hanke et al., "Doxorubicin eliminates myeloid-derived suppressor cells and enhances the efficacy of adoptive T cell transfer in breast cancer," *Cancer Research*, 2013.

[110] E. Terpos, J. Berenson, N. Raje, and G. D. Roodman, "Management of bone disease in multiple myeloma," *Expert Review of Hematology*, vol. 7, no. 1, pp. 113–125, 2014.

[111] G. J. Morgan, F. E. Davies, W. M. Gregory et al., "Long-term follow-up of MRC Myeloma IX trial: survival outcomes with bisphosphonate and thalidomide treatment," *Clinical Cancer Research*, vol. 19, no. 21, pp. 6030–6038, 2013.

[112] A. Avilés, N. Neri, J. Huerta-Guzmán, and M. J. Nambo, "Randomized clinical trial of zoledronic acid in multiple myeloma patients undergoing high-dose chemotherapy and stem-cell transplantation," *Current Oncology*, vol. 20, no. 1, pp. e13–e20, 2013.

[113] T. E. Witzig, K. M. Laumann, M. Q. Lacy et al., "A phase III randomized trial of thalidomide plus zoledronic acid versus zoledronic acid alone in patients with asymptomatic multiple myeloma," *Leukemia*, vol. 27, no. 1, pp. 220–225, 2013.

[114] M. R. Porembka, J. B. Mitchem, B. A. Belt et al., "Pancreatic adenocarcinoma induces bone marrow mobilization of myeloid-derived suppressor cells which promote primary tumor growth," *Cancer Immunology, Immunotherapy*, vol. 61, no. 9, pp. 1373–1385, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 534969, 16 pages
http://dx.doi.org/10.1155/2014/534969

Review Article

Microenvironment, Oncoantigens, and Antitumor Vaccination: Lessons Learned from BALB-neuT Mice

Laura Conti, Roberto Ruiu, Giuseppina Barutello, Marco Macagno, Silvio Bandini, Federica Cavallo, and Stefania Lanzardo

Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Torino, Via Nizza 52, 10126 Torino, Italy

Correspondence should be addressed to
Federica Cavallo; federica.cavallo@unito.it

Received 10 April 2014; Accepted 12 May 2014; Published 3 June 2014

Academic Editor: Zhiqiang Meng

The tyrosine kinase human epidermal growth factor receptor 2 (*HER2*) gene is amplified in approximately 20% of human breast cancers and is associated with an aggressive clinical course and the early development of metastasis. Its crucial role in tumor growth and progression makes HER2 a prototypic oncoantigen, the targeting of which may be critical for the development of effective anticancer therapies. The setup of anti-HER2 targeting strategies has revolutionized the clinical outcome of $HER2^+$ breast cancer. However, their initial success has been overshadowed by the onset of pharmacological resistance that renders them ineffective. Since the tumor microenvironment (TME) plays a crucial role in drug resistance, the design of more effective anticancer therapies should depend on the targeting of both cancer cells and their TME as a whole. In this review, starting from the successful know-how obtained with a $HER2^+$ mouse model of mammary carcinogenesis, the BALB-neuT mice, we discuss the role of TME in mammary tumor development. Indeed, a deeper knowledge of antigens critical for cancer outbreak and progression and of the mechanisms that regulate the interplay between cancer and stromal cell populations could advise promising ways for the development of the best anticancer strategy.

1. Introduction

Solid tumors are currently considered to be organ-like structures, composed of cancer cells and other cells that support tumor development. While deep understanding of cancer cells has been reached, less light has been shed on the cell populations that make up the tumor microenvironment (TME), as they have been ostracized for several decades and are only now being reappraised as a driving force for tumor pathogenesis. TME is composed of cells—such as inflammatory cells, mesenchymal stem cells (MSCs), endothelial cells (ECs), cancer-associated fibroblasts (CAFs), and adipocytes (CAAs)—and soluble factors, cytokines, and the extracellular matrix (Figure 1) that bidirectionally communicate with cancer cells. This continuous and finely tuned interplay can promote cancer outbreak, sustain tumor development and invasion, defend a tumor from host immunity, foster therapeutic resistance, and provide niches for cancer stem cells (CSCs) and dormant metastases [1]. In this respect, TME is now considered to be a good target for anticancer therapies, as it provides the opportunity to perturb the delicate balance that promotes tumor progression. In fact, similarly to tumor cells [2], TME is now thought of as the source of a broad range of targets, of which the most promising are tumor-associated antigens that play a key role in cancer development and progression, called oncoantigens (OAs) [3]. We have recently classified OAs according to cellular localization [4]: Class I (cancer cell surface antigens), Class II (soluble antigens and antigens expressed in the TME), and Class III (intracellular proteins expressed by cancer cells). They are currently emerging as ideal targets for a very specific anticancer treatment, as demonstrated by several studies in preclinical models [3].

HER2 represents the prototypic Class I OA and is found to be overexpressed in a variety of human cancers [5]. HER2 amplification or overexpression is found in 15–20% of all

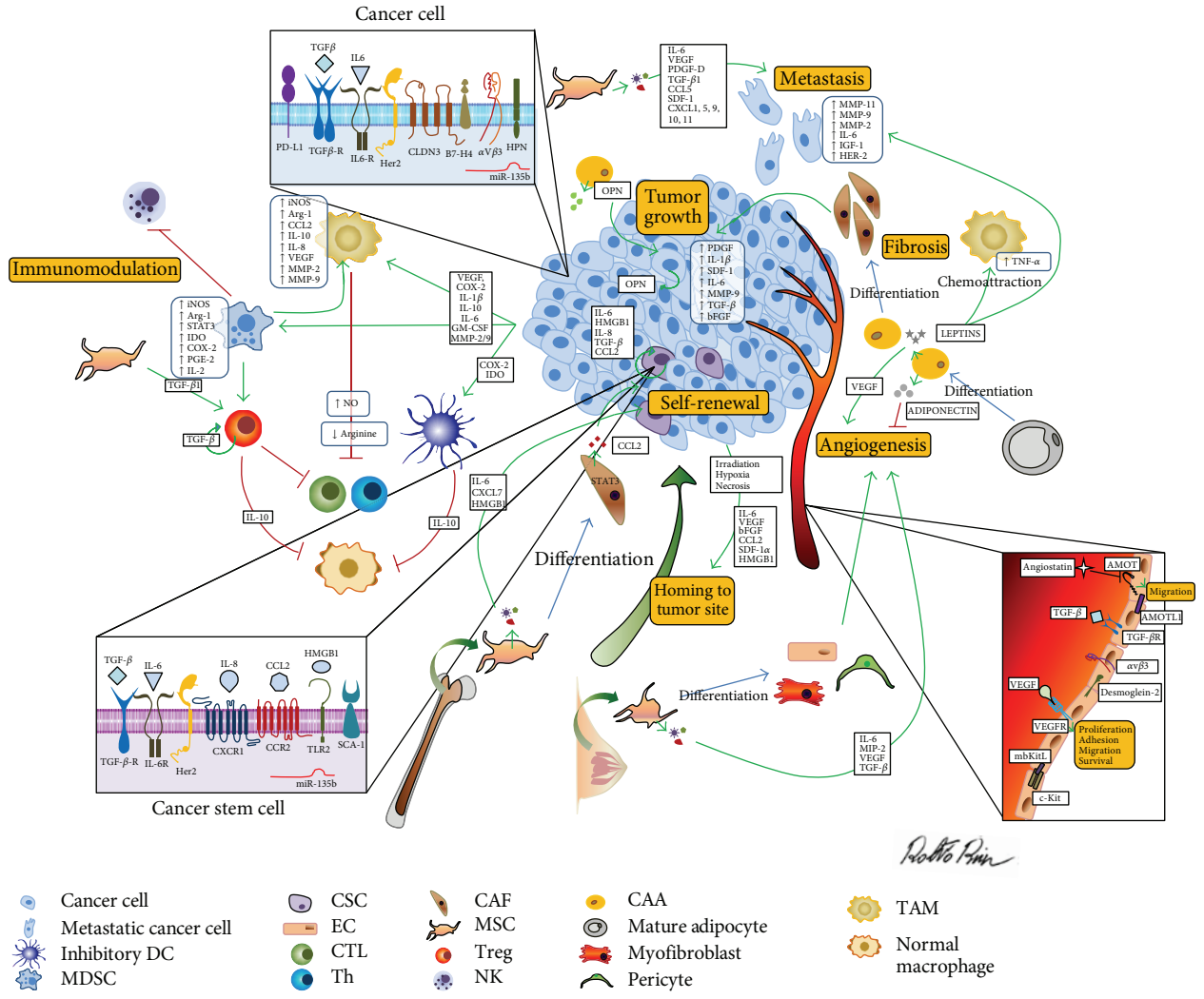


FIGURE 1: Interconnections between the population present in TME in breast tumors. Elevated levels of cytokines and growth factors produced by tumor and stromal cells orchestrate tumor development and progression. Abbreviations: mesenchymal stem cell (MSC), endothelial cell (EC), cancer-associated fibroblast (CAF), cancer stem cell (CSC) adipocyte (CAA), dendritic cell (DC), natural killer (NK), regulatory T (Treg) cell, myeloid derived suppressor cell (MDSC), tumor associated macrophages (TAMs), cytotoxic T lymphocytes (CTL), T helper (Th), interleukin (IL), toll-like receptor (TLR) 2, high mobility group box (HMGB) 1, vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP), stromal cell-derived factor- (SDF-) 1, transforming growth factor- (TGF-)β, chemokine (C-C motif) ligand (CCL)2, angiomotin (Amot), angiomotin-like (AmotL) 1, membrane-bound KitL (mbKitL), tumor necrosis factor- (TNF-)α, interferon- (IFN-)γ, nitric oxide synthase (iNOS), arginase (Arg) 1, indoleamine-2, 3-dioxygenase (IDO), cyclooxygenase- (COX-) 2, transcription factor signal transducer and activator of transcription (STAT) 3, programmed death (PD) 1, osteopontin (OPN), prostaglandin E- (PGE-) 2, platelet-derived growth factor (PDGF), macrophage inflammatory protein- (MIP-) 2, fibroblast growth factor (FGF), Insulin-like growth factor- (IGF-) 1, and tyrosine kinase human epidermal growth factor receptor (HER) 2.

new breast cancer cases and is a prognostic marker of poor outcome [6]. Currently, the identification of HER2 positivity in tumor tissue specimens allows for patient stratification and a more reasonable therapeutic strategy. Indeed, a number of tyrosine kinase inhibitors or monoclonal antibodies (mAbs) that are directed against HER2 are available, while others are currently under investigation in several phase I to III clinical trials [7]. Humanized mAb Trastuzumab is the standard of care in breast cancer treatment in both preoperatory and metastatic settings, whether it is used as a single agent or in association with chemotherapy. Despite initial responsiveness, the majority of patients that suffer from either primary or metastatic breast cancer develop drug resistance within one year, rendering Trastuzumab completely ineffective [8]. Similarly, prolonged exposition to anti-HER2 tyrosine kinase inhibitors often results in the development of HER-2-negative tumor variants [9]. The mechanisms that underlie primary and acquired resistance to HER2-targeted therapies are still under investigation. However, both CSCs and TME seem to play a crucial role in these phenomena [10]. This fact

emphasizes the need to consider cancer cells and their TME as a whole when designing effective anticancer therapies and tells us that targeting a single OA is not sufficient to freeze tumor progression, a possibility that can only be explored thanks to the availability of appropriate *in vivo* cancer models.

The identification of appropriate murine models that are able to mimic most of the features of a human cancer offers considerable potential to give advantages in the race towards the clinic. In particular, the availability of tumor-transplantable models and genetically engineered mammary cancer-prone mice has allowed laboratories to decipher the most important mechanisms involved in mammary tumor development and progression, thus permitting current therapies to be refined. A great deal of data has been obtained by our group from transgenic mice, called BALB-neuT, that overexpress the rat HER2 (neu) oncogene under the mouse mammary tumor virus (MMTV) promoter [11], with this very fact in mind. These mice spontaneously develop mammary carcinomas with 100% penetrance [12] and display a histopathologically [13] and transcriptionally [14] well characterized course that closely recapitulates many features of human breast carcinogenesis. In virtue of the high homology of BALB-neuT tumors to human HER2 positive breast cancer, this is an ideal model to use when setting up new anticancer therapies. Actually, BALB-neuT mice and the cell line derived from a BALB-neuT adenocarcinoma (TUBO cells) have provided us with a fascinating tool and one that is used in many laboratories worldwide to deepen current knowledge of the pathogenic mechanisms that promote HER2 positive tumor growth and consequently elaborate more efficacious antitumor strategies. We herein discuss the lessons learned about TME, HER2, and other OAs from BALB-neuT mice and how this knowledge can help develop a winning strategy against cancer.

2. The Urgency of Defining the Most Promising TME-Associated OAs

Neoplastic transformation is a multistep process which involves specific proteins and regulatory pathways at each stage. The identification of the genes that constitute the driving force of cancer progression is an extraordinary opportunity to gain an advantage over cancer. HER2 represents a paradigm of this conception; its expression at the neoplastic stage, its overexpression in established tumors, and its causal role in cancer progression [14] make it the ideal immunological target. This observation has paved the way for the development of new immunologically based therapies against neoplastic cells that overexpress HER2, which have made some important clinical achievements [15]; the U.S. Food and Drug Administration (FDA) has approved mAbs that target HER2, such as Trastuzumab and Pertuzumab, and several drugs (i.e., TDM1 and ARRY-380) [16], which have prolonged the disease-free survival rates in patients with metastatic HER2 positive breast cancer [17] and are currently under investigation in clinical trials. However, the majority of patients treated with these agents develop resistance within one year of treatment, resulting in disease progression, recurrence, and reduced overall survival [18]. Similar results have also been obtained using active immunotherapy against HER2 in preclinical models [19]. The efficacy of DNA vaccines targeting HER2 in BALB-neuT mice [20] relies mostly on the direct activity of vaccine-elicited Abs [21–23] and is strictly dependent on the tumor stage at the time of vaccination; the sooner the vaccination is performed, the better the outcome [24]. When the vaccine is administered to a still healthy BALB-neuT mouse, repeated boosts keep it tumor free for a period of time that may well equate to its natural life span. However, when the same vaccine is administered to a mouse in a more advanced stage of microscopic lesions, the appearance of palpable tumors is only slightly delayed. This suggests that targeting a single oncoantigen is not sufficient to freeze tumor progression, especially when it is applied to patients that suffer from advanced cancer, as commonly happens in the clinical setting [12].

This partial failure of anti-HER2 treatment suggests that some key elements that drive mammary carcinogenesis must still be sought out and not only on the tumor cells themselves; the best chance of defeating cancer that we have is offered by targeting both cancer cells and TME. TME can dynamically control cancer progression thanks to its continuous interplay with cancer cells [25]. Therefore, the identification of additional OAs that are expressed by either tumor or stromal cells surrounding HER2 positive lesions is urgently needed if we are to develop a combined and more efficient anticancer approach which may prevent the development of the very resistance to anti-HER2 therapy that is responsible for tumor relapse [26].

To address this point, we performed a transcription profile analysis of BALB-neuT preneoplastic and invasive lesions, integrated with a meta-analysis of data obtained from healthy human and neoplastic specimens. Of the 46 putative OAs identified [27], B7-H4 [28], Claudin 3 [29], Hepsin [30], CD52 [31], and Desmoglein 2 [32] are Class I OAs, expressed on the plasma membrane of cancer and TME cells and therefore constitute promising targets for vaccination. Class II OAs are another group of identified OAs and includes cytokines and chemokines copiously released in the TME. These molecules play important roles in establishing the strictly tuned relationship between tumor and stromal cells whose balance is critical for tumor development and progression, as will be discussed in the following sections of this review. Moreover, this analysis led us to identifying many Class III OAs that belong to signal transduction pathways reported to be deregulated in breast and other cancers, such as mitogen activated protein kinase (MAPK) [33], Survivin [34], Aurora kinase [35], and *src* pathway molecules [36]. It is worth noting that some of these networks seem to be regulatory keys of therapeutic resistance, such as Survivin [37], Topoisomerase II α [38], Desmoglein 2 [39], BCL2-interacting killer [40], and ribonucleotide reductase M2 polypeptide [41]. In addition, several identified proteins have a role in CSC self-renewal, which has been demonstrated in the cases of maternal embryonic leucine zipper kinase [42], transcription factor AP-2 γ [43], the microtubule associated TPX2 protein [44], and Aurora kinase A [44]. At present, our

efforts are focused on the characterization of some of these targets and our final goal is the setup of new DNA vaccines that will be tested in BALB-neuT mice in association with anti-HER2 vaccination, in order to improve the vaccination's efficacy against advanced tumor and metastases. A more detailed analysis of OAs that are selectively expressed by the various populations that constitute TME may end up providing us with a sort of tumor Rosetta Stone which could help unveil the reciprocal connection between tumor, CSCs, and stroma.

As reported in several clinical studies, the expression of noncoding genes, such as microRNAs (miRNAs), correlates with cancer relapse and metastasis formation [45]. Several miRNAs contribute to tumor progression in virtue of their ability to posttranscriptionally modulate the expression of oncogenes or oncosuppressors. They can act directly on TME, regulating both the survival of more differentiated cancer cells and the maintenance of a CSC phenotype [46] and controlling neoangiogenesis during tumor progression [47]. Results from experimental studies, which have been strengthened by the human cancer miRNA expression profile, have led researchers to the identification of miRNAs as potent regulators of the crosstalk between cancer and stromal cells [48]. Even if miRNAs cannot be considered oncoantigens because of their lack of immunogenicity, the identification of miRNAs, which are differentially expressed in the tumor, can lead to the identification of their target genes as potential oncoantigens or oncosuppressors, nevertheless [19].

Of note among the miRNAs that have recently been identified is the strong upregulation of miR-135b which has been found in invasive mammary BALB-neuT carcinomas; acting on its targets, midline 1 (MID1) and mitochondrial carrier homolog 2 (MTCH2), it regulates CSC stemness *in vitro* and cancer cell metastatization *in vivo* [49]. This newly unveiled role for miR-135b in mammary carcinogenesis, as observed in other tumors such as colon cancer [50], osteosarcoma [51], ependymoma [52], and hepatocellular carcinoma [53], can provide the basis for the exploration of miR-135b, MID1, and MTCH2's potential as new therapeutic targets in mammary carcinogenesis.

3. CSCs on Stage

The scientific spotlight has very recently been pointed on CSCs, the subpopulation of cells endowed with self-renewal potential and refractoriness to chemo- and radiotherapy that are capable of sustaining tumor growth and progression by giving rise to the heterogeneous population of tumor cells found within a tumor [54]. Even though the initial idea of CSCs as static entities [55] has been overtaken [56], it is well accepted that they control cancer development and progression in a manner that is guided by environmental factors [57]. CSCs are thought to reside in a highly specialized niche that is made up of stromal, endothelial, and more differentiated tumor cells that stimulate CSC survival and stemness via cell to cell contact, paracrine, and other signals [58]. A central role is played here by interleukin- (IL-) 6, which is produced by CSCs and noncancerous cells, MSCs, and immune cells. IL-6 promotes CSC self-renewal, the recruitment of MSCs and immune cells, and the preservation of an inflammatory state that favors tumor growth. Moreover, IL-6 promotes the conversion of more differentiated tumor cells into CSCs, inducing the epithelial-to-mesenchymal transition (EMT). Recently, it has been shown that HER2 overexpression in breast CSCs increases IL-6 secretion [59] which is involved in Trastuzumab resistance [60].

We have recently demonstrated that an autocrine loop involving toll-like receptor 2/high mobility group box-1/NFκB (TLR2/HMGB1/NFκB) induces the enhanced secretion of vascular endothelial growth factor (VEGF) and IL-6 in $Sca1^+$ [61] CSCs, derived from BALB-neuT TUBO cells, that in turn activates the transcription factor signal transducer and activator of transcription 3 (STAT3), thus promoting CSC self-renewal [62]. This pathway also induces the secretion of transforming growth factor- (TGF-)β, a cytokine that induces EMT and the secretion of matrix components that favor metastatization [63]. Moreover, TGF-β recruits endothelial cells and promotes their proliferation, enhancing angiogenesis [64]. Therefore, HER2 positive CSCs promote their own self-renewal, by upregulating TLR2 and secreting its endogenous ligand HMGB1, and generate a favorable microenvironment for tumor progression. This is a very important observation since HMGB1 is not only secreted by CSCs but also secreted by activated dendritic cells (DCs) [65] and necrotic cells [66] and thus is one of the most important molecules driving tumor escape from cytotoxic treatment.

IL-6 stimulates CSCs, MSCs, and fibroblasts and causes them to secrete IL-8, another key cytokine that promotes CSC self-renewal. It is worth noting that HER2 positive CSCs overexpress IL-8 receptors CXCR1/2 [67], which in turn induce HER2 phosphorylation and the activation of its downstream signaling pathway, generating a positive feedback mechanism that promotes CSC expansion [68]. The inhibition of CXCR1, either by mAbs or specific inhibitors, reduces CSC self-renewal, induces cell apoptosis, and inhibits metastatization in breast cancer, indicating that this receptor may be a promising target for combined anticancer therapies [69]. Similar IL-6-dependent upregulation is observed in the chemokine (C-C motif) ligand (CCL) 2 (also known as monocyte chemotactic protein-1, MCP-1), whose production is induced by IL-6 in both tumor cells and stromal cells and that supports the expansion of the CSC compartment by activating the Notch1 signaling pathway [70]. We demonstrated, by microarray analysis, that CCL2 expression increases in BALB-neuT mice as carcinogenesis progresses [71], and its causal role in cancer development was further supported by the observation that BALB-neuT mice, which were knocked-out (KO) for CCL2, displayed prolonged survival over BALB-neuT mice wild-type (WT) for this chemokine [72].

The characterization of all the cytokine networks that connect CSCs, tumor cells, and stromal cells may pave the way for new therapeutic strategies and provide diagnostic and prognostic markers for patients. In this regard, many clinical studies have shown that high serum levels of IL-8 and IL-6 correlate with poor prognosis in breast cancer

patients [73, 74]. Therefore, the design of specific cytokine receptor inhibitors and the assessment of their efficacy in clinical settings may be a source of great potential for future research.

4. Fighting against Proangiogenic OAs

Vascular ECs thoroughly govern angiogenesis, a process that supports the growth of many kinds of solid tumors including breast cancer, providing nutrients and oxygen to proliferating cells, thereby allowing cancer cells to invade tissues and develop metastases. Tumor cells have been observed to preferentially align towards and associate with ECs, even prior to the angiogenic switch [75]. Thanks to this strategic tidiness, ECs and tumor cells can bidirectionally communicate through a complex network of both soluble and insoluble signaling molecules that drive cellular differentiation and find ways to foster the tumor. Moreover, ECs are the most important interface between circulating blood cells, tumor cells, and extracellular matrix and play a pivotal role in controlling leukocyte recruitment and tumor cell behavior during angiogenesis.

A great deal of effort has been poured into attempts to block tumor angiogenesis. In this respect, VEGF-A is nowadays the most renowned therapeutic target. The interaction between VEGF ligands and their EC expressed receptors stimulates angiogenesis and promotes EC permeability, survival, migration, and the invasive potential of cancer cells [76]. Bevacizumab is a recombinant humanized mAb developed against VEGF-A [77], which has been broadly studied in phase III clinical trials and is now FDA-approved for the treatment of metastatic colorectal cancer, nonsmall cell lung cancer, and breast cancer [78]. Other drugs that inhibit the tyrosine kinase activity of VEGFRs, like sunitinib [79], sorafenib [80], axitinib [81], pazopanib [82], vandetanib [83], cabozantinib [84], tivozatinib [85], and linifanib [86], have been developed. Sorafenib has been approved for the treatment of unresectable hepatocellular carcinoma and advanced renal cell carcinoma (RCC), whereas sunitinib has been approved for the treatment of gastrointestinal stromal tumors and metastatic RCC, but only modest benefit has been observed in other types of cancer [87].

Despite many steps forward in the setup of antiangiogenic protocols being made, the development of tumor resistance and the occurrence of relapse in a high percentage of patients have prompted clinicians and researchers to join forces and find new targets for the development of more efficacious therapies. For these reasons, the immune-targeting of OAs expressed on ECs seems to be a successful direction to move towards. As described below, we have tested various DNA vaccination strategies that target tumor angiogenesis; all these vaccines have demonstrated high efficacy without any toxic effect, further stressing the therapeutic potential of targeting tumor ECs in HER2 positive tumors.

Of the class II OAs found to be overexpressed in tumor ECs during BALB-neuT cancer progression [88], the most promising is angiomotin (Amot), a member of the Motin protein family. Using a construct that encodes the kringle domains 1–4 of angiostatin to screen a yeast two-hybrid placenta cDNA library for angiostatin-binding peptides [89], Amot was originally identified as one of the angiostatin receptors. Amot is normally expressed on ECs, where it exerts its proangiogenic activity and stimulates EC migration during angiogenesis [90]. Amot is overexpressed compared to normal tissues in human breast tumors and its presence correlates with poor prognosis and metastatic disease [90]. These findings suggest that Amot has an important role to play during breast tumor progression and may be an optimal target for anticancer therapy [91]. In virtue of these features, we decided to elicit an immunological response against Amot, by means of DNA vaccination, in mice that bear microscopic invasive mammary cancers. This strategy was successfully applied in BALB-neuT mice as well as in the PyMT mouse model of breast cancer, in which carcinogenesis is driven by the polyoma middle T oncoantigen [92]. The therapeutic effect of anti-Amot vaccination was mediated by the induction of specific antibodies that induced increased tumor vessel permeability, which, in turn, resulted in both an increase in chemotherapy efficacy and major epitope spreading, which was accompanied by the induction of a specific anti-HER2 antibody response that further contrasted tumor growth [93].

Another member of the Motin family, angiomotin-like 1 (AmotL1), is an attractive target for antitumor interventions. AmotL1 is endowed with proangiogenic properties that affect EC polarization, directional migration, and the stability of tight junctions during angiogenic sprouting; it may compensate for the absence of Amot and vice versa [94]. Even though our preliminary data indicate that DNA vaccination against AmotL1 is not effective in the prevention of mammary tumor appearance in BALB-neuT mice, encouraging data have come from a combined DNA vaccine against HER2 and AmotL1. Even more promising results have been obtained using a combined DNA vaccine against HER2, Amot, and AmotL1 (Barutello G et al., unpublished data). This kind of vaccination exploits the synergistic effect which stems from the combined action of antibodies which target both the ECs of neoformed tumor vessels and the tumor cells themselves.

Membrane-bound KitL (mbKitL), which is involved in the c-Kit/KitL system required for tumor angiogenesis [95], is an additional promising target for antiangiogenic cancer immunotherapy. mbKitL is expressed on tumor ECs and is essential for providing them with survival signals, as is clearly exploited in the role that c-Kit signaling network plays in maintaining breast cancer cells [96]. A DNA vaccine that targets mbKitL is able to inhibit the growth of a mouse HER2 positive transplantable tumor; vaccination impairs tumor vessel formation and stabilization and thus interferes with tumor cell-derived VEGF bioavailability [97].

Besides representing good targets for anticancer therapies, antigens expressed on tumor ECs may also be exploited for tumor diagnosis. In this context, we have recently demonstrated that both ECs and cancer cells in mammary tumors arising in BALB-neuT mice express $\alpha_v\beta_3$ integrin, a receptor for several extracellular matrix proteins which harbor an arginine-glycine-aspartic acid (RGD) sequence [98]. $\alpha_v\beta_3$ integrin is widely considered to be a marker of

the angiogenesis, tumor progression, and invasion of different types of cancer. Since its level of expression correlates with cancer progression [99], we have developed a probe for the optical imaging detection of $\alpha_v\beta_3$ integrin and have shown that it can successfully detect microscopic *in situ* carcinomas in BALB-neuT mice, therefore proving itself to be a promising tool for the early diagnosis of breast cancer [98].

5. The Controversial Role of Inflammation and Immune Cells in the TME

Despite the fact that natural immune surveillance mechanisms are activated during the early stages of BALB-neuT carcinogenesis [100–103], tumors finally acquire the three immune hallmarks required to progress: the ability to thrive in a chronically inflamed TME, to suppress immune reactivity, and to evade immune recognition [104]. The fight between natural immune surveillance mechanisms and these acquired capabilities is mirrored by the important, yet controversial, role that immune cell infiltrates play in the TME. The tumor stroma of BALB-neuT mice is infiltrated by CD4 and CD8 T lymphocytes and a few B, natural killer (NK), and $\gamma\delta$ T lymphocytes, but mostly by regulatory T (Tregs) cells, myeloid derived suppressor cells (MDSCs), and tumor associated macrophages (TAMs) that are recruited into TME in response to inflammatory molecules and cytokines being released in the tumor milieu [105, 106].

