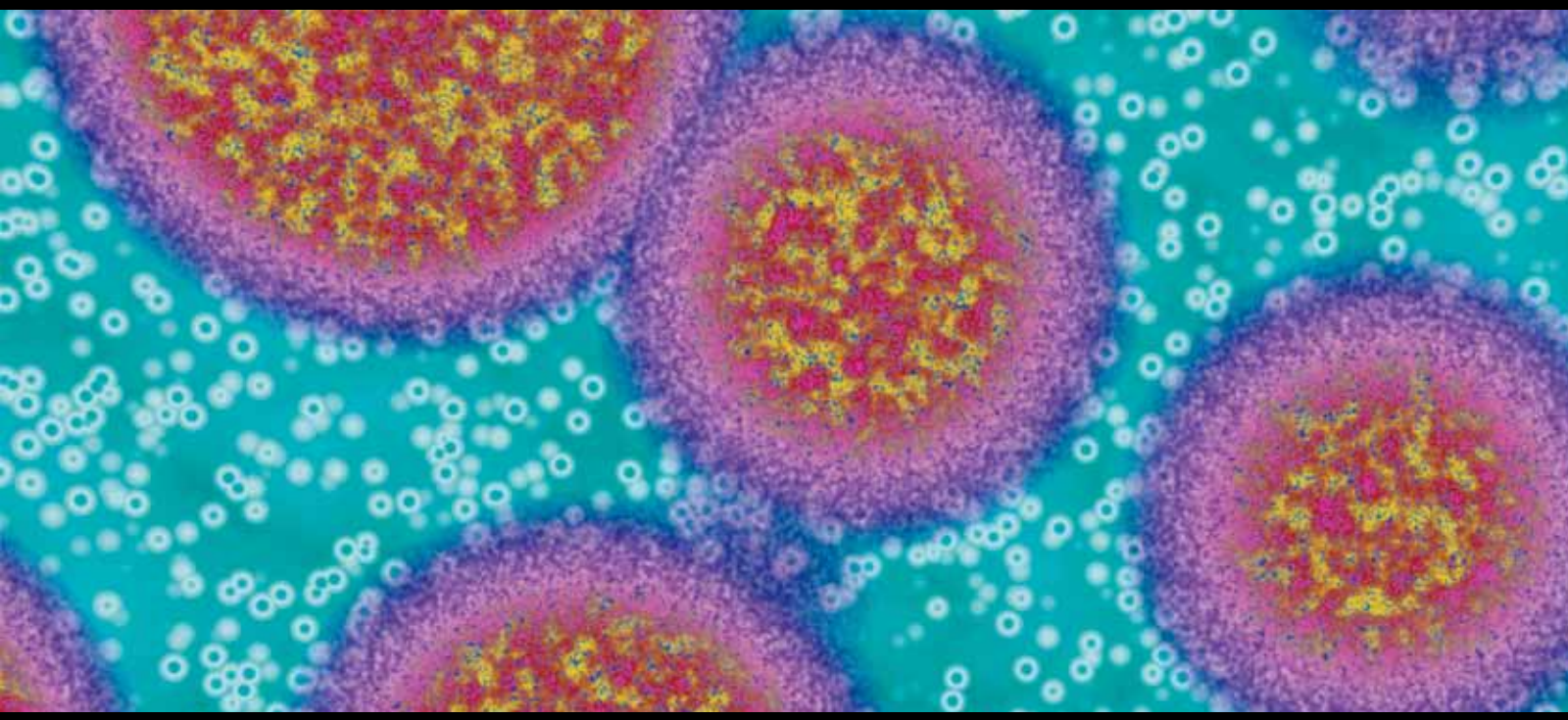
International Journal of Cell Biology

Cell Adhesion in Cancer

Guest Editors: Eok-Soo Oh, Motoharu Seiki, Martin Gotte, and Jun Chung



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Editorial

Cell Adhesion in Cancer

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During cancer progression, cells lose their original tissue contacts, move through the extracellular matrix (ECM), enter into the lymphatic and/or blood system, extravasate, and ultimately form new tumors. Therefore, tumor cells inevitably experience alterations in cell-cell and cell-ECM adhesion and the transformation activities of tumor cells are highly influenced by cell adhesion via adhesion receptors such as cadherins, integrins, cell surface proteoglycans, and tetraspanins. These adhesion receptors, together with extracellular ligands in the tumor microenvironment, couple the extracellular environment to intracellular signals, thereby enhancing cancer cell migration, invasion, proliferation, and survival. Therefore, knowledge of the role of cell adhesion in cancer is key to understanding the development of cancer, and such knowledge could potentially form the basis for effective approaches to cancer treatment. Therefore, in this issue, we have invited several authors to address such issues.

The special issue will be composed of three parts. A set of four papers discuss regulatory mechanisms in the light of interesting issues that have arisen recently, such as the role of cell-ECM interactions in genetic mutations in tumors, as well as the important roles of lipid rafts, turnover of focal adhesions, and anoikis resistance in cancer cell adhesion and migration. All of these characteristics have been shown to be important in cancer cell adhesion, and recent findings provide potential mechanisms of tumor cell interactions and metastatic activities.