The acquired ability of BALB-neuT tumors to thrive in a chronically inflamed microenvironment has been highlighted by microarray analyses that have shown the occurrence of an upregulation in four transcriptional networks, in advanced as compared to preneoplastic lesions, whose hub genes code for proinflammatory cytokines IL-1β, tumor necrosis factor- (TNF-)α, interferon- (IFN-)γ, and CCL2 [71]. The final outcome of the activation of these four networks is tumor promotion; however, how each individual network influences tumor progression is neither simple nor unequivocal. For instance, increased IFN-γ release in TME during tumor progression appears to play a major tumor inhibitory role and is a marker of the M1 TAMs that express immunostimulatory, antiangiogenic, and tumoricidal functions [107]. Accordingly, IFN-γ KO BALB-neuT mice display faster tumor progression, associated with a more intense tumor angiogenesis [71, 108, 109]. Moreover, chronic systemic administration of recombinant IL-12 in BALB-neuT mice induced high and sustained IFN-γ production, as detected in the sera of treated mice that in turn caused a delay in tumor onset and a reduction in the number of mammary glands affected by the tumor [109, 110]. The role that the other three networks play in tumor progression is the opposite. They can initially show antitumor activity, but the incipient tumor soon uses them to provide itself with a shortcut for progression. In reality, the activation of CCL2 is directly associated with enhanced progression [72], as discussed above. Similarly, increases in IL-1β and TNF-α in TME may favor cancer progression either directly [71, 111] or by recruiting suppressor cells [112, 113].

A tumor's ability to exploit inflammation to its own benefit is strictly related to the second immune hallmark of cancer, the capability to suppress the immune response directly or via the recruitment of suppressor cells [104]. IL-1β released by stromal cells together with other tumor-derived factors, including granulocyte macrophage colony-stimulating factor (GM-CSF), cyclooxygenase 2 (COX-2), IL-6, and VEGF, induce the accumulation and expansion of MDSCs [112, 113] by triggering Janus kinase (JAK)/STAT3 pathways [114]. MDSCs are a phenotypically heterogeneous population with an immunosuppressive capacity that are, in normal conditions, generated from the bone marrow and rapidly differentiates into mature DCs, macrophages, or granulocytes, while, in cancer bearing patients, present a partial block of maturation [115]. In BALB-neuT tumors, VEGF was detected in the supernatant from primary tumor cultures and from tumor cell lines as well as in the sera of BALB-neuT tumor-bearing mice. A possible explanation may lie in the increase of matrix metalloproteinase-(MMP-) 9 within the tumor mass, as previously shown [116], that mediates the release of growth factors, such as VEGF, stromal cell-derived factor- (SDF-) 1, and mbKitL [117]. Accordingly, any interference with VEGF or mbKitL activity, besides hampering the angiogenic process [97, 118], has been reported to induce MDSC shrinkage [97, 119].

MDSCs exhibit immunosuppressive functions that occur via multiple mechanisms, such as inducible nitric oxide synthase (iNOS) and arginase-1 (Arg-1) production, which suppresses the T-cell immune response in TME via the release of nitric oxide and reactive oxygen species that cause T cell receptor (TCR) nitration and T cell apoptosis and the depletion of L-arginine required for T cell functions [120]. As indoleamine-2, 3-dioxygenase (IDO) appears to be involved in MDSC-mediated T cell inhibition [121] and cyclooxygenase- (COX-) 2 is required to induce, via prostaglandin E- (PGE-) 2, Arg-1 expression by MDSCs [122, 123], a considerable amount of effort is going into inhibiting these molecules [124]. In this respect, we are testing a therapeutic protocol that consists of the concomitant administration of anti-HER2 DNA vaccines and plasmids that code for IDO [125] or COX-2 or short hairpin (sh)RNAs in BALB-neuT mice [24].

In order to curb the significant MDSC contribution to suppressing the immune system, we have looked for additional targets that these cells express both in tumor bearing mice and in cancer patients. As discussed above, B7-H4, a member of the B7 family, has been identified as being overexpressed in BALB-neuT mouse invasive lesions and appears to be an excellent target candidate, thanks to its critical role in the regulation of antigen specific immune responses [3]. Indeed, within TME, the expression of B7-H4 by tumor cells and MDSCs seems to be involved in the inhibition of the T cell response to tumor associated antigens [126]. In the light of these considerations, we are developing DNA plasmids that code for both HER2 and B7-H4 shRNAs, and we propose an evaluation of their efficacy in the inhibition of mammary carcinogenesis (Macagno M, unpublished data). Another important pathway that contributes to tumor mediated immune suppression is found in

the CD28 family member, programmed death 1 (PD-1) and its ligand PD-L1 [127]. PD-L1 is expressed by both MDSCs and tumor cells [128] and its interaction with activated T cell expressed PD-1 promotes T cell tolerance by suppressing their cytotoxic capacity and cytokine secretion [127]. We were among the first to show that the PD-1 blockade results in an increased response to antitumor vaccination. In these experiments BALB-neuT mice were vaccinated against HER2 and concomitantly treated with the administration of anti-PD-1 mAb BAT [129].

In response to IL-1β stimulation, MDSCs also produce the suppressive cytokine IL-10 [130] which acts on TAMs inducing their reprogramming and polarization towards an M2 phenotype. M2 TAMs support tumor progression through the release of immunosuppressive (i.e., CCL2 and IL-10), proangiogenic (i.e., IL-8 and VEGF), and tissue remodeling (i.e., MMP-2 and MMP-9) factors. Their expansion in breast cancer tissues has been correlated with poor prognosis [131]. In BALB-neuT mice M2 TAMs are the main tumor infiltrating population [105]. The administration of zoledronic acid to BALB-neuT mice can revert M2 polarization by interfering with the mevalonate pathway and thus hamper IL-10 and VEGF production, recovering the release of IFN-γ in the mammary glands of treated mice [105].

HER2 and the other OAs expressed by mammary tumors in BALB-neuT mice are self-molecules toward which the immune system is tolerant [132]. As a consequence, the predominant effector T-cells in the TME are presumably constituted of low avidity OA-specific T cells whose activity is inhibited by Tregs that first expand in the spleen and tumor draining lymph nodes during cancer progression and in TME in later phases [100, 132, 133]. This situation reproduces what normally happens in tumor bearing patients [134] and is part of the ability to suppress immune reactivity that the tumor acquires during progression [104]. Indeed, natural immune surveillance somehow counteracts Treg expansion in the early phases of carcinogenesis in BALB-neuT mice. In complement C3 KO BALB-neuT mice, tumor progression occurs earlier and this is associated with the increased expansion of Treg cells over complement competent BALB-neuT mice [102]. This increased Treg expansion is prompted by a lack of C3a and C5a, whose receptor signaling is required during the early events of effector T cell activation [135] and negatively modulates Treg function by inducing FoxP3 downregulation [136]. Its absence in BALB-neuT C3 KO mice deflects naïve T cells into Treg [137] and potentiates their function [136].

The down modulation of MHC class I (MHC I) [138] is the mechanism most frequently exploited by tumor cells to escape from immune recognition [139]. It is intriguing that an inverse correlation exists between HER2 overexpression and the expression of MHC I and of the components of the antigen-processing machinery [140]. MHC I down modulation, albeit incomplete, means that cancer cells are more susceptible to NK cell-mediated lysis, if NK receptor activating ligands are present. This may have an impact on cancer progression at least in the initial stages of carcinogenesis. The fundamental role that NK cells play in hampering the expansion of incipient BALB-neuT tumors has been investigated in perforin (PFP) KO BALB-neuT mice, as the majority of NK mediated protection relies on the release of PFP on target cells. In fact, both female [103] and male [141] BALB-neuT PFP KO mice show fourfold increases in mammary carcinoma incidence. Nevertheless, preliminary results also indicate that advanced BALB-neuT tumors downregulate the expression of ligands that activate NK receptors (Lanzardo S, unpublished data), suggesting that advanced tumors reach a balance between a loss of sensitivity to $CD8^+$ T cell killing and the maintenance of NK-cell-inhibitory specificities. We are now evaluating the expression of MHC I and of some NK ligands in TUBO-derived CSCs to assess whether NK cells recognize and more efficiently kill CSCs than their differentiated counterparts, as has already been shown for colon cancer-derived CSCs [142].

6. Role of Adipocytes and Fibroblasts in Breast Cancer Progression

While immune cells are well recognized as major players in the orchestration of a permissive TME, other cell populations have only recently been recognized as active parts of the tumor promoting ability of TME. These include CAAs and FACs.

Besides its classical definition as a fat reservoir, adipose tissue is now considered to be a fully functioning endocrine organ [143] that secretes growth factors and cytokines, known as adipokines, which are involved in angiogenesis, immunity, and endocrine signaling [144]. Adipocytes enshroud the mammary gland, regulating epithelial cell growth during the hormonally controlled courses of mammary gland development, from pubertal maturation to involution after lactation [145].

The understanding of the important, but still underestimated, role of adipocytes in cancer stems from several studies which highlight the anatomical proximity of many tumors to adipose tissues and point to the positive correlation between obesity and higher cancer risk [146–149]. Adipocytes can, under the pressure of cancer cell stimuli, abdicate their physiological role in favor of tumor promoting activities in breast cancers that grow in an adipose tissue dominated context. In this way they become CAAs that exhibit decreased lipid content, reduced adipocytes marker expression, and an overexpression of proinflammatory cytokines and MMPs, such as MMP-11 and MMP-9 [150, 151]. It is worth noting that MMP-9 has been identified as being overexpressed in BALB-neuT mammary cancer which would seem to point to its important role during tumor progression.

A number of studies have shown that CAAs support and expedite breast cancer progression [152–154] by providing proinflammatory cytokines, such as IL-6, TNF-α, and reactive oxygen species [155]. On the other hand, IL-6 in breast TME seems to stimulate the proinvasive effects of CAAs, besides promoting CSC self-renewal as discussed above [150]. Moreover, CAAs in TME can differentiate in fibroblast-like cells that, together with other stromal cell populations, participate in the generation of dense collagenous stroma, the

so called desmoplastic response, typically observed in breast cancer [156].

CAAs functions are mainly mediated by leptin and adiponectin, two functionally opposite members of the adipokine family, that seem to play a pivotal role in cancer progression [157]. Leptin promotes tumor growth, eliciting the activity of several signaling pathways such as insulin-like growth factor-1 (IGF-1) and HER2 and inducing the expression of MMP-2, MMP-9, and VEGF, which finally promote cell migration and metastatic spreading [158, 159]. Furthermore, leptin exerts a chemoattractant effect on macrophages and monocytes [160] and stimulates them to produce the inflammatory cytokine TNF-α that in turn manifests proangiogenic activity [161]. On the other hand, adiponectin acts as an antiangiogenic and anti-inflammatory factor that is able to repress proliferation and induce apoptosis in breast cancer cells [147, 162]. Interestingly, some studies have found that caloric restriction can exert an anticancer effect via alterations in systemic IGF-1 and NF-κB levels [163].

Altogether these data suggest that the recently discovered therapeutic potential of adipocytes could open new and promising perspectives in breast cancer treatment. One example of this comes from the preclinical experience gained with adipokine osteopontin (OPN), also called "early T cell-activation gene 1," a multifunctional component of the extracellular matrix that has been linked to a plethora of autoimmune diseases [164]. OPN has very recently been rediscovered as a diagnostic and prognostic marker in HER2 positive breast cancer [165] and one whose abnormal expression in patients is linked to poor prognosis [166]. It has also been proposed that the autocrine production of OPN by tumor cells may be an important factor that allows invasion and survival to occur [167]. In fact, the interaction between extracellular matrix deposited OPN and cell adhesion molecules, such as $\alpha_v\beta_3$ integrins which are overexpressed in BALB-neuT tumors [98], increases both the expression of VEGF in ECs, allowing neovascularization, and the activation of connective tissue growth factor and cysteine-rich angiogenic inducer 61(CYR61), which enhances neovascularization and mammary tumor growth *in vivo* [168].

As previously mentioned, CAAs can differentiate into fibroblast-like cells that share many properties with CAFs [169]. CAFs promote tumor growth and invasion secreting proangiogenic factors (i.e., VEGF-A and MMP-9) [170], proinflammatory molecules (i.e., SDF-1, IL-6, and IL-1β) [171], and several growth factors (i.e., TGF-β, platelet-derived growth factor, PDGF, and basic fibroblast growth factor, bFGF) [172, 173]. In particular, the aberrant production of IL-6 and CCL2 in mammary cancer activates STAT3 in CAFs, which finally sustains tumor-associated inflammation and is required for breast cancer cell migration [174]. Certainly, in BALB-neuT mice this network seems to be particularly interesting, as in a BALB-neuT mice knock-in for a constitutively active Stat3 *allele*, we observed an earlier and more invasive onset of mammary tumors [175].

7. MSCs Are Key Players in the TME Orchestra

Adult multipotent MSCs make for a fascinating TME population which is able to control the interplay between cancer cells and tumor stroma. Physiologically, MSCs are located predominantly in the bone marrow and contribute to the maintenance and regeneration of a variety of connective tissues [176]. During injury and inflammation, they are recruited to damaged sites via the release of soluble molecules and operate in tissue remodeling [177].

MSCs also localize into different types of solid tumors which they first migrate towards then integrate into the tumor-associated stroma [178]. Recent studies have provided direct evidence that MSCs are recruited in TME by a broad range of soluble factors which are secreted by cancer cells and CSCs, including IL-6 [179], VEGF and bFGF [180], CCL2 [181], SDF-1α [182], and HMGB1 [183]. Moreover, stressful conditions, such as irradiation [184], hypoxia [185] and, cellular damage [183], can enhance the recruitment of MSCs to the site of growing tumors. Once there, MSCs contribute to the development of an active TME, in which bone marrow-derived MSCs generate CAFs, while local adipose tissue-derived MSCs contribute mainly to the vascular and fibrovascular stroma (pericytes, myofibroblasts, and ECs) [186]. In addition, MSCs interact with tumor cells and with all other stromal cells through a broad range of signaling molecules, generating complex crosstalk whose net effect is to stimulate tumor progression. For example, MSCs can promote breast cancer neoangiogenesis, possibly thorough the secretion of macrophage inflammatory protein 2 (MIP-2), VEGF, TGF-β, and IL-6 [187] and display potent immunomodulatory properties [188] that enable them to inhibit CTLs and NK cells by stimulating Tregs through the release of TGF-β1 [189].

Conflicting data have led to the hypothesis that two opposing immunological MSC phenotypes exist, one proinflammatory and one immunosuppressive, which are dependent on the engagement of specific TLRs [190]. The role of TLR2 is still debated, with some studies claiming that TLR2 activation on MSCs inhibits their immunosuppressive properties [191], while others argue that TLR2 stimulation does not affect this capability [192]. Notably, these considerations are mostly based on *in vitro* experiments. Therefore, BALB-neuT mice may well be a suitable tool for the difficult task of definitely clarifying the role of TLR2 in MSCs. Starting from our observation that TLR2 drives mammary CSC self-renewal [62], we are developing BALB-neuT mice that are KO for TLR2, in which we would like to characterize the role of TLR2 not only in CSCs but also in MSCs and other stromal populations.

MSCs are thought to contribute to CSC niche generation, thus regulating cancer cell stemness through multiple pathways and secreted factors (i.e., IL-6 and CXCL7 [193], PGE-2 [194], EGF, bFGF, bone morphogenic protein (BMP) 4, TGF-β1, SDF-1α, and CCL5 [195], among others) that increase CSC self-renewal and expand the CSC population.

Furthermore, MSCs promote various malignant features; they control the metastatic ability of breast cancer cells by inducing EMT through the secretion of PDGF-D [196], TGF-β1 [197], IL-6, and VEGF [198] and promote cancer cell

migration through the release of a plethora of chemokines such as CCL5 [199], CXCL1 and CXCL5 [200], CXCL9, CXCL10, and CXCL11 [201] or SDF-1 [202]. For all these reasons, MSCs represent an attractive target when considering the design of new and promising anticancer treatments. However, the lack of specific markers that discriminate MSCs from other cell types makes the direct targeting of the MSC population an unrealistic approach. An attempt to disrupt signaling pathways between MSCs and CSCs is more feasible. In fact, the experience we have gained with the BALB-neuT model suggests that some of the molecules released by MSCs, such as IL-6, TGF-β, and HMGB1, are key molecules in CSC self-renewal and cancer progression [62]. The targeting of these molecules or their receptors, which are somehow redundant in different malignant processes, may be a means by which to interfere with tumor pathogenesis on multiple levels.

In recent years, there has been growing interest in the use of MSCs as a tool for the target-specific delivery of therapeutic agents, because their avid tumor tropism means that they can act as a sort of Trojan horse. MSCs can be genetically engineered to express antitumor cytokines, such as IFN-β [203], IL-12 [204], and TRAIL [205], or prodrugs such as cytosine deaminase [206], which are then released directly into the tumor milieu, thus greatly reducing their systemic toxicity. These approaches have been shown to be effective in the management of various preclinical tumor models. However, these killer MSCs may still maintain all the protumoral features here described and some concerns still exist about the potential conversion of MSCs into cancer cells themselves [207]. Therefore, the actual exploitation of MSCs as a tool for anticancer therapy still needs more study, and BALB-neuT mice represent a good model through which to evaluate the feasibility of this approach, in the context of HER2 positive breast cancers.

8. Conclusions

The growth and progression of breast cancer cells depend not only on their intrinsic malignant potential but also on a mutual and continuous dialogue between cancer cells and stromal, immune, and endothelial cells within TME. Multidirectional interactions between several substances, such as cytokines, MMPs, and growth factors, secreted by all these populations closely cooperate for the generation of a permissive TME that is crucial for successful cancer progression. This complex and finely tuned interplay between cancer and stromal cells during breast cancer development is summarized in Figure 1.

Experimental studies, conducted on preclinical models, have provided significant hints as to how TME affects tumor progression and response to therapy. BALB-neuT mice are an emblematic example in this regard. Over the years, the exploitation of this model has allowed the identification of novel molecular targets to be carried out and has prompted us to develop new, promising therapeutic approaches. On the other hand, it has provided evidence that the direct targeting of cancer cells is not enough to obtain complete disease remission. This highlights the need to extend antitumor intervention beyond the tumor bulk, as targeting both cancer cells and other TME cell populations may be a more complete and effective strategy.

Given the significant role that CSCs play in the various steps of tumor development and TME modulation, we have recently focused on the identification of pathways that regulate CSC self-renewal and influence, on TME as well as on the investigation of CSC-specific antigens. Another promising field of study can be found in action on tumor angiogenesis; in particular, strategies that modulate vessel permeability may also stabilize tumor vessels and favor both the distribution of traditional drugs into the tumor milieu and immune cell accessibility. As in the BALB-neuT model, the tumor infiltrate is mainly composed of immunosuppressive cells. The addition of immunomodulatory strategies to standard anticancer approaches could be essential for a therapeutic success.

Other TME cell populations, which are still almost unexplored in this model and whose involvement in tumor pathogenesis is still in its infancy, are found in CAAs and CAFs. Given the tissue organization of mammary glands and of the tumor within, which is rich in adipose cells and fibrous tissue, the identification of markers that are overexpressed by CAFs and CAAs may lead to the eradication of these cells which favor cancer progression through the production of various cytokines and extracellular matrix proteins. The blockade of these soluble molecules or their receptors may be an interesting option, as the disruption of the TME signaling network may make cancer cells themselves more amenable to traditional approaches. The drugs used in these combined treatments may be successfully delivered to TME by exploiting the avid tropism of MSCs, which may be engineered in order to produce molecules that inhibit the different populations present into the TME.

In conclusion, the targeting of multiple TME populations may represent the best strategy for setting up innovative anticancer treatments that significantly improve patient survival and shrink the development of drug resistance; in this regard, BALB-neuT mice provide a suitable experimental setting, thanks to the high translational value of this model.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Federica Cavallo and Stefania Lanzardo equally contributed to this paper.

Acknowledgments

This work was supported by Grants from the Italian Association for Cancer Research (IG 11675), Fondazione Ricerca Molinette Onlus, the University of Turin, and the Compagnia di San Paolo (Progetti di Ricerca Ateneo/CSP). This work has

been supported by Fondazione Veronesi that granted L.C. in the 2014 Pink is Good Program. The authors thank Dr. Dale Lawson for critically reading the paper.

References

[1] H. Korkaya, S. Liu, and M. S. Wicha, "Breast cancer stem cells, cytokine networks, and the tumor microenvironment," *Journal of Clinical Investigation*, vol. 121, no. 10, pp. 3804–3809, 2011.

[2] P. Zhou, D. R. Shaffer, D. A. Alvarez Arias et al., "In vivo discovery of immunotherapy targets in the tumour microenvironment," *Nature*, vol. 506, no. 7486, pp. 52–57, 2014.

[3] F. Cavallo, R. A. Calogero, and G. Forni, "Are oncoantigens suitable targets for anti-tumour therapy?" *Nature Reviews Cancer*, vol. 7, no. 9, pp. 707–713, 2007.

[4] M. Iezzi, E. Quaglino, A. Amici, P. L. Lollini, G. Forni, and F. Cavallo, "DNA vaccination against oncoantigens: a promise," *Oncoimmunology*, vol. 1, no. 3, pp. 316–325, 2012.

[5] S. Ménard, P. Casalini, M. Campiglio, S. Pupa, R. Agresti, and E. Tagliabue, "HER2 overexpression in various tumor types, focussing on its relationship to the development of invasive breast cancer," *Annals of Oncology*, vol. 12, supplement 1, pp. S15–S19, 2001.

[6] D. J. Slamon, G. M. Clark, and S. G. Wong, "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene," *Science*, vol. 235, no. 4785, pp. 177–182, 1987.

[7] D. L. Nielsen, M. Andersson, and C. Kamby, "HER2-targeted therapy in breast cancer. Monoclonal antibodies and tyrosine kinase inhibitors," *Cancer Treatment Reviews*, vol. 35, no. 2, pp. 121–136, 2009.

[8] J. C. Thery, J. P. Spano, D. Azria, E. Raymond, and F. Penault Llorca, "Resistance to human epidermal growth factor receptor type 2-targeted therapies," *European Journal of Cancer*, vol. 50, no. 5, pp. 892–901, 2014.

[9] X.-R. Ren, J. Wei, G. Lei et al., "Polyclonal HER2-specific antibodies induced by vaccination mediate receptor internalization and degradation in tumor cells," *Breast Cancer Research*, vol. 14, no. 3, p. R89, 2012.

[10] F. M. Frame and N. J. Maitland, "Cancer stem cells, models of study and implications of therapy resistance mechanisms," *Advances in Experimental Medicine and Biology*, vol. 720, pp. 105–118, 2011.

[11] S. Rovero, A. Amici, E. Di Carlo et al., "DNA vaccination against rat Her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice," *Journal of Immunology*, vol. 165, no. 9, pp. 5133–5142, 2000.

[12] F. Cavallo, R. Offringa, S. H. van der Burg, G. Forni, and C. J. M. Melief, "Vaccination for treatment and prevention of cancer in animal models," *Advances in Immunology*, vol. 90, pp. 175–213, 2006.

[13] E. Quaglino, S. Rolla, M. Iezzi et al., "Concordant morphologic and gene expression data show that a vaccine halts HER-2/neu preneoplastic lesions," *Journal of Clinical Investigation*, vol. 113, no. 5, pp. 709–717, 2004.

[14] F. Cavallo, A. Astolfi, M. Iezzi et al., "An integrated approach of immunogenomics and bioinformatics to identify new Tumor Associated Antigens (TAA) for mammary cancer immunological prevention," *BMC Bioinformatics*, vol. 6, no. 4, article S7, 2005.

[15] A. M. Scott, J. P. Allison, and J. D. Wolchok, "Monoclonal antibodies in cancer therapy," *Cancer Immunity*, vol. 12, p. 14, 2012.

[16] G. D. Phillips, C. T. Fields, G. Li et al., "Dual targeting of HER2-positive cancer with trastuzumab emtansine and pertuzumab: critical role for neuregulin blockade in antitumor response to combination therapy," *Clinical Cancer Research*, vol. 20, no. 2, pp. 456–468, 2014.

[17] J. Baselga, J. Cortés, S.-B. Kim et al., "Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer," *The New England Journal of Medicine*, vol. 366, no. 2, pp. 109–119, 2012.

[18] V. Guarneri, E. Barbieri, M. V. Dieci, F. Piacentini, and P. Conte, "Anti-HER2 neoadjuvant and adjuvant therapies in HER2 positive breast cancer," *Cancer Treatment Reviews*, vol. 36, supplement 3, pp. S62–S66, 2010.

[19] P. L. Lollini, F. Cavallo, C. De Giovanni, and P. Nanni, "Preclinical vaccines against mammary carcinoma," *Expert Review of Vaccines*, vol. 12, no. 12, pp. 1449–1463, 2013.

[20] E. Quaglino, M. Iezzi, C. Mastini et al., "Electroporated DNA vaccine clears away multifocal mammary carcinomas in her-2/neu transgenic mice," *Cancer Research*, vol. 64, no. 8, pp. 2858–2864, 2004.

[21] A. Porzia, S. Lanzardo, A. Citti et al., "Attenuation of PI3K/Akt-mediated tumorigenic signals through PTEN activation by DNA vaccine-induced anti-ErbB2 antibodies," *Journal of Immunology*, vol. 184, no. 8, pp. 4170–4177, 2010.

[22] E. Quaglino, C. Mastini, A. Amici et al., "A better immune reaction to Erbb-2 tumors is elicited in mice by DNA vaccines encoding rat/human chimeric proteins," *Cancer Research*, vol. 70, no. 7, pp. 2604–2612, 2010.

[23] E. Quaglino, F. Riccardo, M. Macagno et al., "Chimeric DNA vaccines against ErbB2+ carcinomas: from mice to humans," *Cancers*, vol. 3, no. 3, pp. 3225–3241, 2011.

[24] E. Bolli, E. Quaglino, M. Arigoni et al., "Oncoantigens for an immune prevention of cancer," *The American Journal of Cancer Research*, vol. 1, no. 2, pp. 255–264, 2011.

[25] L. Vera-Ramirez, P. Sanchez-Rovira, C. L. Ramirez-Tortosa et al., "Gene-expression profiles, tumor microenvironment, and cancer stem cells in breast cancer: latest advances towards an integrated approach," *Cancer Treatment Reviews*, vol. 36, no. 6, pp. 477–484, 2010.

[26] A. Goltsov, D. Faratian, S. P. Langdon, P. Mullen, D. J. Harrison, and J. Bown, "Features of the reversible sensitivity-resistance transition in PI3K/PTEN/AKT signalling network after HER2 inhibition," *Cellular Signalling*, vol. 24, no. 2, pp. 493–504, 2012.

[27] R. A. Calogero, E. Quaglino, S. Saviozzi, G. Forni, and F. Cavallo, "Oncoantigens as anti-tumor vaccination targets: the chance of a lucky strike?" *Cancer Immunology, Immunotherapy*, vol. 57, no. 11, pp. 1685–1694, 2008.

[28] Y. Qian, L. Shen, L. Cheng, Z. Wu, and H. Yao, "B7-H4 expression in various tumors determined using a novel developed monoclonal antibody," *Clinical and Experimental Medicine*, vol. 11, no. 3, pp. 163–170, 2011.

[29] S. Lu, K. Singh, S. Mangray et al., "Claudin expression in high-grade invasive ductal carcinoma of the breast: correlation with the molecular subtype," *Modern Pathology*, vol. 26, no. 4, pp. 485–495, 2013.

[30] P. Xing, J.-G. Li, F. Jin et al., "Clinical and biological significance of hepsin overexpression in breast cancer," *Journal of Investigative Medicine*, vol. 59, no. 5, pp. 803–810, 2011.

[31] B. Bisig, P. Gaulard, and L. De Leval, "New biomarkers in T-cell lymphomas," *Best Practice and Research: Clinical Haematology*, vol. 25, no. 1, pp. 13–28, 2012.

[32] W. K. Fang, W. Gu, L. D. Liao et al., "Prognostic significance of desmoglein 2 and desmoglein 3 in esophageal squamous cell carcinoma," *Asian Pacific Journal of Cancer Prevention*, vol. 15, no. 2, pp. 871–876, 2014.

[33] K. K. Haagenson and G. S. Wu, "Mitogen activated protein kinase phosphatases and cancer," *Cancer Biology and Therapy*, vol. 9, no. 5, pp. 337–340, 2010.

[34] L. Klampfer, "The role of signal transducers and activators of transcription in colon cancer," *Frontiers in Bioscience*, vol. 13, no. 8, pp. 2888–2899, 2008.

[35] G. Vader and S. M. A. Lens, "The Aurora kinase family in cell division and cancer," *Biochimica et Biophysica Acta*, vol. 1786, no. 1, pp. 60–72, 2008.

[36] E. L. Mayer and I. E. Krop, "Advances in targeting Src in the treatment of breast cancer and other solid malignancies," *Clinical Cancer Research*, vol. 16, no. 14, pp. 3526–3532, 2010.

[37] B. N. Rexer and C. L. Arteaga, "Optimal targeting of HER2-PI3K signaling in breast cancer: mechanistic insights and clinical implications," *Cancer Research*, vol. 73, no. 13, pp. 3817–3820, 2013.

[38] N. Shafee, C. R. Smith, S. Wei et al., "Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors," *Cancer Research*, vol. 68, no. 9, pp. 3243–3250, 2008.

[39] I. Beyer, H. Cao, J. Persson et al., "Coadministration of epithelial junction opener JO-1 improves the efficacy and safety of chemotherapeutic drugs," *Clinical Cancer Research*, vol. 18, no. 12, pp. 3340–3351, 2012.