Another set of five papers of this special issue address the molecular level regulatory role of cell adhesion molecules such as integrins and the immunoglobulin super family and adhesion-related regulatory molecules such as plakoglobin, HIC-5, and prostaglandins. All of these molecules have received considerable attention in cell adhesion research and have distinct role(s) in different aspects of cancer progression, but all are important regulators of human carcinogenesis through their capacity to regulate cancer cell adhesion. However, because their roles in cancer cell adhesion and metastasis are still under investigation, this special issue addresses their functional significance, particularly in the context of cancer cell adhesion.

A set of two papers address the possibility of suppressing tumors by regulating cell adhesion status using ECM-derived functional peptides or a physiological regulator such as heparin. Research in this area will improve the understanding of cancer progression and may provide the basis for a new strategy for treating cancer.

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Review Article

Plakoglobin: Role in Tumorigenesis and Metastasis

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Plakoglobin (γ-catenin) is a member of the Armadillo family of proteins and a homolog of β-catenin. As a component of both the adherens junctions and desmosomes, plakoglobin plays a pivotal role in the regulation of cell-cell adhesion. Furthermore, similar to β-catenin, plakoglobin is capable of participating in cell signaling. However, unlike β-catenin that has well-documented oncogenic potential through its involvement in the Wnt signaling pathway, plakoglobin generally acts as a tumor/metastasis suppressor. The exact roles that plakoglobin plays during tumorigenesis and metastasis are not clear; however, recent evidence suggests that it may regulate gene expression, cell proliferation, apoptosis, invasion, and migration. In this paper, we describe plakoglobin, its discovery and characterization, its role in regulating cell-cell adhesion, and its signaling capabilities in regulation of tumorigenesis and metastasis.

1. Introduction

Plakoglobin (also known as γ-catenin) is a member of the Armadillo family of proteins and a structural and functional homolog of β-catenin. These catenin proteins have two major roles in the cell: the mediation of cell-cell adhesion and cell signaling. As adhesive proteins, both β-catenin and plakoglobin interact with the cytoplasmic domain of cadherins, thereby tethering the cadherin proteins to the cytoskeleton. In addition to their cell-cell adhesive functions, both β-catenin and plakoglobin interact with a number of intracellular partners including signaling proteins and transcription factors, which accounts for their involvement in cellular signaling [1–4]. Despite these similarities, a major difference between β-catenin and plakoglobin emerges when considering their signaling functions. While β-catenin has a well-defined oncogenic potential as the terminal component of the Wnt signaling pathway [5–7], plakoglobin is typically associated with tumor/metastasis suppressor activity [8–10]. However, the mechanisms that underlie this activity remain undefined. In this paper, we have focused on the potential roles of plakoglobin during tumorigenesis and metastasis in an attempt to define how this often overlooked protein contributes to these complex processes.

2. Plakoglobin: Initial Identification and Early Characterization

Plakoglobin was initially identified as an 83 kDa protein component of the desmosomal plaque [11]. Subsequently, using monoclonal antibodies, cDNA cloning, and a combination of biochemical, morphological, and molecular approaches, Cowin et al. [12] demonstrated that this 83 kDa protein was present in both desmosomes and the adherens junction and was given the name plakoglobin.

Although plakoglobin was identified as a junctional protein, the role that it played in these junctional complexes was unclear, and the partners with which plakoglobin interacted were not identified. It was not until several years later that coimmunoprecipitation experiments showed that plakoglobin interacted with the desmosomal cadherin desmoglein, thereby confirming plakoglobin as a constituent of the desmosomes [13]. In addition, several groups showed that E-cadherin (initially known as uvomorulin) immunoprecipitates contained three distinct proteins, which became known as α-, β-, and γ-catenin [14–16]. These studies showed that these three catenin proteins, with molecular weights of approximately 102, 88, and 80 kDa, respectively, interacted with the cytoplasmic domain of E-cadherin.

Further work analyzing the formation and stability of the E-cadherin-catenin complexes suggested that the E-cadherin-β-catenin complex was formed immediately after E-cadherin synthesis and was very stable. Interestingly, these studies also found that α-catenin could not be found in association with E-cadherin independent of β-catenin, suggesting that β-catenin was a physical link between E-cadherin and α-catenin. However, since γ-catenin was found to be only loosely associated with E-cadherin, it was determined that the main adhesive complexes consisted of E-cadherin, β-catenin, and α-catenin, although the existence of a separate E-cadherin-γ-catenin complex could not be ruled out [16].

At this time there was some confusion as to the identity of the catenin proteins and their relationship to plakoglobin. It soon became evident that plakoglobin was a homolog of β-catenin, a 92 kDa E-cadherin-associated protein [17]. However, it was not until the work of Knudsen and Wheelock [18] that it became clear that the 80 kDa protein that was associated with E-cadherin was indeed plakoglobin. In this study, the authors showed that plakoglobin interacted with both E- and N-cadherin and that it was a distinct protein from β-catenin [18]. This finding was confirmed by work from other groups demonstrating that plakoglobin and γ-catenin were indeed the same protein [1, 19].