[40] H. Zhou, Y. Zhang, Y. Fu, L. Chan, and A. S. Lee, "Novel mechanism of anti-apoptotic function of 78-kDa glucose-regulated protein (GRP78): endocrine resistance factor in breast cancer, through release of B-cell lymphoma 2 (BCL-2) from BCL-2-interacting killer (BIK)," *Journal of Biological Chemistry*, vol. 286, no. 29, pp. 25687–25696, 2011.

[41] K. N. Shah, K. R. Mehta, D. Peterson, M. Evangelista, J. C. Livesey, and J. S. Faridi, "AKT-induced tamoxifen resistance is overturned by RRM2 inhibition," *Molecular Cancer Research*, vol. 12, no. 3, pp. 394–407, 2014.

[42] L. W. Hebbard, J. Maurer, A. Miller et al., "Maternal embryonic leucine zipper kinase is upregulated and required in mammary tumor-initiating cells in vivo," *Cancer Research*, vol. 70, no. 21, pp. 8863–8873, 2010.

[43] T.-H. Hsieh, C.-F. Tsai, C.-Y. Hsu et al., "Phthalates stimulate the epithelial to mesenchymal transitionthrough an HDAC6-dependent mechanism in human breastepithelial stem cells," *Toxicological Sciences*, vol. 128, no. 2, pp. 365–376, 2012.

[44] J. Regan, T. Sourisseau, K. Soady et al., "Aurora A kinase regulates mammary epithelial cell fate by determining mitotic spindle orientation in a Notch-dependent manner," *Cell Reports*, vol. 4, no. 1, pp. 110–123, 2013.

[45] V. S. Nair, L. S. Maeda, and J. P. A. Ioannidis, "Clinical outcome prediction by MicroRNAs in human cancer: a systematic review," *Journal of the National Cancer Institute*, vol. 104, no. 7, pp. 528–540, 2012.

[46] S. F. Tavazoie, C. Alarcón, T. Oskarsson et al., "Endogenous human microRNAs that suppress breast cancer metastasis," *Nature*, vol. 451, no. 7175, pp. 147–152, 2008.

[47] C. Urbich, A. Kuehbacher, and S. Dimmeler, "Role of microRNAs in vascular diseases, inflammation, and angiogenesis," *Cardiovascular Research*, vol. 79, no. 4, pp. 581–588, 2008.

[48] J. A. Wright, J. K. Richer, and G. J. Goodall, "MicroRNAs and EMT in mammary cells and breast cancer," *Journal of Mammary Gland Biology and Neoplasia*, vol. 15, no. 2, pp. 213–223, 2010.

[49] M. Arigoni, G. Barutello, F. Riccardo et al., "MiR-135b coordinates progression of ErbB2-driven mammary carcinomas through suppression of MID1 and MTCH2," *The American Journal of Pathology*, vol. 182, no. 6, pp. 2058–2070, 2013.

[50] Y. X. Wang, X. Y. Zhang, B. F. Zhang, C. Q. Yang, X. M. Chen, and H. J. Gao, "Initial study of microRNA expression profiles of colonic cancer without lymph node metastasis," *Journal of Digestive Diseases*, vol. 11, no. 1, pp. 50–54, 2010.

[51] R. R. Lulla, F. F. Costa, J. M. Bischof et al., "Identification of differentially expressed microRNAs in osteosarcoma," *Sarcoma*, vol. 2011, Article ID 732690, 6 pages, 2011.

[52] F. F. Costa, J. M. Bischof, E. F. Vanin et al., "Identification of micrornas as potential prognostic markers in ependymoma," *PLoS ONE*, vol. 6, no. 10, Article ID e25114, 2011.

[53] S. Liu, W. Guo, J. Shi et al., "MicroRNA-135a contributes to the development of portal vein tumor thrombus by promoting metastasis in hepatocellular carcinoma," *Journal of Hepatology*, vol. 56, no. 2, pp. 389–396, 2012.

[54] N. Y. Frank, T. Schatton, and M. H. Frank, "The therapeutic promise of the cancer stem cell concept," *Journal of Clinical Investigation*, vol. 120, no. 1, pp. 41–50, 2010.

[55] L. V. Nguyen, R. Vanner, P. Dirks, and C. J. Eaves, "Cancer stem cells: an evolving concept," *Nature Reviews Cancer*, vol. 12, no. 2, pp. 133–143, 2012.

[56] F. Cordero, M. Beccuti, C. Fornari et al., "Multi-level model for the investigation of oncoantigen-driven vaccination effect," *BMC Bioinformatics*, vol. 14, supplement 6, p. S11, 2013.

[57] E. Fessler, F. E. Dijkgraaf, F. De Sousa E Melo, and J. P. Medema, "Cancer stem cell dynamics in tumor progression and metastasis: is the microenvironment to blame?" *Cancer Letters*, vol. 341, no. 1, pp. 97–104, 2013.

[58] A. R. Chin and S. E. Wang, "Cytokines driving breast cancer stemness," *Molecular and Cellular Endocrinology*, vol. 382, no. 1, pp. 598–602, 2014.

[59] Z. C. Hartman, X.-Y. Yang, O. Glass et al., "HER2 overexpression elicits a proinflammatory IL-6 autocrine signaling loop that is critical for tumorigenesis," *Cancer Research*, vol. 71, no. 13, pp. 4380–4391, 2011.

[60] H. Korkaya, G.-I. Kim, A. Davis et al., "Activation of an IL6 inflammatory loop mediates trastuzumab resistance in $HER2^+$ breast cancer by expanding the cancer stem cell population," *Molecular Cell*, vol. 47, no. 4, pp. 570–584, 2012.

[61] C. Grange, S. Lanzardo, F. Cavallo, G. Camussi, and B. Bussolati, "SCA-1 identifies the tumor-initiating cells in mammary tumors of BALB-neuT trangenic mice," *Neoplasia*, vol. 10, no. 12, pp. 1433–1443, 2008.

[62] L. Conti, S. Lanzardo, M. Arigoni et al., "The noninflammatory role of high mobility group box 1/Toll-like receptor 2 axis in the self-renewal of mammary cancer stem cells," *The FASEB Journal*, vol. 27, no. 12, pp. 4731–4744, 2013.

[63] M. Pickup, S. Novitskiy, and H. L. Moses, "The roles of TGFbeta in the tumour microenvironment," *Nature Reviews Cancer*, vol. 13, no. 11, pp. 788–799, 2013.

[64] A. Chiechi, D. L. Waning, K. R. Stayrook, J. T. Buijs, T. A. Guise, and K. S. Mohammad, "Role of TGF- in breast cancer bone

metastases," *Advances in Bioscience and Biotechnology*, vol. 4, no. 10C, pp. 15–30, 2013.

[65] M. E. Bianchi and A. A. Manfredi, "High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity," *Immunological Reviews*, vol. 220, no. 1, pp. 35–46, 2007.

[66] P. Scaffidi, T. Misteli, and M. E. Bianchi, "Release of chromatin protein HMGB1 by necrotic cells triggers inflammation," *Nature*, vol. 418, no. 6894, pp. 191–195, 2002.

[67] J. Singh, B. M. Simões, S. J. Howell, G. Farnie, and R. B. Clarke, "Recent advances reveal IL-8 signalling as a potential key to targeting breast cancer stem cells," *Breast Cancer Research*, vol. 15, no. 4, p. 210, 2013.

[68] J. K. Singh, G. Farnie, N. J. Bundred et al., "Targeting CXCR1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting HER2 via HER2-dependent and -independent mechanisms," *Clinical Cancer Research*, vol. 19, no. 3, pp. 643–656, 2013.

[69] C. Ginestier, S. Liu, M. E. Diebel et al., "CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts," *Journal of Clinical Investigation*, vol. 120, no. 2, pp. 485–497, 2010.

[70] A. Tsuyada, A. Chow, J. Wu et al., "CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells," *Cancer Research*, vol. 72, no. 11, pp. 2768–2779, 2012.

[71] R. A. Calogero, F. Cordero, G. Forni, and F. Cavallo, "Inflammation and breast cancer. Inflammatory component of mammary carcinogenesis in ErbB2 transgenic mice," *Breast Cancer Research*, vol. 9, no. 4, article 211, 2007.

[72] I. Conti and B. J. Rollins, "CCL2 (monocyte chemoattractant protein-1) and cancer," *Seminars in Cancer Biology*, vol. 14, no. 3, pp. 149–154, 2004.

[73] I. H. Benoy, R. Salgado, P. Van Dam et al., "Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival," *Clinical Cancer Research*, vol. 10, no. 21, pp. 7157–7162, 2004.

[74] O. I. Ahmed, A. M. Adel, D. R. Diab, and N. S. Gobran, "Prognostic value of serum level of interleukin-6 and interleukin-8 in metastatic breast cancer patients," *The Egyptian Journal of Immunology*, vol. 13, no. 2, pp. 61–68, 2006.

[75] C.-Y. Li, S. Shan, Q. Huang et al., "Initial stages of tumor cell-induced angiogenesis: evaluation via skin window chambers in rodent models," *Journal of the National Cancer Institute*, vol. 92, no. 2, pp. 143–147, 2000.

[76] D. J. Hicklin and L. M. Ellis, "Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis," *Journal of Clinical Oncology*, vol. 23, no. 5, pp. 1011–1027, 2005.

[77] K. J. Kim, B. Li, J. Winer et al., "Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo," *Nature*, vol. 362, no. 6423, pp. 841–844, 1993.

[78] A. Grothey and L. M. Ellis, "Targeting angiogenesis driven by vascular endothelial growth factors using antibody-based therapies," *Cancer Journal*, vol. 14, no. 3, pp. 170–177, 2008.

[79] D. B. Mendel, A. Douglas Laird, X. Xin et al., "In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship," *Clinical Cancer Research*, vol. 9, no. 1 I, pp. 327–337, 2003.

[80] L. Adnane, P. A. Trail, I. Taylor, and S. M. Wilhelm, "Sorafenib (BAY 43-9006, Nexavar), a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature," *Methods in Enzymology*, vol. 407, pp. 597–612, 2005.

[81] L. J. Wilmes, M. G. Pallavicini, L. M. Fleming et al., "AG-013736, a novel inhibitor of VEGF receptor tyrosine kinases, inhibits breast cancer growth and decreases vascular permeability as detected by dynamic contrast-enhanced magnetic resonance imaging," *Magnetic Resonance Imaging*, vol. 25, no. 3, pp. 319–327, 2007.

[82] G. Sonpavde and T. E. Hutson, "Pazopanib: a novel multitargeted tyrosine kinase inhibitor," *Current Oncology Reports*, vol. 9, no. 2, pp. 115–119, 2007.

[83] A. J. Ryan and S. R. Wedge, "ZD6474—a novel inhibitor of VEGFR and EGFR tyrosine kinase activity," *British Journal of Cancer*, vol. 92, supplement 1, pp. S6–S13, 2005.

[84] U. Vaishampayan, "Cabozantinib as a novel therapy for renal cell carcinoma," *Current Oncology Reports*, vol. 15, no. 2, pp. 76–82, 2013.

[85] D. A. Nosov, B. Esteves, O. N. Lipatov et al., "Antitumor activity and safety of tivozanib (AV-951) in a phase II randomized discontinuation trial in patients with renal cell carcinoma," *Journal of Clinical Oncology*, vol. 30, no. 14, pp. 1678–1685, 2012.

[86] D. H. Albert, P. Tapang, T. J. Magoc et al., "Preclinical activity of ABT-869, a multitargeted receptor tyrosine kinase inhibitor," *Molecular Cancer Therapeutics*, vol. 5, no. 4, pp. 995–1006, 2006.

[87] S. Giuliano and G. Pagès, "Mechanisms of resistance to anti-angiogenesis therapies," *Biochimie*, vol. 95, no. 6, pp. 1110–1119, 2013.

[88] M. Ernkvist, O. Birot, I. Sinha et al., "Differential roles of p80- and p130-angiomotin in the switch between migration and stabilization of endothelial cells," *Biochimica et Biophysica Acta*, vol. 1783, no. 3, pp. 429–437, 2008.

[89] B. Troyanovsky, T. Levchenko, G. Månsson, O. Matvijenko, and L. Holmgren, "Angiomotin: an angiostatin binding protein that regulates endothelial cell migration and tube formation," *Journal of Cell Biology*, vol. 152, no. 6, pp. 1247–1254, 2001.

[90] A. Bratt, W. J. Wilson, B. Troyanovsky et al., "Angiomotin belongs to a novel protein family with conserved coiled-coil and PDZ binding domains," *Gene*, vol. 298, no. 1, pp. 69–77, 2002.

[91] W. G. Jiang, G. Watkins, A. Douglas-Jones, L. Holmgren, and R. E. Mansel, "Angiomotin and angiomotin like proteins, their expression and correlation with angiogenesis and clinical outcome in human breast cancer," *BMC cancer*, vol. 6, article 16, 2006.

[92] E. Y. Lin, J. G. Jones, P. Li et al., "Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases," *The American Journal of Pathology*, vol. 163, no. 5, pp. 2113–2126, 2003.

[93] M. Arigoni, G. Barutello, S. Lanzardo et al., "A vaccine targeting angiomotin induces an antibody response which alters tumor vessel permeability and hampers the growth of established tumors," *Angiogenesis*, vol. 15, no. 2, pp. 305–316, 2012.

[94] Y. Zheng, S. Vertuani, S. Nyström et al., "Angiomotin-like protein 1 controls endothelial polarity and junction stability during sprouting angiogenesis," *Circulation Research*, vol. 105, no. 3, pp. 260–270, 2009.

[95] Q. Li, G. Kondoh, S. Inafuku, Y. Nishimune, and A. Hakura, "Abrogation of c-kit/Steel factor-dependent tumorigenesis by kinase defective mutants of the c-kit receptor: c-kit Kinase

defective mutants as candidate tools for cancer gene therapy," *Cancer Research*, vol. 56, no. 19, pp. 4343–4346, 1996.

[96] J. L. Regan, H. Kendrick, F.-A. Magnay, V. Vafaizadeh, B. Groner, and M. J. Smalley, "C-Kit is required for growth and survival of the cells of origin of Brca1-mutation-associated breast cancer," *Oncogene*, vol. 31, no. 7, pp. 869–883, 2012.

[97] C. Olgasi, P. Dentelli, A. Rosso et al., "DNA vaccination against membrane-bound Kit ligand: a new approach to inhibiting tumour growth and angiogenesis," *European Journal of Cancer*, vol. 50, no. 1, pp. 234–246, 2014.

[98] L. Conti, S. Lanzardo, M. Iezzi et al., "Optical imaging detection of microscopic mammary cancer in ErbB-2 transgenic mice through the DA364 probe binding $\alpha v\beta 3$ integrins," *Contrast Media and Molecular Imaging*, vol. 8, no. 4, pp. 350–360, 2013.

[99] P. C. Brooks, R. A. F. Clark, and D. A. Cheresh, "Requirement of vascular integrin $\alpha v\beta 3$ for angiogenesis," *Science*, vol. 264, no. 5158, pp. 569–571, 1994.

[100] E. Ambrosino, M. Spadaro, M. Iezzi et al., "Immunosurveillance of Erbb2 carcinogenesis in transgenic mice is concealed by a dominant regulatory T-cell self-tolerance," *Cancer Research*, vol. 66, no. 15, pp. 7734–7740, 2006.

[101] J. M. Park, M. Terabe, D. D. Donaldson, G. Forni, and J. A. Berzofsky, "Natural immunosurveillance against spontaneous, autochthonous breast cancers revealed and enhanced by blockade of IL-13-mediated negative regulation," *Cancer Immunology, Immunotherapy*, vol. 57, no. 6, pp. 907–912, 2008.

[102] S. Bandini, C. Curcio, M. Macagno et al., "Early onset and enhanced growth of autochthonous mammary carcinomas in C3-deficient Her2/neu transgenic mice," *Oncoimmunology*, vol. 2, no. 9, Article ID e26137, 2013.

[103] S. E. A. Street, N. Zerafa, M. Iezzi et al., "Host perforin reduces tumor number but does not increase survival in oncogene-driven mammary adenocarcinoma," *Cancer Research*, vol. 67, no. 11, pp. 5454–5460, 2007.

[104] F. Cavallo, C. De Giovanni, P. Nanni, G. Forni, and P.-L. Lollini, "2011: the immune hallmarks of cancer," *Cancer Immunology, Immunotherapy*, vol. 60, no. 3, pp. 319–326, 2011.

[105] M. Coscia, E. Quaglino, M. Iezzi et al., "Zoledronic acid repolarizes tumour-associated macrophages and inhibits mammary carcinogenesis by targeting the mevalonate pathway," *Journal of Cellular and Molecular Medicine*, vol. 14, no. 12, pp. 2803–2815, 2010.

[106] C. Mastini, P. D. Becker, M. Iezzi et al., "Intramammary application of non-methylated-CpG oligodeoxynucleotides (CpG) inhibits both local and systemic mammary carcinogenesis in female BALB/c Her-2/neu transgenic mice," *Current Cancer Drug Targets*, vol. 8, no. 3, pp. 230–242, 2008.

[107] A. Sica, T. Schioppa, A. Mantovani, and P. Allavena, "Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy," *European Journal of Cancer*, vol. 42, no. 6, pp. 717–727, 2006.

[108] M. Spadaro, E. Ambrosino, M. Iezzi et al., "Cure of mammary carcinomas in Her-2 transgenic mice through sequential stimulation of innate (neoadjuvant interleukin-12) and adaptive (DNA vaccine electroporation) immunity," *Clinical Cancer Research*, vol. 11, no. 5, pp. 1941–1952, 2005.

[109] L. Cifaldi, E. Quaglino, E. Di Carlo et al., "A light, non-toxic interleukin 12 protocol inhibits HER-2/neu mammary carcinogenesis in BALB/c transgenic mice with established hyperplasia," *Cancer Research*, vol. 61, no. 7, pp. 2809–2812, 2001.

[110] M. Vagliani, M. Rodolfo, F. Cavallo et al., "Interleukin 12 potentiates the curative effect of a vaccine based on interleukin 2-transduced tumor cells," *Cancer Research*, vol. 56, no. 3, pp. 467–470, 1996.

[111] R. J. Moore, D. M. Owens, G. Stamp et al., "Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis," *Nature Medicine*, vol. 5, no. 7, pp. 828–831, 1999.

[112] X. Song, Y. Krelin, T. Dvorkin et al., "CD11b+/Gr-1+ immature myeloid cells mediate suppression of T cells in mice bearing tumors of IL-1β-secreting cells," *Journal of Immunology*, vol. 175, no. 12, pp. 8200–8208, 2005.

[113] C. Melani, C. Chiodoni, G. Forni, and M. P. Colombo, "Myeloid cell expansion elicited by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice suppresses immune reactivity," *Blood*, vol. 102, no. 6, pp. 2138–2145, 2003.

[114] J. Bromberg, "Stat proteins and oncogenesis," *Journal of Clinical Investigation*, vol. 109, no. 9, pp. 1139–1142, 2002.

[115] B. Almand, J. I. Clark, E. Nikitina et al., "Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer," *Journal of Immunology*, vol. 166, no. 1, pp. 678–689, 2001.

[116] C. Melani, S. Sangaletti, F. M. Barazzetta, Z. Werb, and M. P. Colombo, "Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma," *Cancer Research*, vol. 67, no. 23, pp. 11438–11446, 2007.

[117] B. Heissig, K. Hattori, S. Dias et al., "Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of Kit-ligand," *Cell*, vol. 109, no. 5, pp. 625–637, 2002.

[118] I. Dimova, G. Popivanov, and V. Djonov, "Angiogenesis in cancer—general pathways and their therapeutic implications," *Journal of BUON*, vol. 19, no. 1, pp. 15–21, 2014.

[119] S. Kusmartsev, E. Eruslanov, H. Kübler et al., "Oxidative stress regulates expression of VEGFR1 in myeloid cells: link to tumor-induced immune suppression in renal cell carcinoma," *Journal of Immunology*, vol. 181, no. 1, pp. 346–353, 2008.

[120] B. Hoechst, T. Voigtlaender, L. Ormandy et al., "Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor," *Hepatology*, vol. 50, no. 3, pp. 799–807, 2009.

[121] J. Yu, W. Du, F. Yan et al., "Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer," *Journal of Immunology*, vol. 190, no. 7, pp. 3783–3797, 2013.

[122] N. Obermajer, J. L. Wong, R. P. Edwards, K. Odunsi, K. Moysich, and P. Kalinski, "PGE2-driven induction and maintenance of cancer-associated myeloid-derived suppressor cells," *Immunological Investigations*, vol. 41, no. 6-7, pp. 635–657, 2012.

[123] Y.-R. Na, Y.-N. Yoon, D.-I. Son, and S.-H. Seok, "Cyclooxygenase-2 inhibition blocks M2 macrophage differentiation and suppresses metastasis in murine breast cancer model," *PLoS ONE*, vol. 8, no. 5, Article ID e63451, 2013.

[124] B. N. P. Kumar, S. Rajput, K. K. Dey et al., "Celecoxib alleviates tamoxifen-instigated angiogenic effects by ROS-dependent VEGF/VEGFR2 autocrine signaling," *BMC Cancer*, vol. 13, article 273, 2013.

[125] M.-C. Yen, C.-C. Lin, Y.-L. Chen et al., "A novel cancer therapy by skin delivery of indoleamine 2,3-dioxygenase siRNA," *Clinical Cancer Research*, vol. 15, no. 2, pp. 641–649, 2009.

[126] C. He, H. Qiao, H. Jiang, and X. Sun, "The inhibitory role of b7-h4 in antitumor immunity: association with cancer progression and survival," *Clinical and Developmental Immunology*, vol. 2011, Article ID 695834, 8 pages, 2011.

[127] G. J. Freeman, A. J. Long, Y. Iwai et al., "Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation," *Journal of Experimental Medicine*, vol. 192, no. 7, pp. 1027–1034, 2000.

[128] J.-I. Youn, S. Nagaraj, M. Collazo, and D. I. Gabrilovich, "Subsets of myeloid-derived suppressor cells in tumor-bearing mice," *Journal of Immunology*, vol. 181, no. 8, pp. 5791–5802, 2008.

[129] E. Quaglino, C. Mastini, M. Iezzi et al., "The adjuvant activity of BAT antibody enables DNA vaccination to inhibit the progression of established autochthonous Her-2/neu carcinomas in BALB/c mice," *Vaccine*, vol. 23, no. 25, pp. 3280–3287, 2005.

[130] S. K. Bunt, V. K. Clements, E. M. Hanson, P. Sinha, and S. Ostrand-Rosenberg, "Inflammation enhances myeloid-derived suppressor cell cross-talk by signaling through Toll-like receptor 4," *Journal of Leukocyte Biology*, vol. 85, no. 6, pp. 996–1004, 2009.

[131] X. Tang, "Tumor-associated macrophages as potential diagnostic and prognostic biomarkers in breast cancer," *Cancer Letters*, vol. 332, no. 1, pp. 3–10, 2013.

[132] S. Rolla, C. Nicoló, S. Malinarich et al., "Distinct and non-overlapping T cell receptor repertoires expanded by DNA vaccination in wild-type and HER-2 transgenic BALB/c mice," *Journal of Immunology*, vol. 177, no. 11, pp. 7626–7633, 2006.

[133] H. Von Boehmer, "Mechanisms of suppression by suppressor T cells," *Nature Immunology*, vol. 6, no. 4, pp. 338–344, 2005.

[134] S. Occhipinti, L. Sponton, S. Rolla et al., "Chimeric Rat/Human HER2 efficiently circumvents HER2 tolerance in cancer patients," *Clinical Cancer Research*, 2014.

[135] M. G. Strainic, J. Liu, D. Huang et al., "Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells," *Immunity*, vol. 28, no. 3, pp. 425–435, 2008.

[136] M. G. Strainic, E. M. Shevach, F. An, F. Lin, and M. E. Medof, "Absence of signaling into $CD4^+$ cells via C3aR and C5aR enables autoinductive TGF-β1 signaling and induction of $Foxp3^+$ regulatory T cells," *Nature Immunology*, vol. 14, no. 2, pp. 162–171, 2013.

[137] W.-H. Kwan, W. van der Touw, E. Paz-Artal, M. O. Li, and P. S. Heeger, "Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells," *Journal of Experimental Medicine*, vol. 210, no. 2, pp. 257–268, 2013.

[138] P. L. Lollini, G. Nicoletti, L. Landuzzi et al., "Down regulation of major histocompatibility complex class I expression in mammary carcinoma of HER-2/neu transgenic mice," *International Journal of Cancer*, vol. 77, no. 6, pp. 937–941, 1998.

[139] F. Garrido and I. Algarra, "MHC antigens and tumor escape from immune surveillance," *Advances in Cancer Research*, vol. 83, pp. 117–158, 2001.

[140] M. Inoue, K. Mimura, S. Izawa et al., "Expression of MHC Class I on breast cancer cells correlates inversely with HER2 expression," *Oncoimmunology*, vol. 1, no. 7, pp. 1104–1110, 2012.

[141] M. Macagno, S. Bandini, L. Stramucci et al., "Multiple roles of perforin in hampering ERBB-2 (Her-2/neu) carcinogenesis in transgenic male mice," *Journal of Immunology*, vol. 192, no. 11, pp. 5434–5441, 2014.

[142] R. Tallerico, M. Todaro, S. Di Franco et al., "Human NK cells selective targeting of colon cancer-initiating cells: a role for natural cytotoxicity receptors and MHC class i molecules," *Journal of Immunology*, vol. 190, no. 5, pp. 2381–2390, 2013.

[143] P. E. Scherer, "Adipose tissue: from lipid storage compartment to endocrine organ," *Diabetes*, vol. 55, no. 6, pp. 1537–1545, 2006.

[144] A. R. Johnson, J. Justin Milner, and L. Makowski, "The inflammation highway: metabolism accelerates inflammatory traffic in obesity," *Immunological Reviews*, vol. 249, no. 1, pp. 218–238, 2012.

[145] R. C. Hovey and L. Aimo, "Diverse and active roles for adipocytes during mammary gland growth and function," *Journal of Mammary Gland Biology and Neoplasia*, vol. 15, no. 3, pp. 279–290, 2010.

[146] Y. Mao, E. T. Keller, D. H. Garfield, K. Shen, and J. Wang, "Stromal cells in tumor microenvironment and breast cancer," *Cancer and Metastasis Reviews*, vol. 32, no. 1-2, pp. 303–315, 2013.

[147] J. Park, D. M. Euhus, and P. E. Scherer, "Paracrine and endocrine effects of adipose tissue on cancer development and progression," *Endocrine Reviews*, vol. 32, no. 4, pp. 550–570, 2011.

[148] B. Majed, T. Moreau, K. Senouci, R. J. Salmon, A. Fourquet, and B. Asselain, "Is obesity an independent prognosis factor in woman breast cancer?" *Breast Cancer Research and Treatment*, vol. 111, no. 2, pp. 329–342, 2008.

[149] J. Ligibel, "Obesity and breast cancer," *Oncology*, vol. 25, no. 11, pp. 994–1000, 2011.

[150] B. Dirat, L. Bochet, M. Dabek et al., "Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion," *Cancer Research*, vol. 71, no. 7, pp. 2455–2465, 2011.

[151] K. Sun, C. M. Kusminski, and P. E. Scherer, "Adipose tissue remodeling and obesity," *Journal of Clinical Investigation*, vol. 121, no. 6, pp. 2094–2101, 2011.

[152] B. E. Elliott, S.-P. Tam, D. Dexter, and Z. Q. Chen, "Capacity of adipose tissue to promote growth and metastasis of a murine mammary carcinoma: effect of estrogen and progesterone," *International Journal of Cancer*, vol. 51, no. 3, pp. 416–424, 1992.

[153] J. M. Fleming, T. C. Miller, M. J. Meyer, E. Ginsburg, and B. K. Vonderhaar, "Local regulation of human breast xenograft models," *Journal of Cellular Physiology*, vol. 224, no. 3, pp. 795–806, 2010.

[154] J. Yamaguchi, H. Ohtani, K. Nakamura, I. Shimokawa, and T. Kanematsu, "Prognostic impact of marginal adipose tissue invasion in ductal carcinoma of the breast," *The American Journal of Clinical Pathology*, vol. 130, no. 3, pp. 382–388, 2008.

[155] L. M. Berstein, A. Y. Kovalevskij, T. E. Poroshina et al., "Signs of proinflammatory/genotoxic switch (adipogenotoxicosis) in mammary fat of breast cancer patients: role of menopausal status, estrogens and hyperglycemia," *International Journal of Cancer*, vol. 121, no. 3, pp. 514–519, 2007.

[156] L. Bochet, C. Lehuede, S. Dauvillier et al., "Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer," *Cancer Research*, vol. 73, no. 18, pp. 5657–5668, 2013.

[157] M. N. VanSaun, "Molecular pathways: adiponectin and leptin signaling in cancer," *Clinical Cancer Research*, vol. 19, no. 8, pp. 1926–1932, 2013.

[158] N. K. Saxena, L. Taliaferro-Smith, B. B. Knight et al., "Bidirectional crosstalk between leptin and insulin-like growth factor-I

signaling promotes invasion and migration of breast cancer cells via transactivation of epidermal growth factor receptor," *Cancer Research*, vol. 68, no. 23, pp. 9712–9722, 2008.

[159] A. Eisenberg, E. Biener, M. Charlier et al., "Transactivation of erbB2 by short and long isoforms of leptin receptors," *FEBS Letters*, vol. 565, no. 1–3, pp. 139–142, 2004.

[160] M. L. Gruen, M. Hao, D. W. Piston, and A. H. Hasty, "Leptin requires canonical migratory signaling pathways for induction of monocyte and macrophage chemotaxis," *The American Journal of Physiology—Cell Physiology*, vol. 293, no. 5, pp. C1481–C1488, 2007.

[161] J.-P. Bastard, M. Maachi, C. Lagathu et al., "Recent advances in the relationship between obesity, inflammation, and insulin resistance," *European Cytokine Network*, vol. 17, no. 1, pp. 4–12, 2006.

[162] T. Jardé, S. Perrier, M.-P. Vasson, and F. Caldefie-Chézet, "Molecular mechanisms of leptin and adiponectin in breast cancer," *European Journal of Cancer*, vol. 47, no. 1, pp. 33–43, 2011.

[163] A. E. Harvey, L. M. Lashinger, G. Otto, N. P. Nunez, and S. D. Hursting, "Decreased systemic IGF-1 in response to calorie restriction modulates murine tumor cell growth, nuclear factor-κB activation, and inflammation-related gene expression," *Molecular Carcinogenesis*, vol. 52, no. 12, pp. 997–1006, 2013.

[164] D. Chabas, S. E. Baranzini, D. Mitchell et al., "The influence of the proinflammatory cytokine, osteopontin, on autoimmue demyelinating desease," *Science*, vol. 294, no. 5547, pp. 1731–1735, 2001.

[165] M. Mirza, E. Shaughnessy, J. K. Hurley et al., "Osteopontin-c is a selective marker of breast cancer," *International Journal of Cancer*, vol. 122, no. 4, pp. 889–897, 2008.

[166] D. Thorat, A. Sahu, R. Behera et al., "Association of osteopontin and cyclooxygenase-2 expression with breast cancer subtypes and their use as potential biomarkers," *Oncology Letters*, vol. 6, no. 6, pp. 1559–1564, 2013.

[167] S. Meierjohann, M. Schartl, and J.-N. Volff, "Genetic, biochemical and evolutionary facets of Xmrk-induced melanoma formation in the fish Xiphophorus," *Comparative Biochemistry and Physiology C: Toxicology and Pharmacology*, vol. 138, no. 3, pp. 281–289, 2004.

[168] A. C. Cook, A. B. Tuck, S. McCarthy et al., "Osteopontin induces multiple changes in gene expression that reflect the six "hallmarks of cancer" in a model of breast cancer progression," *Molecular Carcinogenesis*, vol. 43, no. 4, pp. 225–236, 2005.

[169] Y.-Y. Wang, C. Lehuédé, V. Laurent et al., "Adipose tissue and breast epithelial cells: a dangerous dynamic duo in breast cancer," *Cancer Letters*, vol. 324, no. 2, pp. 142–151, 2012.

[170] C. H. Stuelten, S. DaCosta Byfield, P. R. Arany, T. S. Karpova, W. G. Stetler-Stevenson, and A. B. Roberts, "Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-α and TGF-β," *Journal of Cell Science*, vol. 118, no. 10, pp. 2143–2153, 2005.

[171] N. Erez, M. Truitt, P. Olson, and D. Hanahan, " Cancer Associated Fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-κB-dependent manner," *Cancer Cell*, vol. 17, no. 2, pp. 135–147, 2010.

[172] K. Pietras and A. Östman, "Hallmarks of cancer: interactions with the tumor stroma," *Experimental Cell Research*, vol. 316, no. 8, pp. 1324–1331, 2010.

[173] R. Kalluri and M. Zeisberg, "Fibroblasts in cancer," *Nature Reviews Cancer*, vol. 6, no. 5, pp. 392–401, 2006.

[174] M. Snyder, X.-Y. Huang, and J. J. Zhang, "Signal Transducers and Activators of Transcription 3 (STAT3) directly regulates cytokine-induced fascin expression and is required for breast cancer cell migration," *Journal of Biological Chemistry*, vol. 286, no. 45, pp. 38886–38893, 2011.

[175] I. Barbieri, S. Pensa, T. Pannellini et al., "Constitutively active Stat3 enhances neu-mediated migration and metastasis in mammary tumors via upregulation of Cten," *Cancer Research*, vol. 70, no. 6, pp. 2558–2567, 2010.

[176] D. J. Prockop, "Marrow stromal cells as stem cells for non-hematopoietic tissues," *Science*, vol. 276, no. 5309, pp. 71–74, 1997.

[177] S. Maxson, E. A. Lopez, D. Yoo, A. Danilkovitch-Miagkova, and M. A. LeRoux, "Concise review: role of mesenchymal stem cells in wound repair," *Stem Cells Translational Medicine*, vol. 1, no. 2, pp. 142–149, 2012.

[178] E. Spaeth, A. Klopp, J. Dembinski, M. Andreeff, and F. Marini, "Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells," *Gene Therapy*, vol. 15, no. 10, pp. 730–738, 2008.

[179] Y. Rattigan, J.-M. Hsu, P. J. Mishra, J. Glod, and D. Banerjee, "Interleukin 6 mediated recruitment of mesenchymal stem cells to the hypoxic tumor milieu," *Experimental Cell Research*, vol. 316, no. 20, pp. 3417–3424, 2010.

[180] E. Ritter, A. Perry, J. Yu, T. Wang, L. Tang, and E. Bieberich, "Breast cancer cell-derived fibroblast growth factor 2 and vascular endothelial growth factor are chemoattractants for bone marrow stromal stem cells," *Annals of Surgery*, vol. 247, no. 2, pp. 310–314, 2008.

[181] R. M. Dwyer, S. M. Potter-Beirne, K. A. Harrington et al., "Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells," *Clinical Cancer Research*, vol. 13, no. 17, pp. 5020–5027, 2007.

[182] Y. Zhang, P. Yang, T. Sun et al., "MiR-126 and miR-126* repress recruitment of mesenchymal stem cells and inflammatory monocytes to inhibit breast cancer metastasis," *Nature Cell Biology*, vol. 15, no. 3, pp. 284–294, 2013.

[183] R. Lotfi, J. Eisenbacher, G. Solgi et al., "Human mesenchymal stem cells respond to native but not oxidized damage associated molecular pattern molecules from necrotic (tumor) material," *European Journal of Immunology*, vol. 41, no. 7, pp. 2021–2028, 2011.

[184] A. H. Klopp, E. L. Spaeth, J. L. Dembinski et al., "Tumor irradiation increases the recruitment of circulating mesenchymal stem cells into the tumor microenvironment," *Cancer Research*, vol. 67, no. 24, pp. 11687–11695, 2007.

[185] P. Chaturvedi, D. M. Gilkes, C. C. Wong et al., "Hypoxia-inducible factor-dependent breast cancer-mesenchymal stem cell bidirectional signaling promotes metastasis," *Journal of Clinical Investigation*, vol. 123, no. 1, pp. 189–205, 2013.

[186] S. Kidd, E. Spaeth, K. Watson et al., "Origins of the tumor microenvironment: quantitative assessment of adipose-derived and bone marrow-derived stroma," *PLoS ONE*, vol. 7, no. 2, Article ID e30563, 2012.

[187] T. Zhang, Y. W. Lee, Y. F. Rui, T. Y. Cheng, X. H. Jiang, and G. Li, "Bone marrow-derived mesenchymal stem cells promote growth and angiogenesis of breast and prostate tumors," *Stem Cell Research and Therapy*, vol. 4, no. 3, article 70, 2013.

[188] J. M. Ryan, F. P. Barry, J. M. Murphy, and B. P. Mahon, "Mesenchymal stem cells avoid allogeneic rejection," *Journal of Inflammation*, vol. 2, article 8, 2005.

[189] S. A. Patel, J. R. Meyer, S. J. Greco, K. E. Corcoran, M. Bryan, and P. Rameshwar, "Mesenchymal stem cells protect breast cancer cells through regulatory T cells: role of mesenchymal stem cell-derived TGF-β," *Journal of Immunology*, vol. 184, no. 10, pp. 5885–5894, 2010.

[190] R. S. Waterman, S. L. Tomchuck, S. L. Henkle, and A. M. Betancourt, "A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype," *PLoS ONE*, vol. 5, no. 4, Article ID e10088, 2010.

[191] J. Lei, Z. Wang, D. Hui et al., "Ligation of TLR2 and TLR4 on murine bonTritschler I.e marrow-derived mesenchymal stem cells triggers differential effects on their immunosuppressive activity," *Cellular Immunology*, vol. 271, no. 1, pp. 147–156, 2011.

[192] M. Pevsner-Fischer, V. Morad, M. Cohen-Sfady et al., "Toll-like receptors and their ligands control mesenchymal stem cell functions," *Blood*, vol. 109, no. 4, pp. 1422–1432, 2007.

[193] S. Liu, C. Ginestier, S. J. Ou et al., "Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks," *Cancer Research*, vol. 71, no. 2, pp. 614–624, 2011.

[194] H.-J. Li, F. Reinhardt, H. R. Herschman, and R. A. Weinberg, "Cancer-stimulated mesenchymal stem cells create a carcinoma stem cell niche via prostaglandin E2 Signaling," *Cancer Discovery*, vol. 2, no. 9, pp. 840–855, 2012.

[195] X.-L. Yan, C.-J. Fu, L. Chen et al., "Mesenchymal stem cells from primary breast cancer tissue promote cancer proliferation and enhance mammosphere formation partially via EGF/EGFR/Akt pathway," *Breast Cancer Research and Treatment*, vol. 132, no. 1, pp. 153–164, 2012.

[196] E. Devarajan, Y.-H. Song, S. Krishnappa, and E. Alt, "Epithelial-mesenchymal transition in breast cancer lines is mediated through PDGF-D released by tissue-resident stem cells," *International Journal of Cancer*, vol. 131, no. 5, pp. 1023–1031, 2012.

[197] Q. Xu, L. Wang, H. Li et al., "Mesenchymal stem cells play a potential role in regulating the establishment and maintenance of epithelial-mesenchymal transition in MCF7 human breast cancer cells by paracrine and induced autocrine TGF-β," *International Journal of Oncology*, vol. 41, no. 3, pp. 959–968, 2012.

[198] A. De Luca, L. Lamura, M. Gallo, V. Maffia, and N. Normanno, "Mesenchymal stem cell-derived interleukin-6 and vascular endothelial growth factor promote breast cancer cell migration," *Journal of Cellular Biochemistry*, vol. 113, no. 11, pp. 3363–3370, 2012.

[199] A. E. Karnoub, A. B. Dash, A. P. Vo et al., "Mesenchymal stem cells within tumour stroma promote breast cancer metastasis," *Nature*, vol. 449, no. 7162, pp. 557–563, 2007.

[200] J. L. Halpern, A. Kilbarger, and C. C. Lynch, "Mesenchymal stem cells promote mammary cancer cell migration in vitro via the CXCR2 receptor," *Cancer Letters*, vol. 308, no. 1, pp. 91–99, 2011.

[201] S. Y. Shin, J.-S. Nam, Y. Lim, and Y. H. Lee, "TNFα-exposed bone marrow-derived mesenchymal stem cells promote locomotion of MDA-MB-231 breast cancer cells through transcriptional activation of CXCR3 ligand chemokines," *Journal of Biological Chemistry*, vol. 285, no. 40, pp. 30731–30740, 2010.

[202] L. V. Rhodes, J. W. Antoon, S. E. Muir, S. Elliott, B. S. Beckman, and M. E. Burow, "Effects of human mesenchymal stem cells on ER-positive human breast carcinoma cells mediated through ER-SDF-1/CXCR4 crosstalk," *Molecular Cancer*, vol. 9, article 295, 2010.

[203] M. Studeny, F. C. Marini, J. L. Dembinski et al., "Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents," *Journal of the National Cancer Institute*, vol. 96, no. 21, pp. 1593–1603, 2004.

[204] X.-C. Chen, R. Wang, X. Zhao et al., "Prophylaxis against carcinogenesis in three kinds of unestablished tumor models via IL12-gene-engineered MSCs," *Carcinogenesis*, vol. 27, no. 12, pp. 2434–2441, 2006.

[205] M. R. Loebinger, A. Eddaoudi, D. Davies, and S. M. Janes, "Mesenchymal stem cell delivery of TRAIL can eliminate metastatic cancer," *Cancer Research*, vol. 69, no. 10, pp. 4134–4142, 2009.

[206] L. Kucerova, V. Altanerova, M. Matuskova, S. Tyciakova, and C. Altaner, "Adipose tissue-derived human mesenchymal stem cells mediated prodrug cancer gene therapy," *Cancer Research*, vol. 67, no. 13, pp. 6304–6313, 2007.

[207] N. Serakinci, U. Fahrioglu, and R. Christensen, "Mesenchymal stem cells, cancer challenges and new directions," *European Journal of Cancer*, vol. 50, no. 8, pp. 1522–1530, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 179486, 10 pages
http://dx.doi.org/10.1155/2014/179486

Review Article

Exosome in Tumour Microenvironment: Overview of the Crosstalk between Normal and Cancer Cells

Catarina Roma-Rodrigues,[1] Alexandra R. Fernandes,[1,2] and Pedro Viana Baptista[1,3]

[1] *Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal*
[2] *Centro de Química Estrutural, Complexo 1, Instituto Superior Técnico, Universidade de Lisboa, Avenida Rovisco Pais, 1049-001 Lisboa, Portugal*
[3] *CIGMH, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal*

Correspondence should be addressed to Alexandra R. Fernandes; ma.fernandes@fct.unl.pt and Pedro Viana Baptista; pmvb@fct.unl.pt

Received 11 April 2014; Accepted 9 May 2014; Published 21 May 2014

Academic Editor: Lijun Jia

Cancer development is a multistep process in which exosomes play important roles. Exosomes are small vesicles formed in vesicular bodies in the endosomal network. The major role of exosomes seems to be the transport of bioactive molecules between cells. Depending on the cell of origin, exosomes are implicated in the regulation of several cellular events, with phenotypic consequences in recipient cells. Cancer derived exosomes (CCEs) are important players in the formation of the tumour microenvironment by (i) enabling the escape of tumour cells to immunological system and help initiating the inflammatory response; (ii) acting in the differentiation of fibroblasts and mesenchymal cells into myofibroblasts; (iii) triggering the angiogenic process; and (iv) enhancing the metastatic evolution of the tumour by promoting epithelial to mesenchymal transformation of tumour cells and by preparing the tumour niche in the new anatomical location. Since the finding that exosomes content resembles that of the cell of origin, they may be regarded as suitable biomarkers for cancer diagnosis, allowing for diagnosis and prognosis via a minimal invasive procedure. Exosome involvement in cancer may open new avenues regarding therapeutics, such as vectors for targeted drug delivery.

1. Introduction

The major modifications to normal cells that result in the formation of a tumour derive from mutational events in oncogenes or tumour suppressor genes that occur in a normal cell and will ultimately lead to uncontrolled growth [1]. This is the case of cell cycle regulatory genes (e.g., *RB1*), cell cycle checkpoint related genes (e.g., *TP53*), genes encoding proteins involved in DNA integrity maintenance and in sustaining propagation of damaged cells (e.g., *MLH1*, *MSH2*, *BRCA1* and 2), and genes involved in inhibition of apoptotic pathways (e.g., bcl2 overexpression) [2]. Evasion of growth and apoptotic control is usually followed by angiogenesis and metastasis. Many obstacles must be overcome, namely, the ability to survive an inhospitable microenvironment, where intercommunication between tumour cells and their surrounding microenvironment is essential for overcoming this obstacle and for tumour progression [3].

Despite the importance of the modifications occurring at the cell level, the tumour microenvironment is also relevant for the development of cancer. In fact, in the case of epithelial tumours, it is the combination of tumorigenic cells and stromal cells that dictates the extracellular matrix composition of the carcinoma [4]. The intracellular and intercellular communication between tumour and stromal cells is accomplished via cell-cell interactions (mediated by gap junction channels), paracrine mechanisms involving growth factors, chemokines, and proteases, as well as by extracellular vesicles [5]. Concerning the biological function of the vesicles involved in cell-cell communication, two main

classes are considered: exosomes and microvesicles. These vesicles are secreted by most normal and malignant cells and share in common an enclosed lipid bilayer. However, while microvesicles are generated by budding from the plasma membrane, exosomes are derived from the endolysosomal pathway [5, 6]. Exosomes are involved not only in the cell-cell communication in "bulk" tumour microenvironment but also between tumour and distant cells, favouring secretion of growth factors, cytokines, and angiopoietic factors by stromal cells, induction of proliferation of endothelial cells, metastasis, and immune responses [7, 8]. Therefore, exosomes constitute valuable biomarkers for cancer diagnosis and prognosis and also constitute either targets or vectors for therapeutic approaches in cancer [9].

In this review we intend to highlight the relevance of exosomes in tumorogenesis, highlighting their biogenesis, composition, and main function, and then focusing on their role in cancer development and progression. Finally, we will address the potential of exosomes as biomarkers and their use for cancer therapy.

2. Exosomes Biogenesis

Exosomes are formed in the endosomal network. The formation of the early endosomes occurs in the plasma membrane by the fusion of endocytic vesicles [10]. The maturation process consists in an acidification of the endosome lumen, via altered protein content and fusion with intraluminal vesicles (ILVs), which are formed by invaginations of the endosomal membrane, randomly engulfing portions of the cytosolic contents. The process of ILVs formation requires specialised units highly enriched with tetraspanins (such as CD9, CD63, CD81, CD82, and CD151) and several complexes called endosomal sorting complex required for transport (ESCRT complex) [10]. The presence of phosphatidylinositol 3-phosphate, ubiquitinated cargos in early endosomes vesicles and the curved membrane topology of the vesicles, which is reached by protein-protein interactions of the tetraspanins, induces the recruitment of ESCRT-I and ESCRT-II [6]. These proteins, together with ESCRT-III, which binds ESCRT-I via the protein Alix, promote the budding of the membrane [6]. Furthermore, this process also involves protein-lipid interactions, including interaction of ESCRT proteins with oxysterols and polyglycerophospholipid BMP (bismonoacylglycerolphosphate) [11].

At the end of the maturation process, the multivesicular bodies (MVBs) composed of late endosomes together with ILVs, are situated close to the nucleus. The fusion of MVBs with the plasma membrane leads to the release of the ILVs to the extracellular environment, which are then referred to as exosomes. The releasing process of exosomes may be accomplished by the outward exosome and microvesicle budding pathway or by an inducible release, a highly regulated process that involves several components of the endocytic machinery, including the Rab GTPases, such as Rab11, Rab35, Rab27a, and Rab27b, cytoskeleton regulatory proteins, heparanase, and SNARES (soluble NSF attachment receptor) for target fusion [12–14]. An increased exosome release was found to be triggered by several types of stress, such as changes in pH membrane, hypoxia, oxidative stress, thermal changes, shear stress, and radiation, as well as by stimulation of sphingomyelinase and subsequent formation of ceramide and activation of the tumour suppressor protein p53 [7, 13, 15–17]. Additionally, a feedback regulatory mechanism for controlling exosome release in breast cancer cells was proposed, being observed that exosomes derived from cancer cells also inhibited the exosome release from normal breast cells and suggested a dominant regulatory effect of cancer cell derived exosomes (CCEs) [18].

3. Exosomes Composition

Exosomes are small vesicles ranging in size between 30 and 120 nm, composed by a lipid bilayer containing membrane proteins that surrounds a lumen comprising proteins and nucleic acids, that vary according to cell type and mechanism of biogenesis [19]. As an example, exosomes isolated from malignant effusions of cancer patients contain tumour specific proteins in their surface, such as Her2/Neu from ovarian cancer ascites and Mart1 from patients with melanoma [20]. Additionally, growth factors, such as tumour necrosis factor-alpha (TNF-α), epidermal growth factor (EGF), and fibroblast growth factor (FGF), have also been found associated with exosomes [21–23].

3.1. Protein Content of Exosomes. Over 4600 different proteins have been associated to exosomes, including proteins from the cytosol, the plasma membrane, Golgi apparatus, and endoplasmic reticulum [24, 25]. Due to a common biogenesis path, the most part of exosomes contain proteins involved in the endosomal network, including (i) membrane transport and fusion proteins, such as GTPases, annexins, Rab proteins, and flotillin; (ii) tetraspanins, such as CD9, CD63, CD81, and CD82; (iii) heat shock proteins (HSPs), such as Hsp60, Hsp70, and Hsp90; (iv) proteins involved in MVBs biogenesis, such as Alix and TSG101; (v) cytoskeletal proteins, such as actin, tubulin, syntenin, and moesin; and (vi) lipid-related proteins and phospholipases [7, 19, 26]. Additionally, metabolic enzymes, signal transduction proteins, the carrier protein albumin, and major histocompatibility complement antigens are also commonly found in exosomes [14]. Due to the higher frequency of these proteins, tetraspanins, Alix, flotillin, TSG101, and Rab5b have been frequently used as markers for identification and confirmation of the presence of exosomes [19].

Several studies have shown that CCEs can alter the extracellular matrix through secretion of matrix metalloproteinases (MMPs) or activators of MMPs, such as HSPs. MMPs are zinc-dependent plasma membrane endopeptidases that can degrade extracellular matrix proteins, such as collagen, proteoglycans, fibronectin, and laminins [7]. Hakulinen and collaborators [27] showed that fibrosarcoma and melanoma derived exosomes can secrete MT1-MMP able to activate pro-MMP2 and to degrade collagen and gelatine. Other studies have demonstrated that Hsp90 is also secreted via exosomes and can activate MMP2 to enhance invasion of cancer cells [28].

3.2. Lipid Content of Exosomes. The exosomal lumen is surrounded by a lipid bilayer enriched in (i) raft-associated lipids such as cholesterol; (ii) diglycerides; (iii) sphingolipids, such as sphingomyelin and ceramide; (iv) phospholipids; (v) glycerophospholipids, such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI); and (vi) polyglycerophospholipids [11]. Interestingly, the content of lipids in exosomes differs substantially from that of the parental cells. While the content of sphingomyelin, PS, PI, ceramides, and cholesterol is highly increased in exosomes, the content of PC is decreased (except in reticulocytes) [7, 11]. Additionally, despite similarities of the transmembrane orientation between exosomes and parental cells, the exosomal PS is found to be randomly distributed between the two membrane leaflets, with an enrichment in the external exosomal membrane, contrary to the viable parental cell membrane, where it is located in the inner leaflet [11]. In general, this lipid composition confers rigidity to the vesicle, which confers stability of exosomes in biological fluids and cell culture mediums [11]. Presence of PS on the outer membrane of exosomes can function in exosome recognition and internalisation by recipient cells [29]. As such, exosomes may function as lipid carriers, allowing the transport of the bioactive lipids they carry to a recipient cell (see Section 4). This process of exosome trafficking, particularly in the context of tumour microenvironment, may lead to an enrichment of certain tumour progressive/immunosuppressive lipids, such as prostaglandins [30]. Conversely, it may also lead to a replacement of harmful exosome lipid contents with beneficial ones, such as docosahexaenoic acid, an omega-3 polyunsaturated fatty acid with many health and anticancer benefits, that could be supplied by exosomes throughout the tumour microenvironment affecting cell-to-cell communication, reducing tumour cell growth, and increasing sensitivity to therapeutic interventions [31].

3.3. Nucleic Acid Content of Exosomes. One of the most distinct features of exosomes is the fact that they carry significant amounts of nucleic acids, including microRNAs (miRNAs) and mRNA, as well as mitochondrial DNA (mtDNA), piwi-RNAs (piRNAs), long noncoding RNAs (lncRNAs), ribosomal RNAs (rRNAs), small-nuclear RNAs (snRNAs), small-nucleolar RNAs (snoRNAs), and transfer RNAs (tRNAs) [32]. Although the exosomal mRNA appeared to be mostly degraded to less than 200 fragments, it was possible the *in vitro* translation of full-length proteins [33, 34], suggesting that after being internalised by target cells, mRNA can be translated into proteins. The presence of miRNAs in exosomes has been subject of several studies [35–39]. miRNA content of exosomes has particular relevance in cancer pathologies, whose type and quantity of miRNAs vary with the cell of origin, and seems to reflect the miRNA content of the parental cells, although it was also found some discrepancies in exosomes derived from cancer cells, suggesting selective packaging of miRNAs [40]. mRNAs and miRNAs can be transferred to a recipient cell located in the tumour microenvironment or at distant sites via fusion of the exosome with the target cell membrane [7]. After internalisation by target cells these miRNAs may function as either tumour suppressors or oncogenes.

3.4. Others. Despite the relevance of glycosylation of the membrane surface in the communication of cells with their extracellular environment, very few studies have been made to characterize the carbohydrate content of exosomes. The analysis of glycosylation patterns of exosomes derived from T cells, melanoma, and colon cancer cells revealed that the glycosylation signature seems to be conserved between exosomes and parental cell membranes [41]. In another study, it was observed an enrichment of the sialoglycoprotein galectin-3-binding protein in ovarian tumour derived exosomes [42].

4. Exosomes Function

The major role of exosomes seems to be the transport of bioactive molecules between cells, with consequences in targeted cell phenotypes, such as mRNA and miRNA related to the transfer of genetic, and sometimes epigenetic, information between cells [13]. Additionally, and as described above, another exosome function includes lipid trafficking [30]. The presence of exosomes in healthy body fluids suggests a role of these vesicles in the normal physiology of the body, including communication in the immune system, tissue repair, and communication within the nervous system [43]. Exosomes have also been associated with infection [44] and several pathological conditions, such as in the progression of neurodegenerative disease, cardiovascular diseases and cancer [24], or, on the other side, in the protection against atherosclerosis [45, 46].

The importance of exosomes in tumorigenesis is emphasised by the general increased content of these vesicles in biological fluids of cancer patients relatively to healthy controls, being observed an increased content of exosomes as the tumour progresses [24]. Interestingly, CCEs cause both antitumorigenic and protumorigenic effects. Studies have shown that bladder cancer cell lines shed exosomes containing proteins important for tumour progression, and these exosomes inhibit tumour cell apoptosis through Akt and ERK pathways [47]. On the other side, CCEs can transport tumour antigens to dendritic cells and induce immune responses [24]. These differences between biological functions observed for exosomes most likely arise from differences in the cargo present either on the surface of the vesicle or internally.

Furthermore, CCEs have been implicated in tumour growth, survival, and spread, as well as in angiogenesis, escape from immune surveillance, stimulating tumour cell migration, conferring invasion ability to normal cells, and the preparation of distal tissues for their metastatic colonisation [13]. The following sections will focus on these subjects.

5. Exosomes Targeting and Uptake by Recipient Cells

The mechanisms underlying interaction and fusion of exosomes with target cells remain undefined. It is believed that

the uptake of exosomes by target cells may occur through three main mechanisms: (i) simple fusion of the exosome with the cellular membrane, directly releasing the content of vesicles into the cytoplasm; (ii) exosome uptake by endocytosis; (iii) uptake dependent on the presence of distinct receptor proteins that enable binding of exosomes to target cells [32]. For the latter, it is generally accepted that the cell of origin and secretion conditions of exosomes seem to determine their cell surface content, and consequently the cell-type-specific adhesion molecules, targeting exosomes to specific cells [48]. Nevertheless, exosomes contain many different cell surface molecules and one single exosome is able to engage many different cell receptors [11, 48]. It was postulated that exosome recognition by cells involve lipid receptors, such as receptors of the TIM family that recognises PS and possibly a G protein coupled receptor family protein, G2A, that recognises LPC located at the surface of the vesicles [11]. Moreover, it is likely that exosomes attachment and internalisation are partially mediated by interactions with heparin sulphate proteoglycans (HSPG) located at the membrane of the receptor cell, possibly by a similar mechanism as for lipoprotein or virus internalisation [49].

6. Role of Exosomes in Tumour Microenvironment Development

As mentioned earlier, cancer development is a multistep process in which somatic cells experience events (such as environmental insults or chronic inflammation), accumulating genetic modifications that will ultimately result in uncontrolled growth of the cell [1]. During tumour development, the extracellular matrix suffers modifications that will support malignant progression. The evolution of the tumour microenvironment is driven (i) by the genetic instability of malignant cells, that are constantly releasing exosomes carrying oncogenes and other bioactive molecules involved in tumour progression; (ii) environmental selection forces, which include endogenous tumour-growth induced stress stimuli, such as hypoxia, acidosis, starvation, or oxidative stress; and (iii) inflammatory and immune responses [1].

An epithelial tumour mass is composed by stromal elements that frequently include an altered extracellular matrix enriched with cytokines and growth factors, fibroblasts, a scaffold composed of immune and inflammatory cells, endothelial cells, pericytes, mesenchymal cells, and in the case of more advanced tumours, blood and lymph vessels and nerves [1]. Every stromal cell type seems to have the ability to support hyperproliferation of cancer cells in a specific context and seems to be different for each type of cancer pathologies [50, 51]. However, the molecular mechanisms related to the recruitment and maturation of stromal cells are not completely defined. Exosomes have important roles in the intercellular communication between cancer and stromal cells that will result in the maturation of the tumour microenvironment and tumour growth and proliferation (Figure 1).

6.1. Role of Exosomes in Intratumour Heterogeneity. A common trait between all tumour pathologies is the phenotypic heterogeneity of the population of cancer cells within tumours. This is mainly due to genetic instability of cancer cells leading to genetic alterations, differential environmental stimuli, and stochastic processes that occur within the tumour microenvironment [52]. It is likely that intratumour heterogeneity may also result from the internalisation of CCES by neighbour healthy epithelial cells. In fact, it was observed an alteration of the phenotype of normal cells after internalisation with exosomes derived from colorectal, lung, and prostate cancer cells [48, 53].