Subsequent analysis of the kinetics of plakoglobin synthesis and associations with cadherins demonstrated that following synthesis, plakoglobin interacted with both desmoglein and E-cadherin in both the soluble and cytoskeleton-associated pools of cellular proteins. In addition, a distinct, cadherin-independent pool of plakoglobin was also observed, suggesting that plakoglobin may have a role in the cell in addition to cell adhesion. Finally, phosphorylation experiments revealed that whereas the insoluble (cadherin-associated) pool of plakoglobin was serine phosphorylated, the soluble pool was serine, threonine, and tyrosine phosphorylated, suggesting that these different pools of plakoglobin are differentially regulated and perform varying functions [20]. Collectively, these studies demonstrated that plakoglobin is a homolog of β-catenin and a unique protein in that it is the only component common to both E-cadherin and desmosomal cadherin-containing junctions.

3. Plakoglobin Functions: Cell-Cell Adhesion

The most documented role of plakoglobin within the cell is in cell-cell adhesion. As such, plakoglobin is found in both adherens junctions and desmosomes (Figure 1). Adherens junctions are a ubiquitous type of intercellular adhesion structure present in both epithelial and nonepithelial cells, whereas desmosomes are adhesive junctions that confer tensile strength and resilience to cells and are present not only in epithelial cells but also in nonepithelial cells that endure mechanical stress, such as cardiac muscle. Both adherens junctions and desmosomes are cadherin based. Cadherins are single-pass transmembrane glycoproteins that form homotypic interactions with cadherin proteins on neighboring cells. Intracellularly, cadherins interact with proteins of the catenin family. At the adherens junction, the C-terminal domain of E-cadherin interacts, in a mutually exclusive manner, with β-catenin or plakoglobin, which then interacts with α-catenin, which is an actin-binding protein. A fourth catenin protein, p120-catenin, interacts with the juxtamembrane domain of E-cadherin and is important for E-cadherin dimerization and stability at the membrane (Figure 1; for reviews see [21, 22]). At the desmosome, the desmosomal cadherins (desmocollins and desmogleins) interact intracellularly with plakophilin and plakoglobin, which interact with desmoplakin, an intermediate filament binding protein (Figure 1; for reviews, see [23, 24]).

The identification of plakoglobin as a constituent of both the adherens junction and the desmosomes suggested that it might play an important role in regulating cell-cell adhesion. However, the observation that the adherens junctions could exist as a complex containing E-cadherin, β-catenin, and α-catenin, independent of plakoglobin [16] questioned the necessity of plakoglobin, at least at the adherens junctions. Regardless, it soon became apparent that plakoglobin does have an essential role in regulating cell-cell adhesion.

It had been previously shown that disruption of E-cadherin-based cell-cell adhesion led to a transformed and/or invasive phenotype while reexpression of E-cadherin in cells lacking its expression resulted in a mesenchymal to epithelial phenotypic transition [25–31]. Furthermore, reduced expression of E-cadherin was known to inversely correlate with the differentiation grade of tumors [32–37]. While it was clear that these E-cadherin-based junctions were important for the maintenance of an "epithelial" phenotype, the role of plakoglobin in this phenomenon was not discerned until it was shown that the expression of E- or P-cadherin alone in murine spindle cell carcinomas that lacked endogenous expression of these proteins was not sufficient to modify the morphology or tumorigenicity of these cells [38]. Although these cadherins were expressed in the cells, localized to the cell membrane, and interacted with both α- and β-catenin, they did not interact with plakoglobin. Further analysis showed that the levels of plakoglobin in these cells were very low, thus accounting for the absence of plakoglobin association with E-cadherin. From this work, the authors suggested that the association of the E-cadherin-catenin complex with plakoglobin may be necessary for its tumor suppressing activity.

Another significant role for plakoglobin in the regulation of cell-cell adhesion was discovered when studies showed that A431 epithelial cells treated with dexamethasone (which resulted in the isolation of fibroblastic A431 cells lacking E-cadherin but expressing desmoglein) were unable to form desmosomes upon exogenous expression of E- or P-cadherin, despite the formation of the adherens junction in these cells [39]. Interestingly, the authors observed that although plakoglobin was present at low levels in these cells, it was not coimmunoprecipitated with the exogenously expressed E-cadherin; in fact, the plakoglobin found in these cells coprecipitated with desmoglein. To examine the possibility that plakoglobin plays a regulatory role in desmosome formation, the authors expressed an E-cadherin-plakoglobin chimeric protein capable of forming stable adherens junctions in the cells and observed desmosome

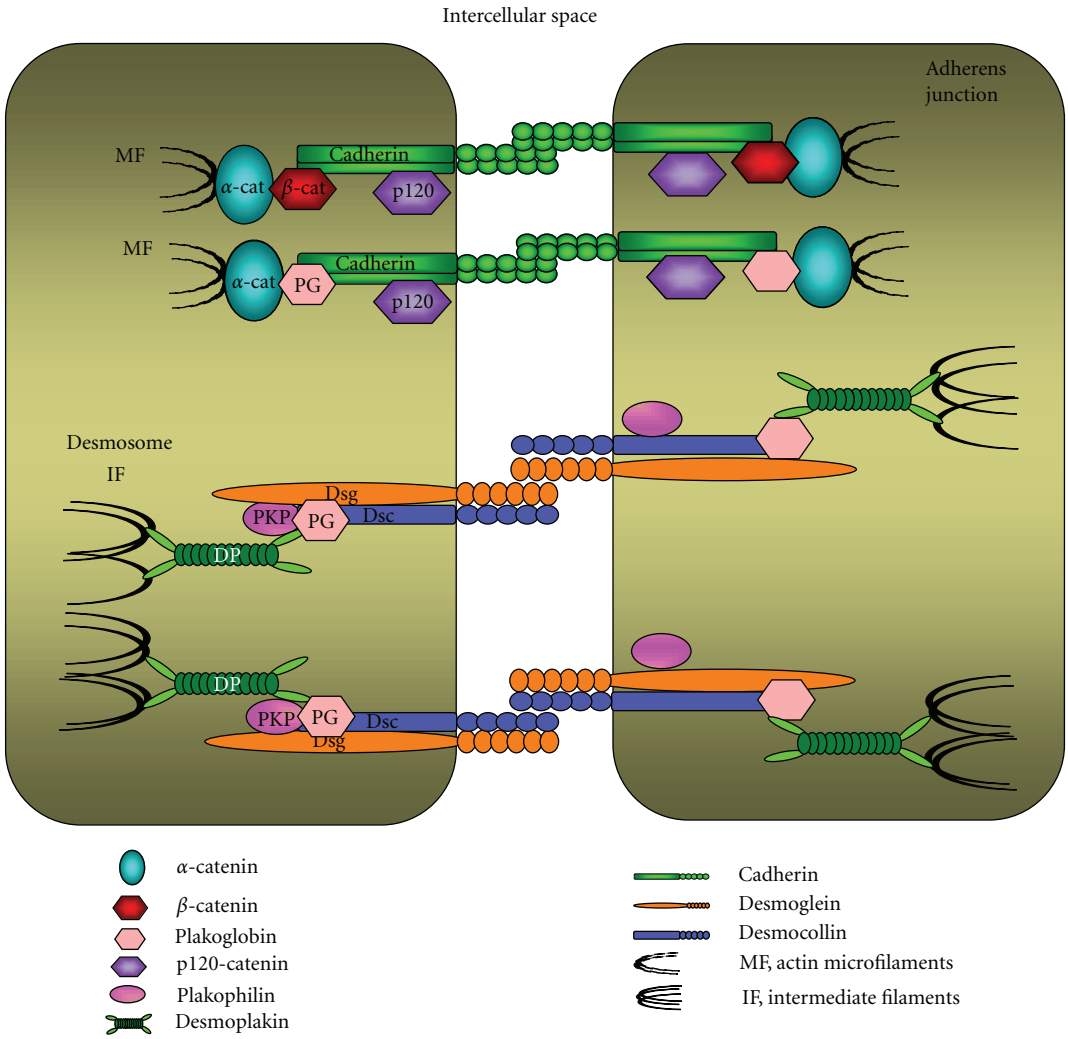

Figure 1: Cell adhesion complexes in epithelial cells. Cell-cell adhesion is maintained in epithelial tissues by the adherens junction and desmosomes. At the adherens junctions, E-cadherin forms extracellular interactions with E-cadherin molecules on neighboring cells. Intracellularly, E-cadherin interacts with either β-catenin or plakoglobin, which then interact with α-catenin, an actin-binding protein. A fourth catenin, p120-catenin, also interacts with E-cadherin and regulates its stability at the membrane. At the desmosome, the desmosomal cadherins (desmoglein and desmocollin) interact with plakoglobin and plakophilin, which interact with desmoplakin, which in turn associates with the intermediate filament cytoskeleton. The basic, core protein composition of the desmosomes is represented here: the exact protein constituents of the desmosomes and their interactions vary between different types of cells and tissues.

formation. While it had been previously observed that adherens junction formation not only preceded, but was also a prerequisite for desmosome formation [40–48], this was the first indication that plakoglobin served as a molecule involved in crosstalk between both junctional complexes in epithelia.

Following this study, our laboratory demonstrated the role of plakoglobin in adhesive junction formation by expressing low/physiological levels of plakoglobin in SCC9 cells, a squamous cell carcinoma cell line that lacks the expression of both plakoglobin and E-cadherin [9, 49]. Following exogenous plakoglobin expression, SCC9 cells underwent a mesenchymal to epidermoid phenotypic transition that was concurrent with the stabilization of N-cadherin and the formation of desmosomes and well-organized N-cadherin-containing adherens junctions [9]. This result confirmed that plakoglobin expression was necessary for desmosome formation and also demonstrated that plakoglobin-N-cadherin interactions could occur prior to desmosome formation. Other studies have further characterized the role of plakoglobin in desmosome assembly and function. Palka and Green [50] demonstrated the role of plakoglobin's C terminus for the proper assembly of the desmosomal plaque, and Acehan et al. showed that plakoglobin is essential for the efficient binding of desmoplakins to the intermediate filaments [51]. Furthermore, plakoglobin was shown to be necessary for the recruitment of plakophilin 3 to the membrane, desmosome formation, efficient cell-cell adhesion, and inhibition of cell migration and invasion

[52, 53]. Finally, work from Birchmeier's laboratory showed that plakoglobin double knockout mice died during embryogenesis as a result of disrupted heart function due to the loss of stable desmosomes in the intercalated discs of cardiac muscle, further confirming the essential role of plakoglobin in desmosome formation and function [48, 54].