6.2. Role of Exosomes in Immunological Responses in Tumour Microenvironment. Generally, early tumour microenvironment resembles the environment of wounds that never heal [54]. In the context of immune response, the tumour microenvironment contains (i) innate immune cells, such as macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells and (ii) adaptive immune cells, such as T and B lymphocytes. However the immune cells more represented in tumour microenvironment are tumour associated macrophages and T lymphocytes [55]. Exosomes play an important role in inter- and intracommunication between the immune cells and cancer cells.

As already referred, the communication between cells of the immunity system is mediated by direct contact or cytokine and chemokine production, which also involves exosomes [6]. It is the balance of the activation level and abundance of the immune mediators and modulators in the tumour microenvironment that dictates if the tumour-inflammatory response or antitumour immunity occurs [4]. Exosomes have important roles in this equilibrium. It was previously observed that, through a still not defined mechanism, exosomes derived from several tumours, including pleural malignant mesothelioma and prostate cancer, inhibited the proliferative response of lymphocytes or natural killer cells [43, 56]. Additionally, miRNA transported by CCEs may act like ligands by binding to Toll-like receptors and trigger the inflammatory response. In fact, it was observed that oncogene miR-21 and miR-29a secreted from exosomes derived from lung cancer cells were able to bind to murine and human TLR [57]. On the other way, exosomes may act in the specialised activation of T lymphocytes against cancer cells by the presentation of membrane proteins, such as HER2/Neu, enriched in tumour cells.

6.3. Role of Exosomes in Production of Cancer Associated Fibroblasts. Cancer associated fibroblasts (CAF) are the most prominent cell type in the tumour microenvironment of many cancers types, including colon, pancreas, and breast, and play critical roles in tumour-stromal interactions. The mechanisms inherent to CAF formation seem to involve the formation of myofibroblasts by differentiation of resident fibroblasts, epithelial and endothelial cells (via epithelial to mesenchymal transition (EMT)), pericytes, bone-marrow-derived circulating fibrocytes and mesenchymal stem cells [58]. This differentiation is mainly promoted by platelet derived- and ECCs containing-tumour growth factor-beta 1 (TGF-β1) and fibroblast growth factor-2 (FGF-2) [58, 59]

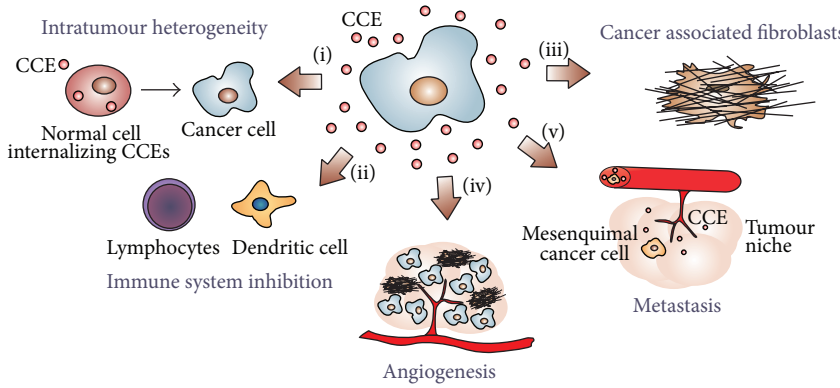


Figure 1: Tumour microenvironment processes mediated by cancer cell derived exosomes (CCEs): (i) intratumour heterogeneity resulting from phenotype modification of normal cells after internalization of CCEs; (ii) inhibition of the immune response against tumour cells by inhibiting the proliferative response of lymphocytes; (iii) activation of the differentiation of fibroblasts into cancer associated fibroblasts; (iv) stimulation of the angiogenic process; and (v) epithelial to mesenchymal transition (EMT) and preparation of a premetastatic niche at the distant location.

and consists in a phenotypic change of the cell mediated by arrangements of the cytoskeleton, pericellular coats, extracellular matrix turnover, and growth factor production. Interestingly, Webber and collaborators observed that differentiation of fibroblasts into myofibroblasts only require cancer cells exosomal TGF-β, stressing the relevance of exosomes as effectors in the alteration of cancer stroma [59]. In addition, two independent studies revealed that exosomes derived from ovarian cancer cells are able to convert adipose tissue-derived mesenchymal stem cells into myofibroblasts-like cells [60, 61].

The altered phenotype of CAFs will result in the increased production of alpha smooth muscle actin with consequences in the increased stiffness of the extracellular matrix [58]. CAFs contribute to the architectural and molecular remodelling of the tumour microenvironment supporting tumour growth, vascularisation, and metastasis [58, 62, 63]. Indeed, as a consequence of the increased stiffness in the tumour microenvironment, epithelial cells may acquire a mesenchymal phenotype by losing the cell-cell junctions and cell polarity [58]. Furthermore, it is known that CAF release bioactive molecules, such as HGF, IL6, PDGF, prostaglandins, proteases and miRNAs to the extracellular matrix, suggesting a role of exosomes in their transport [62]. Additionally, Luga and Wrana observed that exosomes derived from CAFs promote activity, motility, and metastasis of breast cancer cells by activating autocrine Wnt-PCP signalling [63].

Another example of the role of CCEs in tumour progression is the release of exosomes containing the extracellular matrix MMPs inducer (EMMPRIN) by lung carcinoma cells [64]. The release of bioactive EMMPRIN stimulates the expression of the matrix MMPs in fibroblasts, with consequences in tumour metastization. In addition, exosomes released by prostate cancer cells under hypoxic conditions were loaded with a significant higher number of TGF-β, IL6, TNF-1a, and MMPs, that have been implicated in the induction of a stem cell phenotype in the microenvironment of tumour cells and promotion of metastasis [65].

6.4. Role of Exosomes in Angiogenesis. The vascular formation inherent to the cancer progression may be triggered by hypoxic and nutrient depletion conditions in the tumour microenvironment, as well as by inflammatory responses, usually observed in epithelial cell carcinomas [54]. The angiogenic process consists in a neovascular formation from preexisting blood vessels and results from numerous interactions between regulators, mediators, and stimulatory molecules. Endothelial cells and pericytes located at the tumour microenvironment are imperative in this process [66]. Vascular endothelial growth factors (VEGF), FGF, TGF-β, PDGF, and IL-8 are some of the angiogenic factors that act on the regulation of quiescence, migration, and proliferation of endothelial cells, required for the stimulation of angiogenesis [66].

Recent studies have shown that exosomes released under hypoxic conditions contribute to the stimulation of angiogenesis [66]. Melanoma cells derived exosomes containing miRNA-9 were internalised by endothelial cells promoting metastasis and angiogenesis by activation of the JAK-STAT pathway [32]. In another study, exosomes derived from metastatic breast cancer cells contained multiple angiogenic miRNAs, including miRNA-210 whose expression is inversely correlated with overall survival in breast cancer [35]. Moreover, it was observed that extracellular vesicles derived from hypoxic brain tumour glioblastoma multiform cells were enriched with angiogenic stimulatory molecules, such as IL-8 and PDGF [67].

6.5. Role of Exosomes in Metastasis. The increased malignancy of a tumour, with several implications on cancer patient survival, consists in the formation of tumour metastasis. The metastization process is complex and involves several steps including (i) EMT; (ii) breach of the basement membrane barrier; (iii) migration of the cell through the neighbouring tissue; (iv) entry, transport, and exit from blood and lymph vessels; (v) establishment of the cell in a secondary anatomical site; and (vi) growth of the secondary tumour, by

the creation of a new tumour microenvironment favourable to cancer cell growth [3]. The role of exosomes in metastasis process relies mainly on the first, second, and, indirectly, the fifth steps.

During EMT, cancer cells loose the epithelial characteristics towards a more mesenchymal phenotype and acquire motility capabilities. The mechanisms inherent to this event are complex, involving cytoskeletal alterations and downregulation of expression of E-cadherin [65]. Interestingly, it was observed that proteins in exosomes derived from hypoxic prostate cancer cells are involved in the pathways of epithelial adherens junctions and cytoskeleton remodelling, suggesting that the increased invasiveness observed in prostate cancer cells is mediated by exosomes [65]. In another study, Jeppesen and collaborators [68] studied the protein content of exosomes derived from a human bladder carcinoma cell line without metastatic capacity relatively to two isogenic derivate metastatic cell lines formed in the lung and liver of mice. They reported an increased abundance in exosomes derived from metastatic cells, of vimentin, hepatoma-derived growth factor (HDGF), casein kinase II, and annexin A2, which are associated with the EMT process, as well as other proteins involved in cellular movement and cell-cell signalling.

Besides involvement in EMT, exosomes seem also to be involved in the formation and preparation of the premetastatic niche at the new anatomical location. Interestingly, it was reported that exosomes of melanoma cells are preferentially taken up by sentinel lymph nodes and prepare the premetastatic niche by deposition in the extracellular matrix and vascular proliferation in the lymph nodes. Subsequently, free melanoma cells were recruited to the lymph nodes that have taken up the cancer derived exosomes [69]. In another study, Peinado and collaborators suggested that melanoma derived exosomes induce vascular leakiness at premetastatic sites and increase the metastatic behaviour of bone marrow cells through the oncoprotein receptor kinase MET [70].

7. Clinical Relevance of Exosomes in Cancer

7.1. Use of Exosomes as Biomarkers. As already mentioned throughout this review, the content of an exosome depends on the cell of origin. Particularly, exosomes derived from cancer cells are enriched with proteins, mRNA, and miRNA that are more abundant in cancer cells than in normal cells [8]. Hence, exosomes may be used as biomarkers for cancer diagnosis. Several studies including proteomics and transcriptomics have been used to understand the content of exosomes derived from specific tumours, with the purpose of their use as biomarkers [36, 71, 72]. As an example Taylor and Gercel-Taylor observed that the levels of 8 microRNAs (miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, and miR-214) are of diagnostic value in ovarian cancer [71]. They found that these microRNAs are present in circulating exosomes in the sera of patients and the levels of the microRNAs found in these vesicles were similar to those found in the cancer cells, suggesting an effective way to diagnose ovarian cancer even in asymptomatic patients [71].

One of the major advantages of the use of exosomes as biomarkers is the possibility of a rapid pathology prognosis through a minimal invasive procedure. In fact, one of the higher challenges in the battle against tumours is an early and accurate diagnosis [73]. Currently, the tissue biopsy, which is an invasive procedure, with potential damaging side effects, is generally required for a correct diagnosis. The presence in body fluids of exosomes containing biomarkers of subtypes of cancer cells may allow the use of minimal invasive "liquid biopsies" (such as blood collection) for prognosis and diagnosis of cancer. In contrast to the circulating tumour cells (CTCs), which were also suggested as important biomarkers for real-time diagnosis of cancer progression, the modification of the content of exosomes accompanies the development of the tumorigenicity of the cells, allowing the prognosis of cancer since the beginning of the pathology. However, the quantity and heterogeneity of exosomes in body fluids may be a drawback in the use of these vesicles as biomarkers, as it can lead to false negatives or positives in prognosis and diagnosis [73].

7.2. Use of Exosomes for Cancer Therapeutics. From a therapeutically point of view, the natural role of exosomes as carriers of metabolites from donor cells to recipient cells and in inducing a biological response has been investigated by several researchers in a dual manner. Exosomes may be used both as mediators of tumour resistance and as vessels for targeted drug delivery. Figure 2 highlights the therapies that were proposed for treatment of tumours based on exosomes characteristics.

Several studies suggested a role of exosomes in drug resistance, by extruding hydrophilic drugs from cancer cells [74, 75], in resistance to radiation [76] and to immunotherapy [77, 78]. To surpass this negative effect of exosomes in cancer treatment, the removal of exosomes from the blood circulation of patients in a haemodialysis-like procedure was proposed [79].

On the other side, since the first suggestions of the role of exosomes in the immune system performed by Raposo et al. [80] and Zitvogel et al. [81] that exosome based cell-free vaccines could represent an alternative to dendritic cell therapy for suppressing tumour growth. With this in mind, three phase I [82–84] clinical trials were performed using dendritic and ascites derived exosomes. However, it was observed that only a small percentage of patients presented transient stabilisation of the disease. A phase II clinical trial was also described in 2009 that combined the administration of dendritic cell derived exosomes carrying NKg2D ligands and Il-15Rα, in association with Treg cell-inhibiting treatments in patients with non-small-cell lung cancer that has been stabilised by chemotherapy [85].

One other use of exosomes for cancer therapy is the possibility of using these vesicles for targeted delivery. This process may be accomplished by the targeting of cancer cells mediated by specific antibodies or ligands of highly expressed membrane receptors, further internalisation of exosomes and induced apoptosis of the cells, which may be mediated by miRNA, siRNA and anticancer drugs. Several studies have

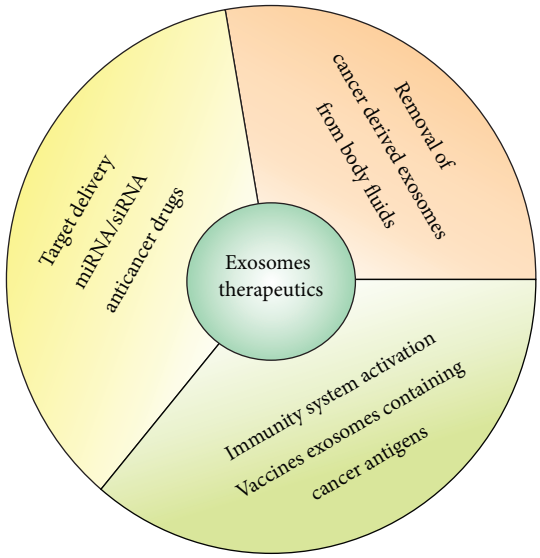


Figure 2: Major groups of exosomes based therapies, which include the removal of cancer derived exosomes, containing bioactive molecules, from the blood (or other body fluids) of cancer patients through a haemodialysis-like process; activation of the immune system against cancer cells by the use of vaccines of exosomes containing proteins with higher expression in tumour cell membranes; and use of exosomes containing microRNA (miRNA), small interference RNA (siRNA), and/or anticancer drugs for targeting delivery to cancer cells.

described the successful delivery and tumour inhibition using this process [86–88]. As an example, Alvarez-Erviti et al. [87] developed a way to introduce siRNA into exosomes by electroporation and to specifically target these siRNA loaded exosomes to neurons. In another study published in 2013, Ohno et al. [88] successfully delivered let-7a miRNA to xenografted breast cancer cells in mice.

8. Conclusions and Future Directions

Current knowledge of CCEs suggests that they can play an important role in the development and progression of cancer through modulation of intercellular communication within the tumour microenvironment by the transfer of protein, lipid, and RNA cargo. The complete understanding of their role in intercellular communication and tumorigenesis will be achieved by further exploration and comparison of their secretion in normal cells and during cancer development and progression, together with assessment of their specific content under these conditions. Exploration of CCEs contents may allow the development of novel diagnostic and therapeutic approaches, with minimally invasive procedures.

The use of exosomes for targeted delivery may also prove to be the answer in iRNA based therapeutics, whose main drawback has been the development of an effective delivery system. Generation of synthetic exosomes—"exosome mimetics"—for drug delivery may also allow selective targeting of cancer cells (for a review see [86]). Although in its infancy, the future possibilities of these natural nanovesicules are tremendous.

Abbreviations

CAF: Cancer associated fibroblasts
CCEs: Cancer cells derived exosomes
EMT: Epithelial to mesenchymal transition.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors acknowledge Fundação para a Ciência e Tecnologia for CIGMH (PEst-OE/SAU/UI0009/2014); PTDC/BBB-NAN/1812/2012; and PTDC/CVT-EPI/4651/2012.

References

[1] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell*, vol. 144, no. 5, pp. 646–674, 2011.

[2] T. Van Dyke and T. Jacks, "Cancer modeling in the modern era: progress and challenges," *Cell*, vol. 108, no. 2, pp. 135–144, 2002.

[3] T. R. Geiger and D. S. Peeper, "Metastasis mechanisms," *Biochimica et Biophysica Acta - Reviews on Cancer*, vol. 1796, no. 2, pp. 293–308, 2009.

[4] S. I. Grivennikov, F. R. Greten, and M. Karin, "Immunity, Inflammation, and Cancer," *Cell*, vol. 140, no. 6, pp. 883–899, 2010.

[5] S. El Andaloussi, I. Mäger, X. O. Breakefield, and M. J. A. Wood, "Extracellular vesicles: biology and emerging therapeutic opportunities," *Nature Reviews Drug Discovery*, vol. 12, no. 5, pp. 347–357, 2013.

[6] J. C. Akers, D. Gonda, R. Kim, B. S. Carter, and C. C. Chen, "Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies," *Journal of Neuro-Oncology*, vol. 113, no. 1, pp. 1–11, 2013.

[7] B. N. Hannafon and W. Q. Ding, "Intercellular communication by exosome-derived microRNAs in cancer," *International Journal of Molecular Sciences*, vol. 14, no. 7, pp. 14240–14269, 2013.

[8] H. G. Zhang and W. E. Grizzle, "Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions," *The American Journal of Pathology*, vol. 184, no. 1, pp. 28–41, 2014.

[9] Y. Tian, S. Li, J. Song, T. Ji, M. Zhu et al., "A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy," *Biomaterials*, vol. 35, no. 7, pp. 2383–2390, 2014.

[10] S. Keller, M. P. Sanderson, A. Stoeck, and P. Altevogt, "Exosomes: from biogenesis and secretion to biological function," *Immunology Letters*, vol. 107, no. 2, pp. 102–108, 2006.

[11] M. Record, K. Carayon, M. Poirot, and S. Silvente-Poirot, "Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies," *Biochimica Et Biophysica Acta*, vol. 1841, no. 1, pp. 108–120, 2014.

[12] S. Pant, H. Hilton, and M. E. Burczynski, "The multifaceted exosome: biogenesis, role in normal and aberrant cellular

function, and frontiers for pharmacological and biomarker opportunities," *Biochemical Pharmacology*, vol. 83, no. 11, pp. 1484–1494, 2012.

[13] A. S. Azmi, B. Bao, and F. H. Sarkar, "Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review," *Cancer Metastasis Reviews*, vol. 32, no. 3-4, pp. 623–642, 2013.

[14] A. Beach, H. G. Zhang, M. Z. Ratajczak, and S. S. Kakar, "Exosomes: an overview of biogenesis, composition and role in ovarian cancer," *Journal of Ovarian Research*, vol. 7, no. 1, p. 14, 2014.

[15] X. Yu, S. L. Harris, and A. J. Levine, "The regulation of exosome secretion: a novel function of the p53 protein," *Cancer Research*, vol. 66, no. 9, pp. 4795–4801, 2006.

[16] S. EL Andaloussi, S. Lakhal, I. Mäger, and M. J. A. Wood, "Exosomes for targeted siRNA delivery across biological barriers," *Advanced Drug Delivery Reviews*, vol. 65, no. 3, pp. 391–397, 2013.

[17] P. Kucharzewska and M. Belting, "Emerging roles of extracellular vesicles in the adaptive response of tumour cells to microenvironmental stress," *Journal of Extracellular Vesicles*, vol. 2, 2013.

[18] A. Riches, E. Campbell, E. Borger, and S. Powis, "Regulation of exosome release from mammary epithelial and breast cancer cells—a new regulatory pathway," *European Journal of Cancer*, vol. 50, no. 5, pp. 1025–1034, 2014.

[19] A. V. Vlassov, S. Magdaleno, R. Setterquist, and R. Conrad, "Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials," *Biochimica et Biophysica Acta—General Subjects*, vol. 1820, no. 7, pp. 940–948, 2012.

[20] F. Andre, N. E. C. Schartz, M. Movassagh et al., "Malignant effusions and immunogenic tumour-derived exosomes," *Lancet*, vol. 360, no. 9329, pp. 295–305, 2002.

[21] H. G. Zhang, C. Liu, K. Su et al., "A membrane form of TNF-alpha presented by exosomes delays T cell activation-induced cell death," *Journal of Immunology*, vol. 176, no. 12, pp. 7385–7393, 2006, Erratum in *The Journal of Immunology*, vol.177, no.3, p.2025, 2006.

[22] M. P. Sanderson, S. Keller, A. Alonso, S. Riedle, P. J. Dempsey, and P. Altevogt, "Generation of novel, secreted epidermal growth factor receptor (EGFR/ErbB1) isoforms via metalloprotease-dependent ectodomain shedding and exosome secretion," *Journal of Cellular Biochemistry*, vol. 103, no. 6, pp. 1783–1797, 2008.

[23] C. Seelenmeyer, C. Stegmayer, and W. Nickel, "Unconventional secretion of fibroblast growth factor 2 and galectin-1 does not require shedding of plasma membrane-derived vesicles," *FEBS Letters*, vol. 582, no. 9, pp. 1362–1368, 2008.

[24] C. Bang and T. Thum, "Exosomes: new players in cell-cell communication," *International Journal of Biochemistry and Cell Biology*, vol. 44, no. 11, pp. 2060–2064, 2012.

[25] S. Mathivanan, H. Ji, and R. J. Simpson, "Exosomes: extracellular organelles important in intercellular communication," *Journal of Proteomics*, vol. 73, no. 10, pp. 1907–1920, 2010.

[26] S. W. Harshman, A. Canella, P. D. Ciarlariello, A. Rocci, K. Agarwal et al., "Characterization of multiple myeloma vesicles by label-free relative quantitation," *Proteomics*, vol. 13, no. 20, pp. 3013–3029, 2013.

[27] J. Hakulinen, L. Sankkila, N. Sugiyama, K. Lehti, and J. Keski-Oja, "Secretion of active membrane type 1 matrix metalloproteinase (MMP-14) into extracellular space in microvesicular exosomes," *Journal of Cellular Biochemistry*, vol. 105, no. 5, pp. 1211–1218, 2008.

[28] J. McCready, J. D. Sims, D. Chan, and D. G. Jay, "Secretion of extracellular hsp90α via exosomes increases cancer cell motility: a role for plasminogen activation," *BMC Cancer*, vol. 10, article 294, 2010.

[29] L. Zakharova, M. Svetlova, and A. F. Fomina, "T cell exosomes induce cholesterol accumulation in human monocytes via phosphatidylserine receptor," *Journal of Cellular Physiology*, vol. 212, no. 1, pp. 174–181, 2007.

[30] X. Xiang, A. Poliakov, C. Liu et al., "Induction of myeloid-derived suppressor cells by tumor exosomes," *International Journal of Cancer*, vol. 124, no. 11, pp. 2621–2633, 2009.

[31] W. Cao, Z. Ma, M. M. Rasenick, S. Yeh, and J. Yu, "N-3 Poly-Unsaturated Fatty Acids Shift Estrogen Signaling to Inhibit Human Breast Cancer Cell Growth," *PLoS ONE*, vol. 7, no. 12, article e52838, 2012.

[32] A. Gajos-Michniewicz, M. Duechler, and M. Czyz, "MiRNA in melanoma-derived exosomes," *Cancer Letters*, vol. 347, no. 1, pp. 29–37, 2014.

[33] H. Valadi, K. Ekström, A. Bossios, M. Sjöstrand, J. J. Lee, and J. O. Lötvall, "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells," *Nature Cell Biology*, vol. 9, no. 6, pp. 654–659, 2007.

[34] D. M. Pegtel, "Oncogenic herpesviruses sending mixed signals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 31, pp. 12503–12504, 2013.

[35] L. Camacho, P. Guerrero, and D. Marchetti, "MicroRNA and protein profiling of brain metastasis competent cell-derived exosomes," *PloS One*, vol. 8, no. 9, article e73790, 2013.

[36] V. N. Aushev, I. B. Zborovskaya, K. K. Laktionov et al., "Comparisons of microRNA patterns in plasma before and after tumor removal reveal new biomarkers of lung squamous cell carcinoma," *PloS One*, vol. 8, no. 10, article e78649, 2013.

[37] P. Jenjaroenpun, Y. Kremenska, V. M. Nair et al., "Characterization of RNA in exosomes secreted by human breast cancer cell lines using next-generation sequencing," *Peer Journal*, vol. 1, article e201, 2013.

[38] R. Garzon, G. A. Calin, and C. M. Croce, "MicroRNAs in cancer," *Annual Review of Medicine*, vol. 60, pp. 167–179, 2009.

[39] W. Filipowicz, S. N. Bhattacharyya, and N. Sonenberg, "MEchanisms of post-transcriptional regulation by microRNAs: are the answers in sight?" *Nature Reviews Genetics*, vol. 9, no. 2, pp. 102–114, 2008.

[40] L. Pigati, S. C. S. Yaddanapudi, R. Iyengar et al., "Selective release of MicroRNA species from normal and malignant mammary epithelial cells," *PLoS ONE*, vol. 5, no. 10, article e13515, 2010.

[41] B. S. Batista, W. S. Eng, K. T. Pilobello, K. D. Hendricks-Muñoz, and L. K. Mahal, "Identification of a conserved glycan signature for microvesicles," *Journal of Proteome Research*, vol. 10, no. 10, pp. 4624–4633, 2011.

[42] C. Escrevente, N. Grammel, S. Kandzia, J. Zeiser, E. M. Tranfield et al., "Sialoglycoproteins and N-glycans from secreted exosomes of ovarian carcinoma cells," *PloS One*, vol. 8, no. 10, article e78631, 2013.

[43] A. Bobrie, M. Colombo, G. Raposo, and C. Théry, "Exosome secretion: molecular mechanisms and roles in immune responses," *Traffic*, vol. 12, no. 12, pp. 1659–1668, 2011.

[44] A. Fleming, G. Sampey, M. C. Chung et al., "The carrying pigeons of the cell: exosomes and their role in infectious diseases caused by human pathogens," *Pathogens and Disease*, 2014.

[45] E. Hergenreider, S. Heydt, K. Tréguer et al., "Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs," *Nature Cell Biology*, vol. 14, no. 3, pp. 249–256, 2012.

[46] H. Zhu, S. Guariglia, R. Y. Yu et al., "Mutation of SIMPLE in charcot-marie-tooth 1C alters production of exosomes," *Molecular Biology of the Cell*, vol. 24, no. 11, pp. 1619–1637, 2013.

[47] C. A. Franzen, P. E. Simms, A. F. Van Huis, K. E. Foreman, P. C. Kuo, and G. N. Gupta, "Characterization of uptake and internalization of exosomes by bladder cancer cells," *BioMed Research International*, vol. 2014, Article ID 619829, 11 pages, 2014.

[48] D. D. Taylor and C. Gercel-Taylor, "The origin, function, and diagnostic potential of RNA within extracellular vesicles present in human biological fluids," *Frontiers in Genetics*, vol. 4, p. 142, 2013.

[49] H. C. Christianson, K. J. Svensson, T. H. van Kuppevelt, J. P. Li, and M. Belting, "Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 43, pp. 17380–17385, 2013.

[50] V. Catalano, A. Turdo, S. Di Franco et al., "Tumor and its microenvironment: a synergistic interplay," *Seminars in Cancer Biology*, vol. 23, no. 6, part B, pp. 522–532, 2013.

[51] R. M. Hoffman, "Stromal-cell and cancer-cell exosomes leading the metastatic exodus for the promised niche," *Breast Cancer Research*, vol. 153, article 310, 2013.

[52] A. Marusyk, V. Almendro, and K. Polyak, "INtra-tumour heterogeneity: a looking glass for cancer?" *Nature Reviews Cancer*, vol. 12, no. 5, pp. 323–334, 2012.

[53] A. Suetsugu, K. Honma, S. Saji, H. Moriwaki, T. Ochiya, and R. M. Hoffman, "Imaging exosome transfer from breast cancer cells to stroma at metastatic sites in orthotopic nude-mouse models," *Advanced Drug Delivery Reviews*, vol. 65, no. 3, pp. 383–390, 2013.

[54] H. F. Dvorak, "Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing," *The New England Journal of Medicine*, vol. 315, no. 26, pp. 1650–1659, 1986.

[55] C. V. Dang, "MYC on the path to cancer," *Cell*, vol. 149, no. 1, pp. 22–35, 2012.

[56] A. Clayton, "Cancer cells use exosomes as tools to manipulate immunity and the microenvironment," *Oncoimmunology*, vol. 1, no. 1, pp. 78–80, 2012.

[57] M. Fabbri, A. Paone, F. Calore et al., "MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 31, pp. E2110–E2116, 2012.

[58] M. Otranto, V. Sarrazy, F. Bonté, B. Hinz, G. Gabbiani, and A. Desmoulière, "The role of the myofibroblast in tumor stroma remodeling," *Cell Adhesion and Migration*, vol. 6, no. 3, pp. 203–219, 2012.

[59] J. Webber, R. Steadman, M. D. Mason, Z. Tabi, and A. Clayton, "Cancer exosomes trigger fibroblast to myofibroblast differentiation," *Cancer Research*, vol. 70, no. 23, pp. 9621–9630, 2010.

[60] J. A. Cho, H. Park, E. H. Lim et al., "Exosomes from ovarian cancer cells induce adipose tissue-derived mesenchymal stem cells to acquire the physical and functional characteristics of tumor-supporting myofibroblasts," *Gynecologic Oncology*, vol. 123, no. 2, pp. 379–386, 2011.