4. Plakoglobin Functions: Cell Signaling

4.1. Initial Observations and Controversy. The first clue that plakoglobin might participate in cell signaling came from studies of the exogenous expression of Wnt-1 in PC12 cells. In these cells, plakoglobin levels were increased, and it underwent membrane redistribution, suggesting that, in addition to β-catenin levels, Wnt-1 can modulate plakoglobin levels and localization [55]. Subsequently, Karnovsky and Klymkowsky [56] demonstrated plakoglobin signaling activity by microinjecting mRNAs-encoding plakoglobin into fertilized *Xenopus* embryos, resulting in dorsalized gastrulation and anterior axis duplication. In this study, the exogenously expressed plakoglobin localized both at the plasma membrane and in punctate nuclear aggregates. Furthermore, the coinjection of mRNAs-encoding plakoglobin as well as the cytoplasmic domain of desmoglein suppressed both dorsalized gastrulation and anterior axis duplication. In these embryos, plakoglobin was localized primarily to the plasma membrane with some perinuclear distribution. These results suggested that plakoglobin has signaling ability similar to β-catenin, but when it is sequestered at the plasma membrane (as part of desmosomes), plakoglobin is unable to participate in cell signaling.

This initial finding suggested that plakoglobin may have signaling functions similar to its homologs β-catenin and the *Drosophila* Armadillo protein. However, subsequent studies from various groups have demonstrated that while plakoglobin does indeed have signaling capabilities, it appears to function as a tumor suppressor rather than a tumor promoter. The first demonstration of this phenomenon occurred when Simcha et al. [8] found that plakoglobin expression in SV40-transformed NIH3T3 cells decreased the ability of these cells to form tumors in syngeneic mice. This growth suppressive effect of plakoglobin was augmented by cotransfection with N-cadherin. The authors also expressed plakoglobin in the renal carcinoma cell line KTCTL 60, which lacks endogenous expression of E-cadherin and desmosomal cadherins, α-catenin, β-catenin, plakoglobin, and desmoplakin and induces tumor formation in mice. Plakoglobin expression in KTCTL 60 cells also inhibited the tumorigenicity of these cells in syngeneic mice. Notably, the authors showed that the majority of the plakoglobin in these cells was Triton X-100 soluble, suggesting that it was not junction associated. This result was of significance because it demonstrated that plakoglobin could suppress tumor formation independent of its role in mediating cell-cell adhesion.

These studies made it clear that plakoglobin was capable of cell signaling and able to act as a tumor suppressor. Numerous subsequent studies have described the signaling function of plakoglobin as primarily one of tumor suppression, although a few reports have suggested that similar to β-catenin, plakoglobin may have oncogenic activity. In the following sections, we will present the experimental evidence for both the tumorigenic and tumor suppressive activities of plakoglobin and propose possible explanations for these observed discrepancies.

4.2. Plakoglobin Oncogenic Activity. Kolligs et al. [57] have shown that the tumor suppressor adenomatous polyposis coli (APC), which was already known to regulate the levels of β-catenin, could also regulate plakoglobin protein levels. In this study, the authors also showed that exogenous expression of plakoglobin in rat RK3E cells, which express considerable amounts of endogenous plakoglobin and β-catenin [57, 58], resulted in a transformed phenotype, which they suggested was dependent on the upregulation of the oncogene c-Myc and activation of Tcf/Lef signaling. More recently, Pan et al. [59] have shown that the exogenous expression of plakoglobin in HCT116 colon carcinoma cells, which express a mutant β-catenin protein that cannot be degraded [60], resulted in genomic instability and increased invasion and migration.

Both of these studies concluded that plakoglobin possessed oncogenic activity. However, it must be noted that several lines of evidence suggest that the oncogenic activity of plakoglobin may be indirect and achieved through modulation of the protein levels and signaling ability of β-catenin [61–67]. Since plakoglobin and β-catenin interact with some of the same proteins and display high sequence homology (Figure 2, [2, 4, 68, 69]), it became evident that plakoglobin may, in fact, be able to promote tumorigenesis by interacting with proteins that would normally sequester β-catenin (e.g., E-cadherin, Axin, APC), which would result in increased levels of cytoplasmic and nuclear β-catenin and in turn enhanced signaling. Indeed, following the observation that plakoglobin expression resulted in *Xenopus* axis duplication [56], the same group showed that this outcome did not depend on the nuclear localization of plakoglobin, since membrane-anchored forms of this protein produced the same axis duplication [70]. This demonstrated that nuclear plakoglobin was inconsequential in inducing a Wnt-like phenotype, since the cytoplasmic plakoglobin induced this same phenotype. At the same time, Salomon et al. [61] showed that overexpression of plakoglobin resulted in the displacement of β-catenin from, and the increased association of plakoglobin with, the N-cadherin-containing adherens junctions. Furthermore, excess cytoplasmic β-catenin was able to translocate into the nucleus. This was supported by other work, which showed that overexpression of plakoglobin in NIH3T3 cells resulted in the nuclear accumulation of β-catenin and that overexpression of the Wnt coactivator Lef-1 in MDCK cells resulted in its preferential interaction with β-catenin (instead of plakoglobin). Subsequently, the β-catenin-Lef complexes were localized to the nucleus [62], suggesting that when both plakoglobin and β-catenin were present within the cell, β-catenin-Lef complexes were more readily formed and transcriptionally active. Further examination of the ability of plakoglobin to

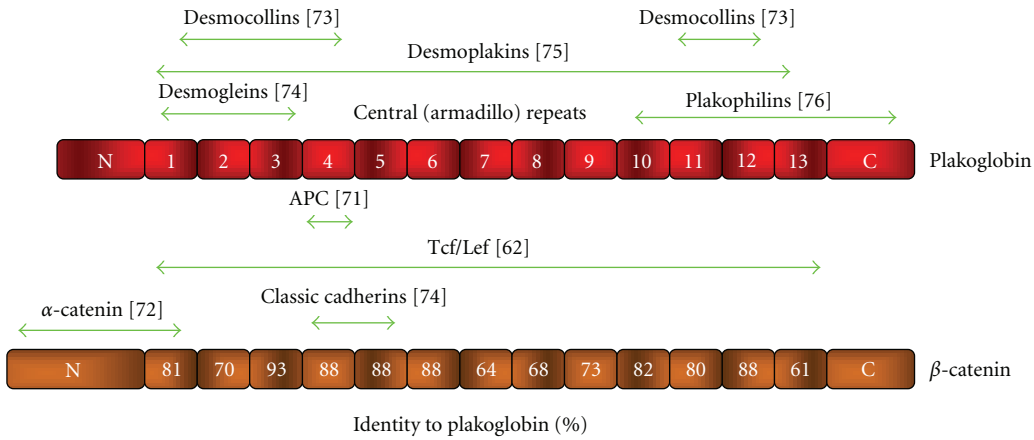

Figure 2: Schematic structure of β-catenin and plakoglobin. Both β-catenin and plakoglobin contain 13 Armadillo repeats that are flanked by N- and C-terminal domains, respectively. The degree of homology between β-catenin and plakoglobin for each Armadillo domain is indicated. Protein partners that interact with plakoglobin and the domains involved in these interactions are indicated. The corresponding references are listed in brackets (see [71–76]).

signal via interactions with the Tcf/Lef family of transcription factors showed that although plakoglobin interacted with Lef-1, this complex was inefficient in binding to DNA, whereas β-catenin-Lef-1 complexes more readily bound DNA [63]. This study also demonstrated that overexpression of plakoglobin resulted in increased β-catenin-Lef-1 complex formation and its association with DNA. Further analysis of the transactivation potential of β-catenin and plakoglobin demonstrated that β-catenin was a much stronger activator of Tcf/Lef target genes than plakoglobin [64].

As mentioned earlier, we have previously shown that the expression of low/physiological levels of plakoglobin in plakoglobin-deficient SCC9 cells induced a mesenchymal to epidermoid change in phenotype, whereas its overexpression resulted in foci formation and decreased apoptosis, which was concurrent with the upregulation of the prosurvival protein Bcl-2 [77]. Using cDNAs-encoding plakoglobin fused to nuclear localization or nuclear export signals (NLS and NES), we subsequently showed that Bcl-2 levels were upregulated in plakoglobin overexpressing SCC9 cells regardless of plakoglobin localization. Furthermore, in these cells, β-catenin-N-cadherin interactions were decreased, and β-catenin accumulated in the nucleus, interacted with Tcf, and its signaling was increased [65], confirming that the overexpressed plakoglobin acted indirectly by enhancing the signaling capability of β-catenin.

The above studies describing the oncogenic potential of plakoglobin may also be as a result of β-catenin. In Kolligs's study [57] where plakoglobin was overexpressed in RK3E cells (which express endogenous β-catenin and plakoglobin [58]), it was not determined if plakoglobin could activate c-Myc expression in the absence of β-catenin or whether either of these catenins was detected in the nucleus in association with the c-Myc promoter. In addition, in Pan's study [59] in which HCT116 cells showed increased genomic instability and migration and invasion upon plakoglobin expression, the endogenous β-catenin was a mutant protein that was unable to be phosphorylated and subsequently degraded [60]. While much of the β-catenin localized to the membrane in these cells [78], plakoglobin expression most likely led to decreased β-catenin-cadherin interactions and increased β-catenin signaling. In support of this prediction, HCT116 cells overexpressing plakoglobin showed increased expression of the oncogenes securin and c-Myc and decreased expression of E-cadherin, all of which are documented β-catenin target genes [79–81]. Taken together, the evidence suggests that although plakoglobin expression may lead to a transformed phenotype, it is likely that this outcome is associated with increased oncogenic β-catenin signaling rather than oncogenic activity due directly to plakoglobin.