[61] J. A. Cho, H. Park, E. H. Lim, and K. W. Lee, "Exosomes from breast cancer cells can convert adipose tissue-derived mesenchymal stem cells into myofibroblast-like cells," *International Journal of Oncology*, vol. 40, no. 1, pp. 130–138, 2012.

[62] F. Thuma and M. Zoller, "Outsmart tumor exosomes to steal the cancer initiating cell its niche," *Seminars in Cancer Biology*, 2014.

[63] V. Luga and J. L. Wrana, "Tumor-stroma interaction: revealing fibroblast-secreted exosomes as potent regulators of Wnt-planar cell polarity signaling in cancer metastasis," *Cancer Research*, vol. 73, no. 23, pp. 6843–6847, 2013.

[64] S. S. Sidhu, A. T. Mengistab, A. N. Tauscher, J. LaVail, and C. Basbaum, "The microvesicle as a vehicle for EMMPRin in tumor-stromal interactions," *Oncogene*, vol. 23, no. 4, pp. 956–963, 2004.

[65] A. Ramteke, H. Ting, C. Agarwal et al., "Exosomes secreted under hypoxia enhance invasiveness and stemness of prostate cancer cells by targeting adherens junction molecules," *Molecular Carcinogenesis*, 2013.

[66] M. Katoh, "Therapeutics targeting angiogenesis: genetics and epigenetics, extracellular miRNAs and signaling networks (Review)," *International Journal of Molecular Medicine*, vol. 32, no. 4, pp. 763–767, 2013.

[67] P. Kucharzewska, H. C. Christianson, J. E. Welch et al., "Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 18, pp. 7312–7317, 2013.

[68] D. K. Jeppesen, A. Nawrocki, S. G. Jensen et al., "Quantitative proteomics of fractionated membrane and lumen exosome proteins from isogenic metastatic and nonmetastatic bladder cancer cells reveal differential expression of EMT factors," *Proteomics*, vol. 14, no. 6, pp. 699–712, 2014.

[69] J. L. Hood, S. San Roman, and S. A. Wickline, "Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis," *Cancer Research*, vol. 71, no. 11, pp. 3792–3801, 2011.

[70] H. Peinado, M. Alečković, S. Lavotshkin et al., "Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET," *Nature Medicine*, vol. 18, no. 6, pp. 883–891, 2012.

[71] D. D. Taylor and C. Gercel-Taylor, "MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer," *Gynecologic Oncology*, vol. 110, no. 1, pp. 13–21, 2008.

[72] S. Dijkstra, I. L. Birker, F. P. Smit et al., "Prostate cancer biomarker profiles in urinary sediments and exosomes," *The Journal of Urology*, vol. 191, no. 4, pp. 1132–1138, 2014.

[73] Y. Li and E. Mustapha Bahassi, "Biofluid-based circulating tumor molecules as diagnostic tools for use in personalized medicine," *Journal of Molecular Biomarkers & Diagnosis*, vol. 5, pp. 157–163, 2013.

[74] K. Shedden, X. T. Xie, P. Chandaroy, Y. T. Chang, and G. R. Rosania, "Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles," *Cancer Research*, vol. 63, no. 15, pp. 4331–4337, 2003.

[75] R. Safaei, B. J. Larson, T. C. Cheng et al., "Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells," *Molecular Cancer Therapeutics*, vol. 4, no. 10, pp. 1595–1604, 2005.

[76] R. Khan, S. Zahid, Y. J. Wan et al., "Protein expression profiling of nuclear membrane protein reveals potential biomarker of

human hepatocellular carcinoma," *Clinical Proteomics*, vol. 10, no. 1, p. 6, 2013.

[77] T. Aung, B. Chapuy, D. Vogel et al., "Exosomal evasion of humoral immunotherapy in aggressive B-cell lymphoma modulated by ATP-binding cassette transporter A3," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 37, pp. 15336–15341, 2011.

[78] V. Ciravolo, V. Huber, G. C. Ghedini et al., "Potential role of HER2-overexpressing exosomes in countering trastuzumab-based therapy," *Journal of Cellular Physiology*, vol. 227, no. 2, pp. 658–667, 2012.

[79] H.-G. Zhang and W. E. Grizzle, "Exosomes and cancer: a newly described pathway of immune suppression," *Clinical Cancer Research*, vol. 17, no. 5, pp. 959–964, 2011.

[80] G. Raposo, H. W. Nijman, W. Stoorvogel et al., "B lymphocytes secrete antigen-presenting vesicles," *The Journal of Experimental Medicine*, vol. 183, no. 3, pp. 1161–1172, 1996.

[81] L. Zitvogel, A. Regnault, A. Lozier et al., "Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes," *Nature Medicine*, vol. 4, no. 5, pp. 594–600, 1998.

[82] B. Escudier, T. Dorval, N. Chaput et al., "Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase 1 clinical trial," *Journal of Translational Medicine*, vol. 3, article 10, 2005.

[83] M. A. Morse, J. Garst, T. Osada et al., "A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer," *Journal of Translational Medicine*, vol. 3, no. 1, article 9, 2005.

[84] S. Dai, D. Wei, Z. Wu et al., "Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer," *Molecular Therapy*, vol. 16, no. 4, pp. 782–790, 2008.

[85] S. Viaud, M. Terme, C. Flament et al., "Dendritic cell-derived exosomes promote natural killer cell activation and proliferation: a role for NKG2D ligands and IL-15Rα," *PLoS ONE*, vol. 4, no. 3, article e4942, 2009.

[86] S. A. A. Kooijmans, P. Vader, S. M. van Dommelen, W. W. van Solinge, and R. M. Schiffelers, "Exosome mimetics: a novel class of drug delivery systems," *International Journal of Nanomedicine*, vol. 7, pp. 1525–1541, 2012.

[87] L. Alvarez-Erviti, Y. Seow, H. Yin, C. Betts, S. Lakhal, and M. J. A. Wood, "Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes," *Nature Biotechnology*, vol. 29, no. 4, pp. 341–345, 2011.

[88] S.-I. Ohno, A. Ishikawa, and M. Kuroda, "Roles of exosomes and microvesicles in disease pathogenesis," *Advanced Drug Delivery Reviews*, vol. 65, no. 3, pp. 398–401, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 941359, 10 pages
http://dx.doi.org/10.1155/2014/941359

Research Article

Prolonged Nitric Oxide Exposure Enhances Anoikis Resistance and Migration through Epithelial-Mesenchymal Transition and Caveolin-1 Upregulation

Pithi Chanvorachote, Varisa Pongrakhananon, and Preedakorn Chunhacha

Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences and Cell-Based Drug and Health Products Development Research Unit, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand

Correspondence should be addressed to Pithi Chanvorachote; pithi_chan@yahoo.com
and Preedakorn Chunhacha; preedakorn.chun@gmail.com

Received 3 March 2014; Accepted 1 May 2014; Published 20 May 2014

Academic Editor: Rutao Cui

Nitric oxide (NO) in tumor microenvironment may have a significant impact on metastatic behaviors of cancer. Noncytotoxic doses of NO enhanced anoikis resistance and migration in lung cancer H23 cells via an increase in lamellipodia, epithelial-mesenchymal transition (EMT) markers including vimentin and snail, and caveolin-1 (Cav-1). However, the induction of EMT was found in Cav-1-knock down cells treated with NO, suggesting that EMT was through Cav-1-independent pathway. These effects of NO were consistently observed in other lung cancer cells including H292 and H460 cells. These findings highlight the novel role of NO on EMT and metastatic behaviors of cancer cells.

1. Introduction

Lung cancer is among the leading causes of cancer-related death worldwide and evidences have suggested that metastasis in such a cancer is a major cause of death [1]. As metastasis is a complicated process, cancer cells must have an ability to overcome several obstacles including anoikis, a process of death mediated after cells detachment [2]. Anoikis is accepted as one important body defense mechanism against cancer dissemination [2]. Like adherent normal cells, most solid tumor cells will die after detachment by anoikis; however, certain population of the cells have a capability to resist anoikis, survive in the blood or lymphatic circulations, reach new sites, and establish secondary tumors. Besides anoikis resistance, a motility behavior of cancer cells was also recognized as a critical factor for success in metastasis as the early step of cancer dissemination involves cell migration and intravasation into blood or lymphatic systems [3]. A number of studies in the cancer research fields have focused on the biological process found in cancer cells called epithelial-mesenchymal transition (EMT) and EMT is believed to enhance metastatic potentials of several cancers [4]. Indeed, EMT is a multistep cellular process that allows an epithelial cell to possess mesenchymal phenotype [5]. Recently, EMT has garnered special attention since many researchers recognized EMT as a hallmark reflecting cancer aggressiveness and poor prognosis [6]. An enhanced metastatic behavior such as an increase in migratory activity was continuously demonstrated in cancer cells exhibiting EMT phenotype [5, 6]. Also, the EMT was shown to be involved with anoikis resistance in lung, melanoma and colon cancer cells [7–9]. Downregulation of E-cadherin, together with upregulation of N-cadherin, vimentin, and snail, was long shown to be a key indicator of EMT process; therefore, the protein alterations were shown to link with the acquisition of anoikis resistance [6, 10–12]. Likewise, caveolin-1 (Cav-1), a major protein component of caveolae, was reported to regulate cancer cell activities. Caveolin-1 expression in lung cancer was shown to be related to poor prognosis and metastasis capability [13]. Our previous study showed that Cav-1 mediated anoikis resistant [14, 15] as well as increased migration and invasion in lung cancer cells [16]. Together, such information leads

to the possible conclusion that EMT and Cav-1 may share overlapping pathways in regulation of metastatic behaviors; however, insights into such regulation remain elusive.

Nitric oxide (NO) is a gaseous biological mediator that frequently reported to be upregulated in lung cancer environments [17]. Our previous works demonstrated that this important mediator affects lung cancer cells in many ways including induced cisplatin [18] and Fas ligand resistance [19]. However, its roles in regulation of EMT remain unknown.

So far, the knowledge regarding the biological mediators that force EMT in lung cancer has been largely unknown. Because more understanding of nature of the cancer cells in response to biological substance may lead to high precision and efficiency in treating the disease, the present study aimed to investigate an effect of long-term NO exposure on EMT characteristics and Cav-1 level in lung cancer cells on the basis that the results gained from the study could benefit the development of therapeutic approaches.

2. Materials and Methods

2.1. Cells and Reagents. The non-small cell lung cancer (NSCLC) cell lines H23, H292, A549, and H460 were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/mL penicillin/streptomycin. The cells were incubated in a 5% CO2 environment at 37°C. For NO exposure, cells were cultured in medium containing DPTA NONOate (at nontoxic concentrations) for 14 days. The culturing medium was replaced by freshly prepared medium containing DPTA NONOate every 2 days. Phalloidin tetramethylrhodamine B isothiocyanate, dipropylenetriamine NONOate (DPTA NONOate), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Hoechst 33342 was obtained from Molecular Probes, Inc. (Eugene, OR). Antibodies for caveolin-1, vimentin, snail, TCF8/ZEB1, E-cadherin, ZO-1, N-cadherin, β-catenin, and β-actin, as well as peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA).

2.2. Cell Viability Assay. Cell viability was determined by the MTT assay. After specific treatments, cells were incubated with 500 μg/mL of MTT at 37°C for 4 h. Then, the MTT solution was removed and 100 μL of DMSO was added to dissolve the formazan crystal. The intensity reading of MTT product was measured at 570 nm using a microplate reader, and the percentage of viable cells was calculated in relation to control cells.

2.3. Cell Morphology and Lamellipodia Characterization. Cell morphology and lamellipodia were investigated by a phalloidin-rhodamine staining assay. The cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at 37°C, permeabilized with 0.1% Triton-X100 in PBS for 4 min, and blocked with 0.2% BSA for 30 min. Then, the cells were incubated with either 1 : 100 phalloidin-rhodamine in PBS or 0.4% sulforhodamine B in 1% acetic acid for 15 min, rinsed 3 times with PBS, and mounted with 50% glycerol. Cell morphology was then assessed by fluorescent imaging (Olympus IX51 with DP70). Filopodia protrusion was represented as the average number of filopodia/cell relative to untreated cells in each field.

2.4. Anoikis Assay. For anoikis evaluation, 6-well tissue culture plates were coated with 200 μL of poly 2-hydroxyethylmethacrylate (poly-HEMA; Sigma) and left for 10 h in a laminar flow hood. Cells were seeded in poly-HEMA-coated plates at the density of 1×10^5 cells/mL and incubated for various times up to 24 h at 37°C. Cell viability was assessed by addition of 1 : 50 resazurin for 1 h at 37°C. Fluorescence intensity of resazurin product (resorufin) was measured at 530 nm (excitation wavelength) and 590 nm (emission wavelength) using a microplate reader. Cell viability was calculated as a percentage relatively to time zero. All analyses were performed in at least three independent replicate experiments.

2.5. Migration Assay. The migration assay was carried out using Transwell cell culture chamber (Corning Costar number 3422, MA, USA). Conditioned media (500 μL media with 10% FBS) were added into the lower compartment of the chamber. P1, A549, H23, and H460 cells at the concentration of 2×10^5 cells in 1% fetal bovine serum containing media were added to the upper compartment of the chamber. After 12 h incubation, the top side of insert membrane was scrubbed with a cotton swab and the bottom side was fixed with ice-cold methanol and stained with Hoechst 33342 and scoring under fluorescence microscope (Olympus IX51 with DP70).

2.6. Plasmid and Transfection. Cav-1 knockdown plasmid short hairpin (sh)RNA-Cav-1 was obtained from Santa Cruz Biotechnology. Stable transfection of Cav-1 knockdown plasmid was generated by culturing cells in a six-well plate until they reached 60% confluence. Fifteen μL of lipofectamine reagent and 2 μg of Cav-1, shRNA-Cav-1, or control plasmid were used to transfect the cells in the absence of serum. After 12 h the medium was replaced with culture medium containing 5% FBS. Approximately 36 h after the beginning of transfection, the cells were digested with 0.03% trypsin, and the cell suspensions were plated onto 75-mL culture flasks and cultured for 24 to 28 days with specific selection. The stable transfectants were pooled, and the expression of Cav-1 protein in the transfectants was confirmed by western blotting. The cells were cultured in antibiotic-free RPMI 1640 medium for at least two passages before they were used in each experiment.

2.7. Western Blotting. After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor cocktail (Roche Molecular Biochemicals,

Basel, Switzerland) for 30 min on ice. Cell lysates were collected and determined for protein content using the Bradford method (Bio-Rad Laboratories, Hercules, CA). An equal amount of proteins of each sample (40 μg) were denatured by heating at 95°C for 5 min with Laemmli loading buffer and subsequently loaded on 10% SDS-polyacrylamide gel electrophoresis. After separation, proteins were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 hour in 5% nonfat dry milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween-20) and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhancement with chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL) and quantified using analyst/PC densitometry software (Bio-Rad).

2.8. Statistical Analysis. The data represented means ± SD from three or more independent experiments. Statistical analysis was performed by Student's t-test at a significance level of $P < 0.05$.

3. Results

3.1. Nitric Oxide Exposure Alters Cell Morphology of Human Non-Small Cell Lung Cancer H23 Cells. To elucidate the effect of NO, a mediator frequently found in tumor microenvironments on metastatic potentials, we first characterized NO donor that causes no toxicity to the cells. H23 cells were incubated with DETA NONOate for 72 h and viability of the cells was determined by MTT-based viability assay. Figure 1(a) shows that the treatment with NO donor (DETA NONOate) at the concentrations ranging from 0–25 μM caused no significant cytotoxic effect on H23 cell viability in comparison to nontreated control. For further investigations, cells were cultured in the normal growth medium supplemented with NO donor at the concentrations of 10 and 25 μM for 14 days. Interestingly, NO-treated cells exhibited dramatically change in terms of cell morphology with cell enlargement in comparison to that of control nontreated cells (Figure 1(b)).

Lamellipodia are a cell protrusion that is widely accepted to be critical for directional migration in many cells [20]. To clarify whether such cell enlargement is caused by an increase of lamellipodia, we performed phalloidin-rhodamine staining assay with Hoechst 33342 nuclear staining. Figure 1(c) indicates that NO-treated cells exhibited increased sheet-like lamellipodia in comparison to those of parental H23 cells. Since lamellipodia were shown to increase in highly motile cells, these results suggested that long-term NO exposure may result in an increase of cell migratory activity.

3.2. Nitric Oxide Exposure Induces Cell Migration and Anoikis Resistance. Having shown that NO treatment has an inductive effect on cell protrusion, we next characterized migratory activity of the cells. NO-treated (14 days) and parental H23 cells were subjected to migration assay as described in Materials and Methods Section. Figure 2 shows that lung cancer cells exposed to NO for 14 days exhibited significantly enhanced migration activity. Even though the dose-dependent effect of NO in this case was not observed, an increase in cell migration approximately 2 folds in both NO-treated conditions strongly indicates potentiating role of NO on motility of these cancer cells. Since migration and anoikis resistance are accepted to be important factors for metastasis, we next elucidated the effect of NO exposure on anoikis susceptibility of the cells. Cells were similarly treated with NO as the aforementioned for 14 days and were determined for anoikis sensitivity by anoikis assay. Figure 2(b) indicates that the NO-treated cells at concentrations of 10 and 25 μM exhibited anoikis resistant phenotype and could survive in detached condition up to 24 h, whereas their nontreated counterparts showed 50% reduction in cell viability after detachment for 24 h. Together, these results suggested that long-term exposure of the cells to NO potentiates anoikis resistance as well as migration ability of H23 cells and may be responsible for the metastasis potentials.

3.3. NO Induces Epithelial to Mesenchymal Transition. Enhanced abilities of cancer cells to metastasis are believed to increase through the process of EMT. Also, EMT was linked to the increasing capability of cancer cells in migrating away and resisting anoikis [9]. The present study further investigated the EMT phenotypes of the long-term NO-treated and H23 cells. The expression levels of EMT markers including vimentin, snail, TCF8/ZEB1, E-cadherin, ZO-1, N-cadherin, and β-catenin were determined using western blot analysis. The cells were exposed to NO donor at the concentrations of 10 and 25 μM for 14 days and EMT markers were evaluated. Figure 3 shows that NO treatment significantly decreased E-cadherin level in the cells. Interestingly, vimentin and snail dramatically upregulated in NO-treated H23 cells in a dose-dependent manner, while other markers TCF8/ZEB1, ZO-1, N-cadherin, and β-catenin were not significantly altered. We found that at the concentration of 10 μM, NO donor induced approximately 2- and 2.5-fold inductions of vimentin and snail, respectively. Moreover, at 25 μM, NO donor induced approximately 4.5 and 4 fold inductions of vimentin and snail, respectively.

Collectively, our results indicated that the long-term treatment of NO induced EMT in lung cancer H23 cells and such EMT may be responsible for the increase of migration and anoikis resistance.

3.4. NO Treatment Increases Caveolin-1 Level. We and others have provided a number of evidences indicating that Cav-1 protein performs a key function in regulation of anoikis resistance as well as cell motility [14–16]. These data lead to the possibility that long-term NO exposure could mediate such cancer cell aggressiveness through Cav-1-dependent mechanism. The parental and NO-treated cells (14 days) were subjected to western blot analysis for Cav-1 determination. The results indicated that after the treatment with NO for 14 days,

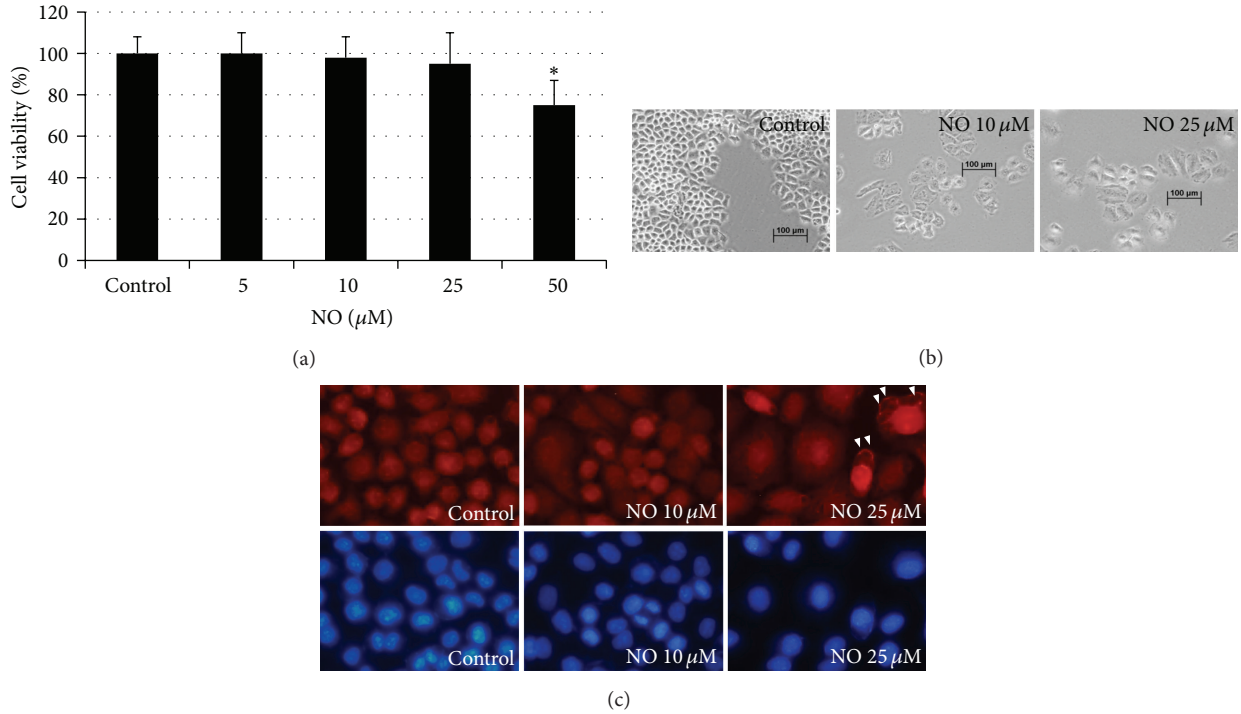


Figure 1: Effect of nitric oxide donor on cytotoxicity in lung carcinoma H23 cells. (a) Effect of DPTA NONOate on cell viability. Lung cancer H23 cells were treated with various concentrations (0–50 μM) of DPTA NONOate for 24 h. The cell viability was analyzed using the MTT assay. The data are the mean ± S.D. ($n = 3$). $^{*}P < 0.05$ versus the nontreated control. (b) Morphology of H23 cells treated with DETA NONOate for 14 days. (c) Lamellipodia formation in H23 cells treated with NO donor. H23 cells were treated with NO donor at concentrations of 10 and 25 μM for 14 days. The cells were then stained with phalloidin-rhodamine and Hoechst33342 dye.

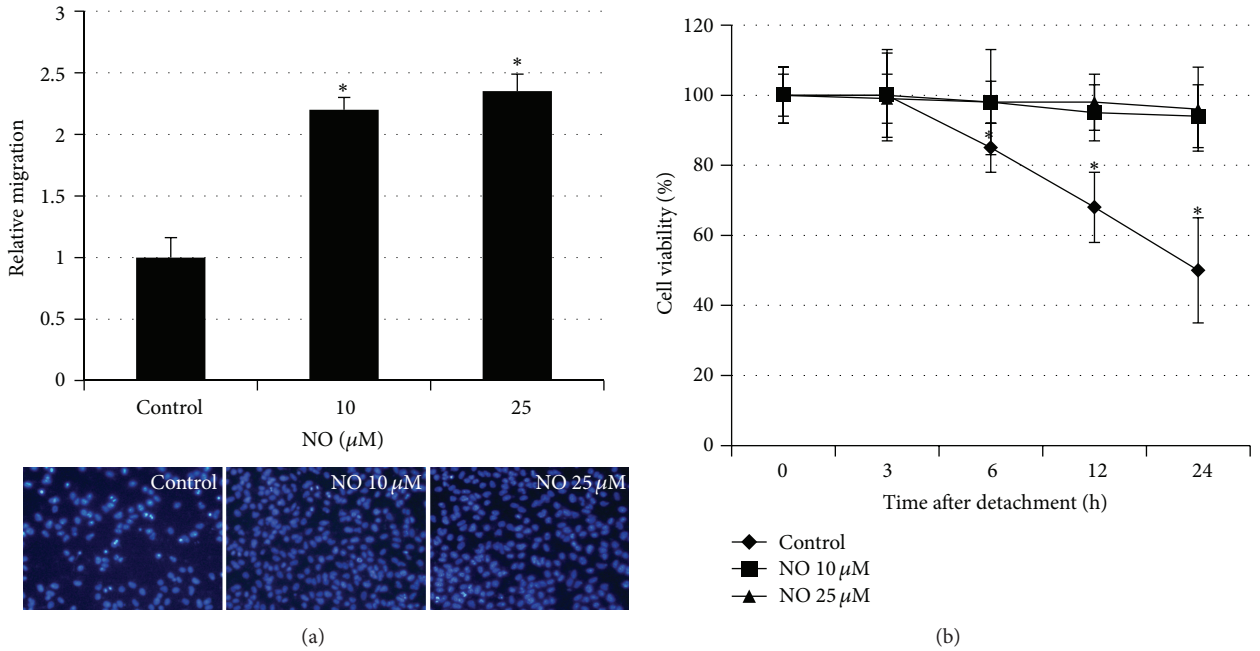


Figure 2: Effect of nitric oxide exposure on cell migration and anoikis response. Cells were exposed to DETA NONOate at various concentrations (10 and 25 μM) for 14 days and subjected to migration and anoikis assays. (a) The relative cell migration was determined as described in Material and Methods Section. (b) Viability of the cells after detachment was evaluated in a time-dependent manner. The data are the mean ± S.D. ($n = 3$). $^{*}P < 0.05$ versus the control cells.

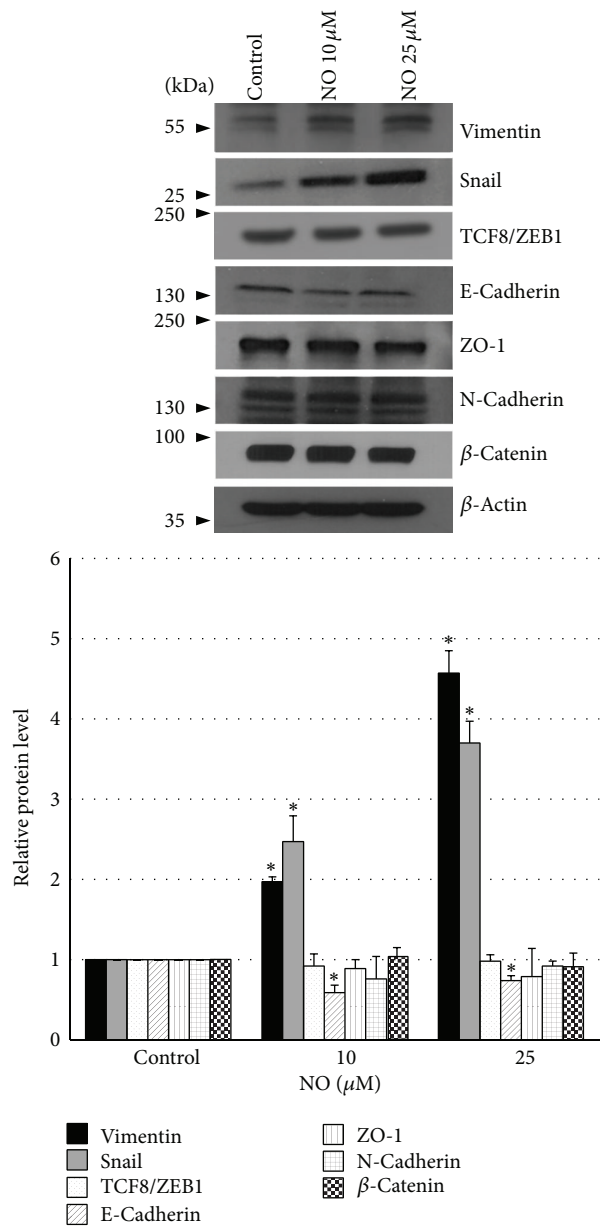


Figure 3: Effect of nitric oxide on epithelial-mesenchymal transition. Cells were exposed to DETA NONOate at various concentrations (10 and 25 μM) for 14 days and subjected to migration and anoikis assays. The expression level of EMT markers including vimentin, snail, TCF8/ZEB1, E-cadherin, ZO-1, N-cadherin, and β-catenin were determined using western blot analysis. To confirm equal loading of the samples, the blots were reprobed with β-actin antibody. The immunoblot signals were quantified by densitometry. The data are the mean ± S.D. ($n = 3$). $^*P < 0.05$ versus the nontreated control.

Cav-1 in H23 cells was significantly upregulated (Figure 4(a)). Further, we provided the evidence demonstrating the roles of Cav-1 on anoikis resistance and cell migration in H23 and other lung cancer cells. Basal Cav-1 level of H23, H292, H460, and A549 was evaluated using western blotting (Figure 4(b)). Further, the anoikis susceptibility and cell migration of all cells were determined. A549 cells exhibiting the highest level of Cav-1 showed the strongest anoikis resistant potential in comparison to that of H23, H292, and H460 lung cancer cells, suggesting the role of Cav-1 in attenuating anoikis process in these cells (Figure 4(c)). Likewise, the level of Cav-1 protein was found to be tightly correlated with migratory activity of the cells (Figure 4(d)). Taken together, our results show that long-term NO exposure increased Cav-1 level in H23 cells and the protein, at least partly, mediated anoikis resistance and increased cell motility.

3.5. NO Induces EMT in a Cav-1-Independent Mechanism. To test NO-mediated EMT in these cells through Cav-1-dependent pathway, H23 cells were stably transfected with ShRNA-Cav-1 plasmid. The stable Cav-1 knock-down cells

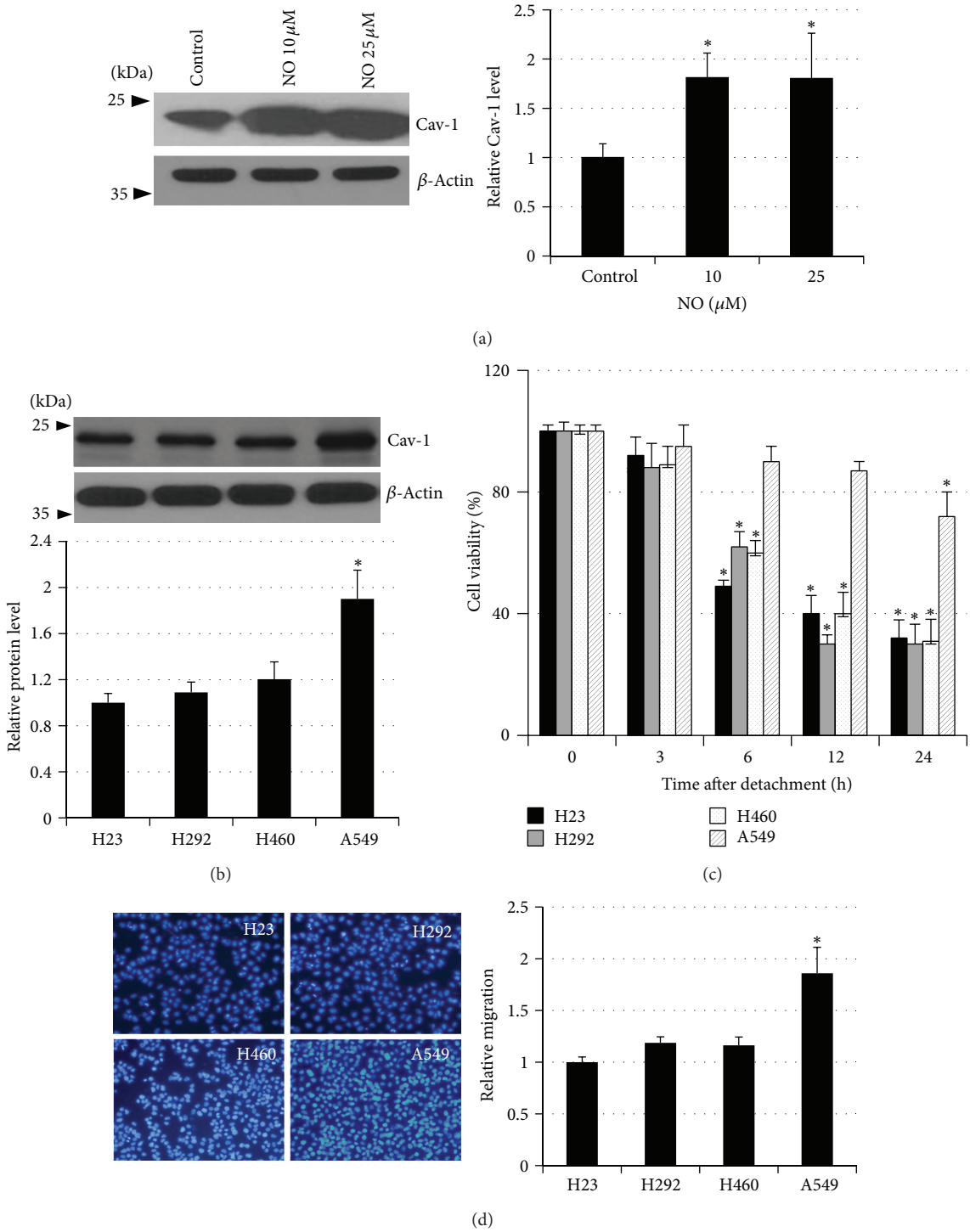


FIGURE 4: Nitric oxide mediated caveolin-1 upregulation, anoikis resistance and increase migration. (a) Cells were exposed to DETA NONOate for 14 days and Cav-1 level was evaluated by western blotting. (b) Basal level of Cav-1 protein in H23, H292, H460, and A549 lung cancer cells was determined and the immunoblot signals were quantified by densitometry. (c) Anoikis susceptibility of H23, H292, H460, and A549 lung cancer cells was determined by anoikis assay. (d) The relative cell migration of H23, H292, H460, and A549 lung cancer cells was determined. The data are the mean ± S.D. ($n = 3$). $^{*}P < 0.05$ versus the H23 control cells.

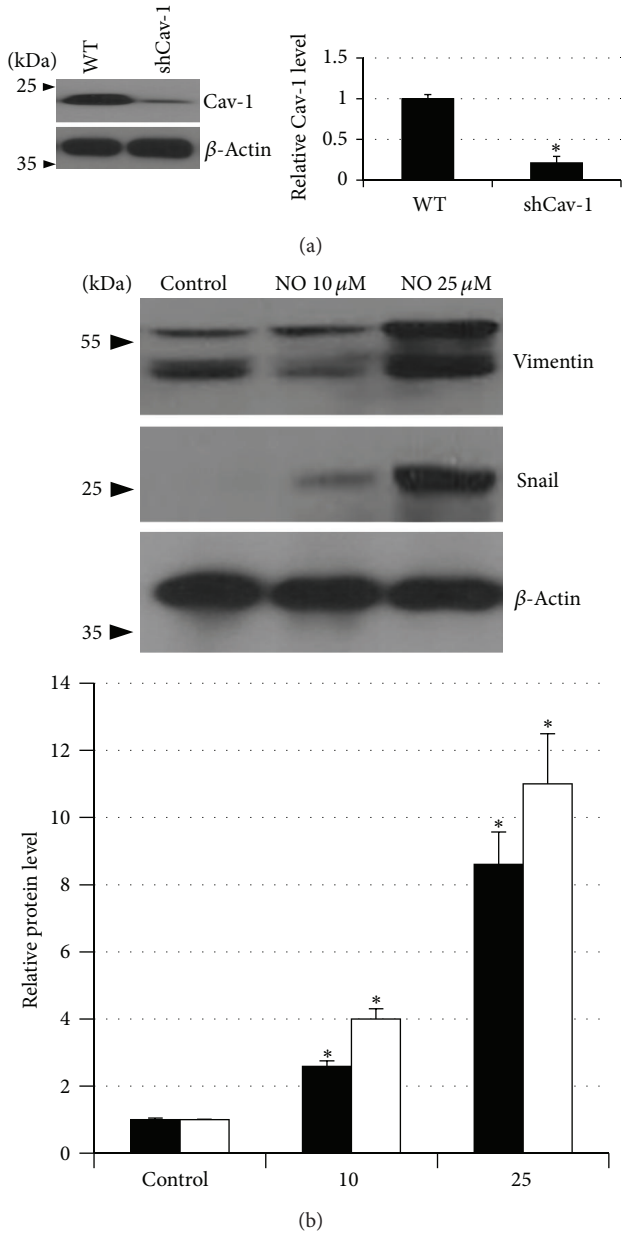


Figure 5: Nitric oxide mediated epithelial-mesenchymal transition via Cav-1-independent mechanism.Stable Cav-1 knock-down (ShCav-1) cells were established as indicated in Section 2. (a) The expression level of Cav-1 protein in the control H23 and ShCav-1 cells was determined by western blotting. (b) The ShRNA-Cav-1 cells were exposed to NO donor for 14 days, and the expression of vimentin and snail was determined. The immunoblot signals of vimentin and snail were quantified by densitometry. The data are the mean ± S.D. ($n = 3$). $^*P < 0.05$ versus the nontreated control.

were tested for the Cav-1 level, and the results indicated that Cav-1 in the cells dramatically decreased (Figure 5(a)). Then, the ShRNA-Cav-1 cells were treated with NO donor as described previously, and EMT markers were evaluated. Even though Cav-1 in the cells was suppressed, vimentin and snail were significantly upregulated in response to long-term NO treatment (Figure 5(b)). These results indicated that the EMT process induced by NO exposure in our system may not mediate through Cav-1-dependent mechanism.

3.6. NO Induces EMT in Other Lung Cancer Cells. Further, we tested whether other lung cancer cells responded to NO exposure in the same way as H23 cells. Lung cancer H292 and H460 cells were exposed to NO at the concentrations of 10 and 25 μM for 14 days and EMT markers and aggressive behaviors of the cells were investigated. Figures 6(a) and 6(d) indicate that H292 cells exhibited a significant increase in Cav-1 and vimentin levels in response to NO treatment, while snail level in these cells was not altered. These results suggested that long-term NO exposure resulted in an increase in both Cav-1 protein and EMT process. Consistently, the migratory activity of these cells significantly enhanced in the cells treated with NO. Taken together, our experiments indicated that long-term NO exposure was able to increase cellular Cav-1 protein and induced EMT.

4. Discussion

The knowledge regarding factors that influence cancer metastasis may benefit the development of novel treating strategy as well as the improved diagnosis sensitivity to this life-threatening disease. Nowadays, evidences suggest that metastasis process of cancers can be enhanced by several factors. Indeed, the increased levels of inflammatory cytokines, reactive oxygen species, and nitric oxide in cancer microenvironment may have an important impact on cell aggressiveness [17, 21, 22]. Recently, we have provided the data indicating that Cav-1 in the detached lung cancer cells could inhibit anoikis process of the cells by sustaining the antiapoptotic Mcl-1 protein [23]. Additionally, in terms of cancer cell migration, Cav-1 protein is shown to be a positive regulator [16]. Among several biological mediators, NO has received an increasing attention in the cancer research field and is believed to be the key factor potentiating cancer progression [21]. However, the evidence in the regulatory role of this substance on EMT and cancer aggressiveness is still limited. Herein, we provide the evidence indicating that the cells that received NO at nontoxic concentration for relatively long period made the cells more capable of spreading by increasing Cav-1 and EMT.

Current evidences explaining the involvement of cancer metastasis and the process of EMT and EMT are accepted to be a mechanism facilitating the cancer cells to spread away [4, 6, 24]. Indeed, the roles of NO on EMT process are intriguing and sometimes contradictory depending on cell type, duration of NO exposure, and dose of NO-modulating agents. Some previous studies suggested that NO attenuates TGF-β1-mediated EMT in alveolar epithelial cells [25] and mouse hepatocytes [26]. Also, NO was shown to suppress EMT in prostate cancer cells when it was used at high concentrations [27]. Based on the fact that EMT is a complex process which is involved in multiple signaling pathways, the distinguishable effect of NO reported by the present study may be due to the difference in type of cells as well as the concentrations of NO.

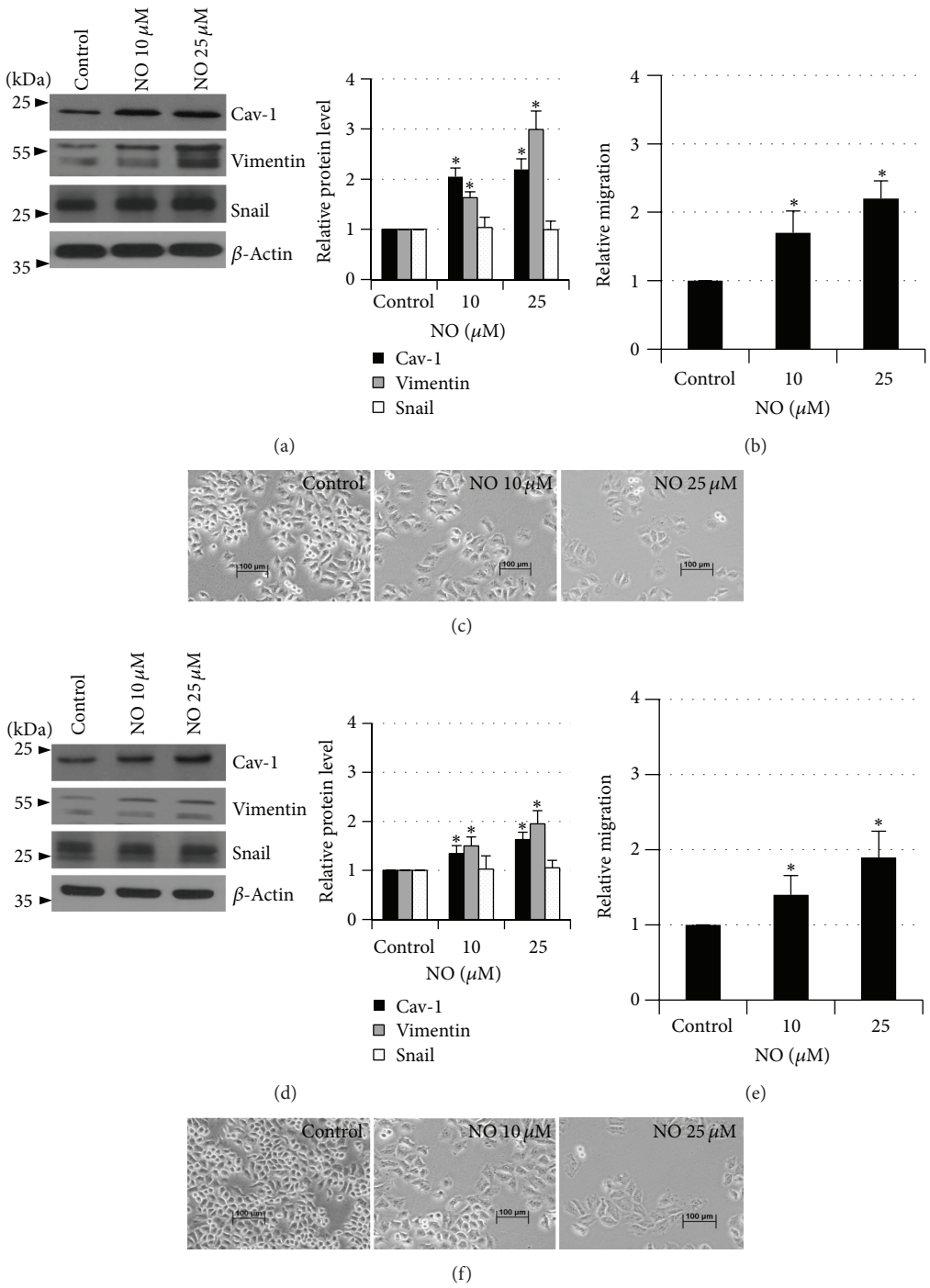


Figure 6: Nitric oxide mediated caveolin-1 upregulation and epithelial-mesenchymal transition in other lung cancer cells. H292 (a) and H460 (d) lung cancer cells were exposed to DETA NONOate for 14 days and expression levels of Cav-1, vimentin, and snail were determined. The immunoblot signals were quantified by densitometry. Migratory activity of H292 (b) and H460 (c) cells was evaluated as described. Morphology of H292 (c) and H460 (f) cells treated with DETA NONOate for 14 days. The data are the mean ± S.D. ($n = 3$). $^{*}P < 0.05$ versus the nontreated control.

We demonstrated that after prolonged NO exposure, the lung cancer H23 cells increased the expression level of vimentin and snail in concomitant with the decrease of E-cadherin. In response to such EMT, the migratory as well as anoikis resistance characteristics in NO-treated cancer cells were enhanced (Figure 2). In addition, in other lung cancer cells the NO mediated EMT and increased metastatic behaviors could be observed (Figure 6). Such information has strengthened the link between NO and EMT process in lung cancer. Together with the fact that not all cancer cells

exhibited high EMT [24], the cells that immerged in NO-rich environment may have a better chance to succeed metastasis through EMT enhancement.

Regarding Cav-1 protein, certain studies have focused on the role of Cav-1 expression in increasing migration and anoikis resistance [15, 16, 22, 23]. Although some evidence has suggested the role of Cav-1 in suppressing cancer [28], in lung cancer, Cav-1 potentiates cancer progression and aggressiveness. Cav-1 expression has been shown to relate to poor prognosis and reduced tumor-free periods in lung cancer patients [29]. Moreover, Cav-1 was shown to facilitate metastasis and induce anoikis resistance in lung carcinoma cell lines [2, 14–16, 30]. Not only does Cav-1 play a role in cell death and survival, but also in cell migration [16], invasion [31], and lipid transportation [32]. However, in our study, NO could induce EMT characteristics even in Cav-1 knockdown cells. This suggests that NO mediated EMT in Cav-1 independent mechanism. Cav-1 may possess an ability to overcome anoikis in the cells by direct interaction with Mcl-1 protein [22]. In addition, Cav-1 was shown to increase cellular level of activated Akt that may directly contribute to the increase cell survival as well as migration [16].

Based on these data, we have provided the novel information that the ability of cancer cells to transition to mesenchymal phenotype could be enhanced by long-term NO exposure. Additionally, NO exposure increases the level of Cav-1, a known protein facilitating anoikis resistance and migration. Our results support the conception that the biological mediators found in cancer microenvironments have a significant impact on the ability of cancer cells to metastasise. This insight may facilitate the better understanding of cancer cell biology.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by Ratchadaphiseksompot Endowment Fund, Chulalongkorn University (RES560530132-HR), and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission. The authors wish to thank Mr. Krich Rajprasit, a proofreader.

References

[1] P. Mehlen and A. Puisieux, "Metastasis: a question of life or death," *Nature Reviews Cancer*, vol. 6, no. 6, pp. 449–458, 2006.

[2] P. Chanvorachote, P. Chunhacha, and P. Pongrakhananon, "Anoikis: a potential target to prevent lung cancer metastasis?" *Lung Cancer Management*, vol. 2, pp. 169–171, 2013.

[3] P. Friedl and D. Gilmour, "Collective cell migration in morphogenesis, regeneration and cancer," *Nature Reviews Molecular Cell Biology*, vol. 10, no. 7, pp. 445–457, 2009.

[4] F. Meng and G. Wu, "The rejuvenated scenario of epithelial-mesenchymal transition (EMT) and cancer metastasis," *Cancer and Metastasis Reviews*, no. 3-4, pp. 455–467, 2012.

[5] R. Kalluri and R. A. Weinberg, "The basics of epithelial-mesenchymal transition," *Journal of Clinical Investigation*, vol. 119, no. 6, pp. 1420–1428, 2009.

[6] M. Yilmaz and G. Christofori, "EMT, the cytoskeleton, and cancer cell invasion," *Cancer and Metastasis Reviews*, vol. 28, no. 1-2, pp. 15–33, 2009.

[7] G. Li, K. Satyamoorthy, and M. Herlyn, "N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells," *Cancer Research*, vol. 61, no. 9, pp. 3819–3825, 2001.

[8] M. E. Minard, L. M. Ellis, and G. E. Gallick, "Tiam1 regulates cell adhesion, migration and apoptosis in colon tumor cells," *Clinical and Experimental Metastasis*, vol. 23, no. 5-6, pp. 301–313, 2006.

[9] P. Chunhacha, V. Sriuranpong, and P. Chanvorachote, "Epithelial-mesenchymal transition mediates anoikis resistance and enhances invasion in pleural effusion-derived human lung cancer cells," *Oncology Letters*, vol. 5, no. 3, pp. 1043–1047, 2013.

[10] M. C. Guadamillas, A. Cerezo, and M. A. del Pozo, "Overcoming anoikis—pathways to anchorageindependent growth in cancer," *Journal of Cell Science*, vol. 124, no. 19, pp. 3189–3197, 2011.

[11] R. B. Hazan, R. Qiao, R. Keren, I. Badano, and K. Suyama, "Cadherin switch in tumor progression," *Annals of the New York Academy of Sciences*, vol. 1014, pp. 155–163, 2004.

[12] H. Ko, S. Kim, C. H. Jin et al., "Protein kinase casein kinase 2-mediated upregulation of N-cadherin confers anoikis resistance on esophageal carcinoma cells," *Molecular Cancer Research*, vol. 10, no. 8, pp. 1032–1038, 2012.

[13] H. J. Joo, D. K. Oh, Y. S. Kim, K. B. Lee, and S. J. Kim, "Increased expression of caveolin-1 and microvessel density correlates with metastasis and poor prognosis in clear cell renal cell carcinoma," *BJU International*, vol. 93, no. 3, pp. 291–296, 2004.

[14] H. Halim, S. Luanpitpong, and P. Chanvorachote, "Acquisition of anoikis resistance up-regulates caveolin-1 expression in human non-small cell lung cancer cells," *Anticancer Research*, vol. 32, no. 5, pp. 1649–1658, 2012.

[15] P. Chunhacha and P. Chanvorachote, "Roles of caveolin-1 on anoikis resistance in non small cell lung cancer," *International Journal of Physiology, Pathophysiology and Pharmacology*, vol. 4, no. 3, pp. 149–155, 2012.

[16] S. Luanpitpong, S. J. Talbott, Y. Rojanasakul et al., "Regulation of lung cancer cell migration and invasion by reactive oxygen species and caveolin-1," *The Journal of Biological Chemistry*, vol. 285, no. 50, pp. 38832–38840, 2010.

[17] C.-Y. Liu, C.-H. Wang, T.-C. Chen, H.-C. Lin, C.-T. Yu, and H.-P. Kuo, "Increased level of exhaled nitric oxide and up-regulation of inducible nitric oxide synthase in patients with primary lung cancer," *The British Journal of Cancer*, vol. 78, no. 4, pp. 534–541, 1998.

[18] P. Chanvorachote, U. Nimmannit, C. Stehlik et al., "Nitric oxide regulates cell sensitivity to cisplatin-induced apoptosis through S-nitrosylation and inhibition of Bcl-2 ubiquitination," *Cancer Research*, vol. 66, no. 12, pp. 6353–6360, 2006.

[19] P. Chanvorachote, U. Nimmannit, L. Wang et al., "Nitric oxide negatively regulates Fas CD95-induced apoptosis through inhibition of ubiquitin-proteasome-mediated degradation of FLICE inhibitory protein," *The Journal of Biological Chemistry*, vol. 280, no. 51, pp. 42044–42050, 2005.

[20] C. Ballestrem, B. Wehrle-Haller, B. Hinz, and B. A. Imhof, "Actin-dependent lamellipodia formation and microtubule-dependent tail retraction control-directed cell migration," *Molecular Biology of the Cell*, vol. 11, no. 9, pp. 2999–3012, 2000.

[21] P. K. Lala and C. Chakraborty, "Role of nitric oxide in carcinogenesis and tumour progression," *The Lancet Oncology*, vol. 2, no. 3, pp. 149–156, 2001.

[22] P. Chanvorachote, U. Nimmannit, Y. Lu, S. Talbott, B.-H. Jiang, and Y. Rojanasakul, "Nitric oxide regulates lung carcinoma cell anoikis through inhibition of ubiquitin-proteasomal degradation of caveolin-1," *The Journal of Biological Chemistry*, vol. 284, no. 41, pp. 28476–28484, 2009.

[23] P. Chunhacha, V. Pongrakhananon, Y. Rojanasakul, and P. Chanvorachote, "Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells," *American Journal of Physi-ology—Cell Physiology*, vol. 302, no. 9, pp. C1284–C1292, 2012.

[24] T. Tsuji, S. Ibaragi, and G.-F. Hu, "Epithelial-mesenchymal transition and cell cooperativity in metastasis," *Cancer Research*, vol. 69, no. 18, pp. 7135–7139, 2009.

[25] S. Vyas-Read, P. W. Shaul, I. S. Yuhanna, and B. C. Willis, "Nitric oxide attenuates epithelial-mesenchymal transition in alveolar epithelial cells," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 293, no. 1, pp. L212–L221, 2007.

[26] X. Pan, X. Wang, W. Lei et al., "Nitric oxide suppresses transforming growth factor-β1—induced epithelial-to-mesenchymal transition and apoptosis in mouse hepatocytes," *Hepatology*, vol. 50, no. 5, pp. 1577–1587, 2009.

[27] S. Baritaki, S. Huerta-Yepez, A. Sahakyan et al., "Mechanisms of nitric oxide-mediated inhibition of EMT in cancer: inhibition of the metastasis-inducer snail and induction of the metastasis-suppressor RKIP," *Cell Cycle*, vol. 9, no. 24, pp. 4931–4940, 2010.

[28] C. Racine, M. Bélanger, H. Hirabayashi, M. Boucher, J. Chakir, and J. Couet, "Reduction of caveolin 1 gene expression in lung carcinoma cell lines," *Biochemical and Biophysical Research Communications*, vol. 255, no. 3, pp. 580–586, 1999.

[29] C.-C. Ho, S.-H. Kuo, P.-H. Huang, H.-Y. Huang, C.-H. Yang, and P.-C. Yang, "Caveolin-1 expression is significantly associated with drug resistance and poor prognosis in advanced non-small cell lung cancer patients treated with gemcitabine-based chemotherapy," *Lung Cancer*, vol. 59, no. 1, pp. 105–110, 2008.

[30] D. Yeh, C. Chen, M.-Z. Sun et al., "Caveolin-1 is an important factor for the metastasis and proliferation of human small cell lung cancer NCI-H446 cell," *Anatomical Record*, vol. 292, no. 10, pp. 1584–1592, 2009.

[31] Y. Tang, X. Zeng, F. He, Y. Liao, N. Qian, and M. Toi, "Caveolin-1 is related to invasion, survival, and poor prognosis in hepatocellular cancer," *Medical Oncology*, vol. 29, no. 2, pp. 977–984, 2012.

[32] A. F. Quest, L. Leyton, and M. Párraga, "Caveolins, caveolae, and lipid rafts in cellular transport, signaling, and disease," *Biochemistry and Cell Biology*, vol. 82, no. 1, pp. 129–144, 2004.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 341654, 7 pages
http://dx.doi.org/10.1155/2014/341654

Research Article

FOXP3 Transcription Factor: A Candidate Marker for Susceptibility and Prognosis in Triple Negative Breast Cancer

Leandra Fiori Lopes,[1] Roberta Losi Guembarovski,[1] Alda Losi Guembarovski,[2] Marina Okuyama Kishima,[2] Clodoaldo Zago Campos,[3] Julie Massayo Maeda Oda,[1] Carolina Batista Ariza,[1] Karen Brajão de Oliveira,[1] Sueli Donizete Borelli,[4] and Maria Angelica Ehara Watanabe[1]

[1] *Laboratory of Molecular Genetics and Immunology, Department of Pathological Sciences, Biological Science Center, State University of Londrina, 86057-970 Londrina, PR, Brazil*
[2] *Department of Pathology, Clinical Analysis and Toxicology, Health Sciences Center, State University of Londrina, 86057-970 Londrina, PR, Brazil*
[3] *Department of Medical Clinic, Health Sciences Center, State University of Londrina, 86057-970 Londrina, PR, Brazil*
[4] *Department of Basic Health Sciences, Maringá State University, 87020-900 Maringá, PR, Brazil*

Correspondence should be addressed to Maria Angelica Ehara Watanabe; maewatuel@gmail.com

Received 20 February 2014; Accepted 9 April 2014; Published 30 April 2014

Academic Editor: Rutao Cui

Triple negative breast cancer (TNBC) is a relevant subgroup of neoplasia which presents negative phenotype of estrogen and progesterone receptors and has no overexpression of the human epidermal growth factor 2 (HER2). FOXP3 (forkhead transcription factor 3) is a marker of regulatory T cells (Tregs), whose expression may be increased in tumor cells. This study aimed to investigate a polymorphism (rs3761548) and the protein expression of FOXP3 for a possible involvement in TNBC susceptibility and prognosis. Genetic polymorphism was evaluated in 50 patients and in 115 controls by allele-specific PCR (polymerase chain reaction). Protein expression was evaluated in 38 patients by immunohistochemistry. It was observed a positive association for homozygous AA (OR = 3.78; 95% CI = 1.02–14.06) in relation to TNBC susceptibility. Most of the patients (83%) showed a strong staining for FOXP3 protein in the tumor cells. In relation to FOXP3-positive infiltrate, 47% and 58% of patients had a moderate or intense intratumoral and peritumoral mononuclear infiltrate cells, respectively. Tumor size was positively correlated to intratumoral FOXP3-positive infiltrate ($P = 0.026$). In conclusion, since FOXP3 was positively associated with TNBC susceptibility and prognosis, it seems to be a promising candidate for further investigation in larger TNBC samples.

1. Introduction

The National Cancer Institute (INCA) estimated 52,680 new cases of breast cancer for 2012 and 2013 in Brazil. It is worth noting that, regardless of nonmelanoma skin cancer, the mammary tumor is the most common among women in many regions of Brazil, accounting high morbidity and mortality among Brazilian women [1].

Breast cancer represents a complex and heterogeneous disease that comprises distinct pathologies, histological features, and clinical outcome. The status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2) has been used as predictive markers to identify a high-risk phenotype and for selection of the most efficient therapies [2, 3]

Triple-negative breast cancer (TNBC) is a subtype characterized by the lack of ER, PR, and HER2 expression and is associated with younger age at diagnosis and often occurs in African-American, premenopausal, and overweighed women (particularly with abdominal obesity) [4]. It represents approximately 12–17% of all breast cancers [5] and encompasses a heterogeneous group of tumors including, but not

limited to, those classified as basal-like. TBNC is aggressive, showing a tendency towards early metastasis and having a poor overall outcome despite being highly responsive to conventional chemotherapy. The aggressive clinical course, poor prognosis, and lack of specific therapeutic options for this subtype of tumor have intensified current interest in this group of patients [6].