4.3. Plakoglobin Signaling in β-Catenin Null Cells. While the oncogenic signaling activity of plakoglobin discussed above can be attributed to the signaling activity of β-catenin rather than plakoglobin itself, this cannot account for all of the observations regarding plakoglobin signaling. Recent studies attempting to discern the signaling activity of plakoglobin independent of β-catenin have used tissue culture cell lines that lack the endogenous expression of β-catenin [82–85]. These studies have shown that in the absence of β-catenin, plakoglobin does indeed have Tcf/Lef-mediated transcriptional activity, although this activity is less than that of β-catenin-Tcf complexes. Interestingly, although these studies have demonstrated that plakoglobin can signal through forming transcriptional complexes with Tcf/Lef transcription factors, they did not assess the tumor-forming properties of these cells, so it remains unclear as to whether these cells possessed transformed or nontransformed properties. To that end, it has been demonstrated that plakoglobin or β-catenin expression in renal carcinomas lacking endogenous β-catenin and plakoglobin resulted in the upregulation of Nr-CAM, a neuronal cell adhesion molecule that can be regulated by both β-catenin and plakoglobin [82]. Furthermore, Nr-CAM expression in NIH3T3 cells conferred a more tumorigenic and invasive phenotype on these cells. Significantly however, although plakoglobin expression

resulted in increased Nr-CAM levels in renal carcinomas and although plakoglobin-Tcf/Lef complexes can regulate Nr-CAM expression, the overall phenotype of these cells upon plakoglobin expression was nontumorigenic [8]. This showed that although plakoglobin may regulate β-catenin-target genes in the absence of β-catenin, it still may suppress tumorigenesis in the same cells. The homology between plakoglobin and β-catenin explains the ability of plakoglobin to signal through Tcf/Lef in the absence of β-catenin. Taken together, indeed, it is not surprising that if β-catenin is completely absent from a cell line, plakoglobin cannot only replace it in junctional complexes, but may also be able to regulate some β-catenin target genes (e.g., Survivin [85]). However, as a final note, it is important to consider that β-catenin-null tumors are extremely rare, and in most tumors and cell lines, plakoglobin signaling activity occurs in the presence of β-catenin.

4.4. Plakoglobin Tumor Suppressor Activity. Despite the observation that plakoglobin overexpression promotes tumorigenesis mediated by the oncogenic signaling of β-catenin, several studies examining the signaling function of plakoglobin have identified it as a tumor suppressor. We have previously shown that expression of physiological levels of plakoglobin in SCC9 cells, which lack endogenous plakoglobin and E-cadherin, resulted in a mesenchymal to epidermoid phenotypic transition, which was concurrent with the stabilization of N-cadherin, the formation of desmosomes, and the downregulation of β-catenin [9]. Furthermore, we have found that plakoglobin-expressing SCC9 cells showed a decreased growth rate compared to parental SCC9 cells. These results, taken together, demonstrated that not only could plakoglobin act as a tumor suppressor, but that potentially it does so by decreasing the levels of β-catenin.

The ability of plakoglobin to inhibit cell growth and proliferation was next observed when Charpentier et al. [10] expressed plakoglobin (under the control of an epidermal-specific promoter) in the basal cells of the epidermis as well as the hair follicles of transgenic mice. These authors showed that plakoglobin expression resulted in a reduced proliferative potential of the epidermal cells and that plakoglobin-expressing hair follicles had a significantly reduced growth phase, with hairs shorter by roughly 30% after plakoglobin expression.

Further evidence suggesting a growth suppressive activity for plakoglobin was provided in lung cancer, when it was shown that while β-catenin was uniformly expressed in various Nonsmall cell lung cancer (NSCLC) cell lines and lung primary tumors, plakoglobin expression was very low or completely absent [86]. The authors showed that exogenous expression of plakoglobin in the low-plakoglobin-expressing NSCLC cells resulted in decreased β-catenin-Tcf signaling, which was concurrent with decreased cell and anchorage-independent growth. This result further supported the idea that plakoglobin can act as a tumor suppressor by inhibiting the oncogenic activity of β-catenin.

Interestingly, when the authors treated these NSCLC cell lines with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (AZA) or the histone deacetylase inhibitor trichostatin A (TSA), plakoglobin levels were increased. Previous analysis of the plakoglobin promoter had described CpG islands within the promoter [87], and while it had been observed that inhibition of DNA methylation could result in increased plakoglobin protein levels in at least one thyroid carcinoma cell line [88], this was the first indication that both DNA methylation and histone deacetylation played important roles in regulating plakoglobin expression.

The occurrence of methylated CpG islands within the plakoglobin promoter as well as histone deacetylation has not been limited to NSCLC cell lines. Various groups have shown that the plakoglobin promoter is methylated in prostate, bladder, trophoblastic, and mammary carcinomas [89–92], which is concurrent with a transformed phenotype. Canes et al. [90] have shown that treatment of bladder carcinoma cells with TSA resulted in increased plakoglobin expression and a decreased ability of these cells to form tumors in mice, once again suggesting a growth inhibitory activity of plakoglobin. Similarly, when mammary carcinoma cell lines were treated with AZA, increased plakoglobin levels were observed, as well as decreased soft agar colony formation and overall cell growth [92], indicative of decreased tumor-forming ability.