Regulatory T cells (Treg) represent a heterogeneous population of cells composed of discrete subsets with different phenotypes and functions [7]. The most specific marker to identify Tregs is *FOXP3*, a member of the forkhead-winged helix family of transcription factors [8, 9] that plays a role in various cellular processes. *FOXP3* expression in tumors was associated with worse overall survival and this gene was also considered a strong prognostic factor for distant metastases-free survival but not for local recurrence risk [10]. Hirata et al. reviewed some well-established molecular markers of therapeutic value in breast cancer and also promising new markers not routinely used in clinical practice, which includes *FOXP3* gene [11].

Promoter regions are potential candidates for the presence of functional single nucleotide polymorphism (SNPs), as they are involved in transcription initiation, and many of the cis-acting elements that regulate gene expression possibly harbor functional polymorphisms [12]. As recently reviewed by Oda et al., FOXP3 polymorphisms occur with high frequency in the general population and have been studied in common multifactorial human diseases, like diabetes, allergic rhinitis, and breast cancer [13]. It is known that SNPs in the promoter region of *FOXP3* gene may affect its expression [14]. Since it has been previously shown that *FOXP3* is involved in breast cancer development [15], several studies have been conducted to investigate a SNP (*rs3761548, C/A)* in the promoter region of *FOXP3* in patients with this neoplasia [16, 17], but its exact role is not yet well understood.

In this context, the present report attempts to investigate if there is an association between genetic polymorphism and protein expression of *FOXP3* gene with clinical outcome, in a search for its involvement in pathogenesis of TNBC.

2. Material and Methods

2.1. Human Subjects. Retrospectively (10 years), clinic and pathological information (tumor size, lymph node involvement, and nuclear grade) and tissue samples of 50 TNBC were obtained at Cancer Hospital of Londrina (HCL), Londrina, Paraná State, Brazil. Clinical staging was determined according to the Union of International Control of Cancer (UICC) classification criteria.

For control group, blood samples from 115 women neoplasia-free were collected in the Blood Center of North Parana, Brazil. The protocol was approved by the Institutional Human Research Ethics Committee of the State University of Londrina, Paraná, Brazil (CAAE No. 0179.0.268-09-CONEP 268).

2.2. DNA Extraction. For patients, the genomic DNA was isolated from formalin-fixed paraffin embedded samples according to Isola et al. [18] protocol. For neoplasia-free control group, the DNA was obtained from peripheral white blood cells using Biopur kit (Biometrix, Curitiba, PR, Brazil). The DNA was resuspended in 50 μL of Milli-Q water and quantified by NanoDrop 2000c Spectrophotometer (NanoDrop Technologies, Wilmington, DE, EUA) at a wavelength of 260/280 nm.

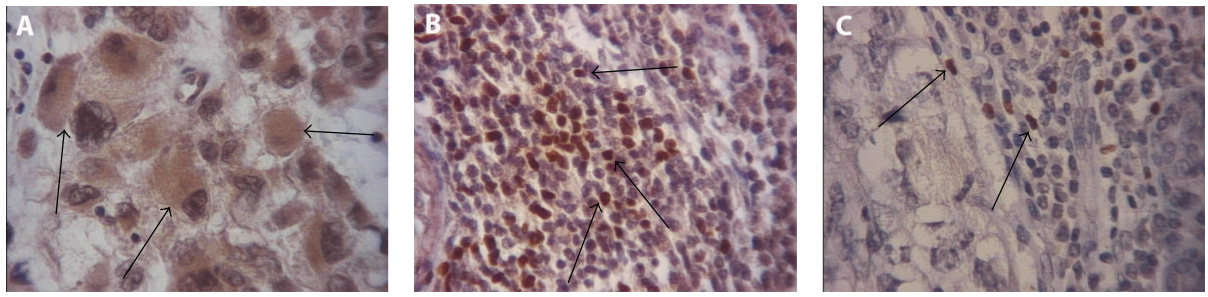
2.3. Genetic Polymorphism of FOXP3 rs3761548. DNA (100 ng) was amplified by polymerase chain reaction (PCR) with specific primers for *FOXP3* following the GenBank accession number NT_079573.4. The samples were amplified using the buffer kit plus 1.25 units Taq polymerase (Invitrogen, Carlsbad, CA, USA).

PCR conditions were 10 min denaturation at 94°C, 35 cycles of 45 s at 94°C, 1 min at 67°C and 1 min at 72°C, and 10 min elongation at 72°C in a thermocycler (PCR-Sprint Hybaid-Guelph, Ontario, Canada). Amplicons of 334 base pairs for A allele and 333 base pairs for C allele were analyzed by electrophoresis on acrylamide gel (10%) and detected by a nonradioisotopic technique using a commercially available silver staining method (Table 1).

2.4. Immunohistochemical Staining. For immunohistochemical analysis, 5 μm of tissue sections was obtained from breast tumors samples. Samples were heated at 56°C, deparaffinized in xylene, and rehydrated in a graded alcohol series. Antigen retrieval was performed with citrate buffer and a mouse/rabbit monoclonal antibody for human *FOXP3* (clone 236A/E7; Abcam, Cambridge, UK; eBioscience) was used. The sections were stabilized at room temperature for 30 min and washed with PBS (phosphate buffered saline) and anti-mouse/rabbit HRP secondary antibody was used as second step (Bio SB Inc. Santa Barbara, CA, USA). The diaminobenzidine (DAB) chromogen system was used (Sigma-Aldrich, USA) and counter staining was performed with Gill's hematoxylin and slide mounts in Canada balsam. Controls were performed to verify the specificity of primary antibody and all analyses were made with at least two pathologists.

2.5. Statistical Analysis. The case control association study was performed using contingency tables to calculate the odds ratios (OR) with a confidence interval (CI) of 95 %. A 3x2 contingency table was constructed, considering wild type genotype (OR = 1.0) as reference, to determine the OR value for heterozygotes and rare genotypes. GraphPad Prism version 5.00 for Windows was used (GraphPad Software, San Diego, CA, USA). The rare homozygous and heterozygotes for *FOXP3* gene were grouped for the presence of at least one allelic variant, in a dominant model of analysis.

Spearman correlation and Chi square statistical tests were used to analyze immunohistochemistry and genetic polymorphism in relation to clinical outcome, using SPSS Statistics 17.0 software (SPSS inc., Chicago, IL, USA). A P value <0.05 was considered statistically significant.



(a) (b) (c)

Figure 1: FOXP3 expression by immunohistochemistry in TNBC tissue samples. (a) FOXP3 cytoplasmic expression in breast tumor cells; (b) FOXP3 intratumoral mononuclear infiltrating cells in breast tumor; and (c) FOXP3 peritumoral mononuclear infiltrating cells in breast tumor. The arrows indicated some strong staining. Magnification 400x.

Table 1: Oligonucleotides and amplicons for *FOXP3* gene.

Gene	Allele	Primer sequence	PCR product
FOXP3 (C/A, rs3761548)	A	5′-CTG GCT CTC TCC CCA ACT GA-3′ 5′-ACA GAG CCC ATC ATC AGA CTC TCT A-3′	334 bp
	C	5′-TGG CTC TCT CCC CAA CTG C-3′ 5′-ACA GAG CCC ATC ATC AGA CTC TCT A-3′	333 bp

3. Results

The mean age of patients was 54 ± 13 years. For some patients specific clinic pathological characteristics were not available. It was observed from patients who had the respective information; 83% presented nuclear grade in stage II or III, 51% had lymph node commitment, and the tumor size mean was 3.5 cm.

3.1. Genetic Polymorphism Analysis. The rs3761548 polymorphism of *FOXP3* gene was evaluated in 50 TNBC patients and in 115 neoplasia free controls. The genotype frequency was 12% (6/50) and 3.48% (4/115) for AA homozygote, 34% (17/50) and 57.39% (66/115) for CA heterozygote, and 54% (27/50) and 39.13% (45/115) for CC homozygote, in patients and controls, respectively (Table 2). Case control study indicated a positive association for AA homozygous genotype in relation to TNBC susceptibility (OR = 3.78, 95% CI = 1.02 to 14.06).

When comparing genotypes of *FOXP3* and clinical outcome, there was no significant association with tumor size ($P = 0.482$; rho = 0.102), lymph node involvement ($P = 0.890$; rho = −0.023), and nuclear grade ($P = 0.682$; rho = −0.062).

3.2. Immunohistochemistry Analysis. In 38 patients analyzed for FOXP3 protein expression, "cytoplasmic" tumoral staining was verified predominantly in all tissue samples analyzed (Figure 1(a)). Most of TNBC patients (83%) had high expression of tumoral FOXP3 protein (two or three crosses). Additionally, for patients who were lymph node free of neoplasia, a strong FOXP3 expression was verified, most of them being with a strength signal (three crosses), despite being not statistically significant ($P = 0.14$).

Tumors sizes and nuclear degrees are equally distributed among the patients according to FOXP3 protein expression ($P = 0.42$ and $P = 0.12$), respectively. Allelic variant showed no correlation with FOXP3 protein expression ($P = 0.792$, rho = −0.046). Therefore, despite being not significant, it was observed that allele A carriers for *FOXP3* gene present higher tumoral expression of this protein by immunohistochemistry ($P = 0.078$).

Table 3 described the protein expression analysis in relation to infiltration of mononuclear cells positive for FOXP3 staining in tumor microenvironment of 38 patients. 47% and 58% of the sample presented a moderate or intense intratumoral and peritumoral infiltrated, respectively (Figures 1(b) and 1(c)). There were no significant results for both mononuclear infiltrated (intratumoral and peritumoral) in relation to lymph node involvement or nuclear grade parameters. Therefore, the results indicated a significant association between intratumoral infiltrated and tumor size ($P = 0.026$). It was observed that this significance was attributed to tumors between 1.5 and 3 cm, since this prognostic parameter was divided into three categories based on clinical criteria (less than 1.5 cm, 1.5 to 3 cm, and more than 3 cm).

4. Discussion

Breast cancer is a complex disease with high clinical morphological and biological heterogeneity. It is known that mammary tumors with similar clinical histology and different prognoses had different therapeutic responses [19–21].

The *FOXP3* gene expressed in CD4+ CD25+ Tregs in normal physiological conditions encodes the FOXP3 protein,

Table 2: Genotype distribution and case control study for *FOXP3* gene in patients and controls.

		Controls ($n = 115$)	Patients ($n = 50$)	OR	IC	P value (χ^2)
FOXP3 rs3761548	CC	45 (39%)	27 (54%)	1.00	—	—
	CA	66 (57%)	17 (34%)	0.38*	0.19–0.76	0.006*
	AA	4 (4%)	6 (12%)	3.78*	1.02–14.06	0.035*
	CA + AA	70 (61%)	23 (46%)	0.55	0.28–1.07	0.077

*$P < 0.05$.

Table 3: FOXP3 protein expression in mononuclear cells in relation to prognostic parameters of TN breast tissues.

FOXP3 protein expression ($n = 38$)	Intensity and prognostic parameters	Frequency (%) or P value
Intratumoral infiltrated of mononuclear cells	Moderate/intense	47%
	Lymph node involvement	0.310
	Nuclear grade	0.531
	Tumor size	0.026*
Peritumoral infiltrated of mononuclear cells	Moderate/intense	58%
	Lymph node involvement	0.679
	Nuclear grade	0.309
	Tumor size	0.598

*$P < 0.05$.

which regulates the activation of T cell, works as a transcriptional repressor and downregulates cytokines expression in T cells [8, 22].

The autoimmune disease that lacks functional *FOXP3*, observed in human and in mice, indicates that this transcription factor has a crucial role in the regulation of T-cell function [23]. Additionally, it has been suggested that *FOXP3*-positive cells in tumors could be a novel therapeutic target that could improve outcomes for such patients [24]. So the high rate of somatic mutations in breast tumors, its conserved sequence, and the regulation of important pathways make *FOXP3* a very plausible candidate for a susceptibility gene in cancer [16].

In this study, we analyzed a *FOXP3* polymorphism (rs3761548) in 50 TNBC patients and in 115 controls free of neoplasia (Table 2). The results indicated a positive association for AA homozygous genotype in relation to TNBC development (OR = 3.78, 95% CI = 1.02 to 14.06). Therefore, we suggested that individuals who had inherited both copies of this allelic variation had a higher susceptibility for developing this subtype of breast cancer than individuals with other genotypes. As far as we researched, there is no articles relating genetic polymorphism of *FOXP3* and TNBC susceptibility in a Brazilian population, but positive associations have been proposed with other diseases such as psoriasis [25] and allergic rhinitis [26]. Raskin et al. [16] investigated three genetic polymorphisms in the *FOXP3* gene in patients with breast cancer, but not triple-negative subtype, and found none significant associations. Additionally, these authors postulated that *FOXP3* gene may be involved with the hereditary breast cancer form, with high penetrance mutations. Our results are not in accordance with these authors, since we found a positive association between a specific *FOXP3* polymorphic mutation and TNBC susceptibility (Table 2). Despite the low number of homozygotes observed in both groups, we found 12% of AA homozygotes in TNBC group versus only 4% in the control group, although the last one is composed of a much larger number of individuals. Thus, although our results deserve caution by the sample size, they indicate a possible role for *FOXP3* gene in TNBC susceptibility.

Initially, it was postulated that *FOXP3* expression was thought to be restricted to hematopoietic tissues. However, although data are scant, *FOXP3* expression in other tissues has also been observed, including human tumor cells [27]. Therefore, biological functions of *FOXP3* in tumor cells and its significance presently remain unclear. According to the same authors, their study clearly demonstrates that FOXP3 expression is not restricted to pancreatic carcinoma cells but seems to characterize many other tumors not only of epithelial (e.g., lung, breast, and colon) but also of other tissue origins (melanoma, leukemia).

Recent data suggest that *FOXP3* expression in tumor cells could be an independent strong prognostic factor for distant metastases in breast cancer [28], but in contrast with these data, *FOXP3* was also recently demonstrated to be a tumor suppressor gene, acting as a transcriptional repressor of *SKP2* and *HER2*, two breast cancer important oncogenes [15, 29]. In the present work, we analyzed the tumoral protein expression of FOXP3 and found that 83% of the patients had a strong expression of this protein in the tumor microenvironment. Other studies found that cancer cells were FOXP3 positive in 57% of HER2+ breast tumors [30] and in 66% of archival samples from human breast cancer patients [10], indicating that our sample of TNBC demonstrated a high expression of this protein and that FOXP3 may have different expression levels in subtypes of breast cancer, with specific prognostic implications.

According to Triulzi et al., in contrast to a putative oncosuppressor role for *FOXP3*, emerging evidence from studies of human cancer samples points to its prometastatic action in vivo, based on the correlation between its expression by tumor cells and poor prognosis. The authors point out that, overall, the associations between FOXP3 expressions in tumor cells are with poor patient's prognosis [31]. Kim et al. found FOXP3 expression in 27.9% of their breast cancer samples and the positive tumors were associated with significantly higher nuclear grade, higher histologic grade, and

a more negative estrogen receptor status. A multivariate analysis with adjustment for patient age and human epidermal growth factor receptor 2 status demonstrated significantly poor survival of FOXP3-strong-positive patients in node-positive patients, which suggest that this protein expression in breast cancer cells is associated with poor prognosis [32].

In our sample we did not observe any associations between protein expression of FOXP3 and clinical outcome parameters, considering tumor size, lymph node involvement, and nuclear grade. Ladoire et al. also found no association with tumor size and lymph node involvement; however, the authors observed a significant result between protein expression and tumor grade ($P = 0.046$), which strengthens prognostic differences for FOXP3 protein expression in mammary tumor subgroups [30].

Another relevant point is that FOXP3 protein expression observed in our TNBC sample was predominantly cytoplasmic. According to the literature data, a cytoplasmic localization was observed in human cancer cells in various tissues [27, 33], including breast carcinoma [10, 25] and breast adenocarcinoma cell line (MCF7) [27]. Also, in a study concerning *FOXP3* expression in prostate cancer cells [34], the authors demonstrated that genetic mutations in this gene could be detected in cancer cells and restrained its expression in the cytoplasm. According to Triulzi et al., in most breast carcinomas, FOXP3 staining was localized predominantly in the cytoplasm, although both cytoplasmic and nuclear expressions were present in some specimens and a few showed only nuclear staining [31]. In this TNBC sample, we also found that most patients had cytoplasmic expression of FOXP3 protein, but some had concomitant perinuclear and/or nuclear expression.

It is noteworthy that experimental evidences show that *FOXP3* downregulates the oncogene *HER2* and other genes in this signaling pathway. Within this context, our results concerning FOXP3 protein expression are in accordance, since we observed a high expression in tumor cells of TNBC patients, who are exactly negative for *HER2* expression by immunohistochemistry. Therefore, Karanikas et al. that found a high expression of FOXP3 in MCF7 and other cell lines said that whether this expression by tumor cells is directly related to carcinogenesis or results indirectly by activation of its normally silent gene is questionable [27].

Although T cells present the most important immunological response in tumor growth in early stages of cancer, they become Tregs after chronic stimulation and interactions with tumor cells, promoting rather than inhibiting cancer development and progression [35]. Karanikas et al. point that the highest mRNA expression levels of FOXP3 observed by tumor cells were with the breast cancer line MCF7, which expressed at least half as much FOXP3 as a Treg clone did and at least ten times more than a population of PHA blasts. This expression level indicates that FOXP3 transcripts are present in a sufficiently high number in tumor cells and caution should be exerted when detection of *FOXP3* mRNA expression in surgical tumor samples is used as an index of tumor infiltration by Tregs [27].

The study of Demir et al. established a predictive and prognostic effect of intratumoral FOXP3 Tregs in locally advanced breast cancer patients. The authors point out that to predict clinical outcome, an evaluation of FOXP3+ Tregs in tumoral tissues before and after neoadjuvant chemotherapy should be considered for these high-risk patients [36]. Concerning infiltrate of mononuclear cells expressing *FOXP3* in the tumor microenvironment, we had the results of 38 patients and observed that 47% and 58% of these had a moderate or intense intratumoral and peritumoral infiltrated, respectively.

Gokmen-Polar et al. found that the number of FOXP3-expressing T regulatory cells does not differ significantly between sentinel nodes with and without metastatic breast carcinoma and also does not affect primary tumor characteristics like tumor type, grade, size, hormone receptor, and HER2 status [37]. Ladoire et al. in their series of HER2+ over expressing breast carcinoma found that the presence of *FOXP3* Treg infiltration had no prognostic behavior [30]. Corroborating these data, we also did not find any significant results when analyzing this parameter in relation to lymph node involvement and nuclear grade.

On the other hand, we observed a significant association with tumor size parameter ($P = 0.026$) and that this significance was attributed to tumor size range from 1.5 to 3 cm. On a multivariate analysis Lee et al. showed that FOXP3-positive Tregs were an independent prognostic factor for overall survival and progression-free survival with hazard ratios of 2.4 (95% CI 1.0–5.6; $P = 0.049$) and 2.0 (95% CI 1.1–3.6; $P = 0.032$), respectively. So these authors concluded that in TNBC patients FOXP3-positive Tregs had stronger prognostic significance. The finding of improved survival associated with highly infiltrating FOXP3-positive Tregs in TNBC contrasted with several other types of solid cancers, but according to them, TNBC may be differently driven by FOXP3 via an immune mechanism [35]. In this context, we hypothesized that Tregs *FOXP3* positive, which were present in the tumor, could act as stimulator of growing in intermediates tumors size (1.5 to 3 cm), in relation to small tumors (less than 1.5 cm). Likewise, another mechanism could stimulate even larger sizes of tumors TNBC (above 3 cm), since, in our sample, the intratumoral infiltrate does not appear to be positively associated with larger tumors.

5. Conclusion

Since we found a significant association between a specific genetic variant in *FOXP3* gene and a high expression of this protein in the tumor microenvironment, which would agree with the fact that TNBC patients do not present the overexpression of *HER2* oncogene, and also a positive correlation between *FOXP3*-positive infiltrate and the prognostic parameter tumor size, we suggest that this transcript factor could be a promising marker of susceptibility and prognosis in human breast cancer pathogenesis, especially in the triple-negative molecular subtype.

Conflict of Interests

The authors declare that there are no conflict of interests.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Londrina State University Coordination for Post-Graduation (PROPPG-UEL). The authors wish to express their gratitude to Fundação Araucária do Paraná (CONV. 300/2012, CONV. 200/2010, and CONV. 1027/2013), CAPES, and Secretaria da Ciência, Tecnologia e Ensino Superior (SETI) for financial support.

References

[1] INCA, *Estimate 2012: Cancer Incidence in Brazil*, Instituto Nacional de Câncer (INCA), Rio de Janeiro, Brazil, 2011.

[2] A. P. Presson, N. K. Yoon, L. Bagryanova et al., "Protein expression based multimarker analysis of breast cancer samples," *BMC Cancer*, vol. 11, p. 230, 2011.

[3] B. Weigelt and J. S. Reis-Filho, "Molecular profiling currently offers no more than tumour morphology and basic immunohistochemistry," *Breast Cancer Research*, vol. 12, supplement 4, p. S5, 2010.

[4] R. Dent, M. Trudeau, K. I. Pritchard et al., "Triple-negative breast cancer: clinical features and patterns of recurrence," *Clinical Cancer Research*, vol. 13, no. 15, part 1, pp. 4429–4434, 2007.

[5] W. D. Foulkes, I. E. Smith, and J. S. Reis-Filho, "Triple-negative breast cancer," *New England Journal of Medicine*, vol. 363, no. 20, pp. 1938–1948, 2010.

[6] E. Y. Cho, M. H. Chang, Y. L. Choi et al., "Potential candidate biomarkers for heterogeneity in triple-negative breast cancer (TNBC)," *Cancer Chemotherapy and Pharmacology*, vol. 68, no. 3, pp. 753–761, 2011.

[7] C. Menetrier-Caux, T. Curiel, J. Faget, M. Manuel, C. Caux, and W. Zou, "Targeting regulatory T cells," *Targeted Oncology*, vol. 7, no. 1, pp. 15–28, 2012.

[8] S. Hori, T. Nomura, and S. Sakaguchi, "Control of regulatory T cell development by the transcription factor *FOXP3*," *Science*, vol. 299, no. 5609, pp. 1057–1061, 2003.

[9] G. Roncador, P. J. Brown, L. Maestre et al., "Analysis of *FOXP3* protein expression in human *CD4+CD25+* regulatory T cells at the single-cell level," *European Journal of Immunology*, vol. 35, no. 6, pp. 1681–1691, 2005.

[10] A. Balsari, A. Merlo, P. Casalini et al., "*FOXP3* expression and overall survival in breast cancer," *Journal of Clinical Oncology*, vol. 27, no. 11, pp. 1746–1752, 2009.

[11] B. K. B. Hirata, J. M. Oda, G. R. Losi, C. B. Ariza, C. E. de Oliveira, and M. A. Watanabe, "Molecular markers for breast cancer: prediction on tumor behavior," *Disease Markers*, 2014.

[12] B. Hoogendoorn, S. L. Coleman, C. A. Guy et al., "Functional analysis of human promoter polymorphisms," *Human Molecular Genetics*, vol. 12, no. 18, pp. 2249–2254, 2003.

[13] J. M. Oda, B. K. B. Hirata, R. L. Guembarovski, and M. A. E. Watanabe, "Genetic polymorphism in *FOXP3* gene: imbalance in regulatory T-cell role and development of human diseases," *Journal of Genetics*, vol. 92, no. 1, pp. 163–171, 2013.

[14] W. M. Bassuny, K. Ihara, Y. Sasaki et al., "A functional polymorphism in the promoter/enhancer region of the *FOXP3*/Scurfin gene associated with type 1 diabetes," *Immunogenetics*, vol. 55, no. 3, pp. 149–156, 2003.

[15] T. Zuo, L. Wang, C. Morrison et al., "*FOXP3* is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene," *Cell*, vol. 129, no. 7, pp. 1275–1286, 2007.

[16] L. Raskin, G. Rennert, and S. B. Gruber, "*FOXP3* germline polymorphisms are not associated with risk of breast cancer," *Cancer Genetics and Cytogenetics*, vol. 190, no. 1, pp. 40–42, 2009.

[17] Y. Liu and P. Zheng, "*FOXP3* and breast cancer: implications for therapy and diagnosis," *Pharmacogenomics*, vol. 8, no. 11, pp. 1485–1487, 2007.

[18] J. Isola, S. DeVries, L. Chu, S. Ghazvini, and F. Waldman, "Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumor samples," *American Journal of Pathology*, vol. 145, no. 6, pp. 1301–1308, 1994.

[19] M. Cianfrocca and W. Gradishar, "New molecular classifications of breast cancer," *CA Cancer Journal for Clinicians*, vol. 59, no. 5, pp. 303–313, 2009.

[20] F. C. Geyer, C. Marchio, and J. S. Reis-Filho, "The role of molecular analysis in breast cancer," *Pathology*, vol. 41, no. 1, pp. 77–88, 2009.

[21] B. Weigelt and J. S. Reis-Filho, "Histological and molecular types of breast cancer: is there a unifying taxonomy?" *Nature Reviews Clinical Oncology*, vol. 6, no. 12, pp. 718–730, 2009.

[22] R. S. McHugh, M. J. Whitters, C. A. Piccirillo et al., "*CD4+CD25+* Immunoregulatory T Cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor," *Immunity*, vol. 16, no. 2, pp. 311–323, 2002.

[23] P. J. Coffer and B. M. T. Burgering, "Forkhead-box transcription factors and their role in the immune system," *Nature Reviews Immunology*, vol. 4, no. 11, pp. 889–899, 2004.

[24] M. A. Watanabe, J. M. Oda, M. K. Amarante, and J. Cesar Voltarelli, "Regulatory T cells and breast cancer: implications for immunopathogenesis," *Cancer and Metastasis Reviews*, vol. 29, no. 4, pp. 569–579, 2010.

[25] L. Gao, K. Li, F. Li et al., "Polymorphisms in the *FOXP3* gene in Han Chinese psoriasis patients," *Journal of Dermatological Science*, vol. 57, no. 1, pp. 51–56, 2010.

[26] L. Zhang, Y. Zhang, M. Desrosiers, C. Wang, Y. Zhao, and D. Han, "Genetic association study of *FOXP3* polymorphisms in allergic rhinitis in a Chinese population," *Human Immunology*, vol. 70, no. 11, pp. 930–934, 2009.

[27] V. Karanikas, M. Speletas, M. Zamanakou et al., "*FOXP3* expression in human cancer cells," *Journal of Translational Medicine*, vol. 6, p. 19, 2008.

[28] A. Balsari, A. Merlo, P. Casalini et al., "*FOXP3* expression and overall survival in breast cancer," *Journal of Clinical Oncology*, vol. 27, no. 11, pp. 1746–1752, 2009.

[29] T. Zuo, R. Liu, H. Zhang et al., "*FOXP3* is a novel transcriptional repressor for the breast cancer oncogene SKP2," *Journal of Clinical Investigation*, vol. 117, no. 12, pp. 3765–3773, 2007.

[30] S. Ladoire, L. Arnould, G. Mignot et al., "Presence of *FOXP3* expression in tumor cells predicts better survival in HER2-overexpressing breast cancer patients treated with neoadjuvant chemotherapy," *Breast Cancer Research and Treatment*, vol. 125, no. 1, pp. 65–72, 2011.

[31] T. Triulzi, E. Tagliabue, A. Balsari, and P. Casalini, "*FOXP3* expression in tumor cells and implications for cancer progression," *Journal of Cellular Physiology*, vol. 228, no. 1, pp. 30–35, 2013.

[32] M. H. Kim, J. S. Koo, and S. Lee, "*FOXP3* expression is related to high Ki-67 index and poor prognosis in lymph node-positive breast cancer patients," *Oncology*, vol. 85, no. 2, pp. 128–136, 2013.

[33] S. Hinz, L. Pagerols-Raluy, H. Oberg et al., "*FOXP3* expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer," *Cancer Research*, vol. 67, no. 17, pp. 8344–8350, 2007.

[34] L. Wang, R. Liu, W. Li et al., "Somatic single hits inactivate the X-linked tumor suppressor *FOXP3* in the prostate," *Cancer Cell*, vol. 16, no. 4, pp. 336–346, 2009.

[35] S. Lee, E. Y. Cho, Y. H. Park, J. S. Ahn, and Y.-H. Im, "Prognostic impact of *FOXP3* expression in triple-negative breast cancer," *Acta Oncologica*, vol. 52, no. 1, pp. 73–81, 2013.

[36] L. Demir, S. Yigit, H. Ellidokuz et al., "Predictive and prognostic factors in locally advanced breast cancer: effect of intratumoral *FOXP3*+ Tregs," *Clinical & Experimental Metastasis*, vol. 30, no. 8, pp. 1047–1062, 2013.

[37] Y. Gokmen-Polar, M. A. Thorat, P. Sojitra, R. Saxena, and S. Badve, "*FOXP3* expression and nodal metastasis of breast cancer," *Cellular Oncology*, vol. 36, no. 5, pp. 405–409, 2013.