Several lines of evidence suggest that plakoglobin plays a role in regulating apoptosis, in addition to acting as a growth suppressor. In their work describing the effects of plakoglobin on hair growth in transgenic mice, Charpentier et al. [10] showed that plakoglobin expression decreased epithelial proliferation. Moreover, this expression also resulted in premature apoptosis, because TUNEL assays showed that the inner root sheath of the plakoglobin-expressing transgenic follicles underwent apoptosis two days earlier than in normal hair follicles. In agreement with these findings, we have previously shown that SCC9 cells expressing physiological levels of plakoglobin were more prone to undergo staurosporine-induced apoptosis when compared to parental SCC9 cells [77]. We have also observed that SCC9 cells expressing plakoglobin exclusively in the nucleus (SCC9-PG-NLS) showed decreased Bcl-2 levels compared to cells with overexpressed wild-type plakoglobin, which suggests that plakoglobin may play a more direct role in regulating the expression of apoptotic genes. More recently, it has been shown that mouse keratinocytes that lack endogenous plakoglobin expression are protected from etoposide-induced apoptosis, whereas plakoglobin-expressing keratinocytes readily undergo apoptosis upon etoposide treatment [93]. In this study, the authors demonstrated that plakoglobin-null keratinocytes were unable to release cytochrome c from the mitochondria and activate caspase 3, suggesting that plakoglobin plays a role in regulating the apoptotic cascade. Furthermore, the mRNA levels of the antiapoptotic protein Bcl-X_L were higher in the plakoglobin null keratinocytes, which could potentially have prevented the translocation of cytochrome c from the mitochondria. Finally, the expression of plakoglobin in the null keratinocytes resulted in decreased Bcl-X_L levels, caspase 3 activation, and apoptosis induction following etoposide treatment. Taken together, these studies have demonstrated that plakoglobin does have some role in apoptosis signaling

and potentially may exert part of its tumor suppressor activity through the modulation of apoptosis.

4.5. Plakoglobin Metastasis Suppressor Activity. As the tumor suppressor activity of plakoglobin began to be revealed, it soon became evident that in addition to inhibiting the growth properties of carcinoma cell lines, plakoglobin also plays a role in regulating the invasive and migratory properties of cancer cells. The initial observation of plakoglobin metastasis suppressor activity was documented in human umbilical vascular endothelial cells (HUVEC), where plakoglobin was typically associated with sites of cell-cell contact [94]. Plakoglobin antisense oligonucleotides increased HUVEC migration, suggesting that the loss of plakoglobin expression led to an increased migratory phenotype. Concurrent with increased migration, the antisense treated HUVEC cells also became more prone to forming tubular structures in Matrigel, suggesting that plakoglobin knock down also promoted angiogenesis.

Mukhina et al. [95] further detailed the metastasis suppressor activity of plakoglobin using MCF-7 cells, which express membrane-localized E-cadherin and plakoglobin, and stable cell junctions. In this study, the authors treated MCF-7 cells with human growth hormone (hGH) and observed a downregulation of plakoglobin, a cytoplasmic distribution of E-cadherin and an increased migratory and invasive phenotype, which was accompanied by an increase in matrix metalloproteinase levels. Furthermore, the authors demonstrated that hGH-mediated invasiveness was dependent on Src kinase and also showed that chemical inhibitors of Src resulted in increased plakoglobin levels and, in turn, decreased invasion and migration. To discern the specific role of plakoglobin in these processes, the authors expressed plakoglobin in the hGH-treated MCF-7 cells, which resulted in both the decreased migration and invasiveness of these cells [95].

The metastasis suppressor activity of plakoglobin has also been described in bladder carcinomas, where the expression of plakoglobin in plakoglobin null cell lines resulted not only in decreased growth and tumorigenicity (as assessed by colony formation in soft agar and tumor formation in nude mice, resp.), but also in decreased invasive and migratory capabilities of the transfectants [96]. Similarly, knock down of plakoglobin using siRNAs resulted in the increased tumorigenic and invasive properties of bladder carcinoma cells relative to their plakoglobin-expressing parental cell lines. This study further demonstrated that plakoglobin expression did not affect Wnt/β-catenin signaling in these bladder carcinomas, which suggested that plakoglobin possessed tumor and metastasis suppressor activities independent of β-catenin.

The ability of plakoglobin to act as a metastasis suppressor independent of its role in cell-cell adhesion has been demonstrated using plakoglobin null keratinocytes [97], which were less adherent to one another and more migratory (as assessed by transwell migration assays). However, when wild-type plakoglobin was expressed in these cells, they became more adherent and less migratory. Using colloidal gold-coated coverslips, the authors were able to assess the migratory abilities of individual cells and observed that individual plakoglobin null keratinocytes were more migratory than their plakoglobin-expressing counterparts. The authors also showed that plakoglobin may regulate single keratinocyte migration by inhibition of Src signaling, which had been previously shown to promote migration and invasion of mammary carcinomas by downregulation of plakoglobin (see above [95]). These results suggested that plakoglobin could suppress migration through the modulation of cell-cell adhesion, as had been previously suggested. However, to determine whether plakoglobin could have an effect in migration independent of its role in cell-cell adhesion, plakoglobin null keratinocytes were transfected with cDNAs encoding mutant plakoglobin, missing either its N- or C-terminus (α-catenin binding and transactivation domain, resp.). The expression of either of these mutant proteins resulted in increased keratinocyte adhesiveness when compared to the plakoglobin null cells, demonstrating that these domains were dispensable for the adhesive function of plakoglobin. Importantly, the authors showed that whereas individual keratinocytes expressing the N-terminal-deleted plakoglobin were not migratory, those that expressed the C-terminal-deleted plakoglobin were migratory. This showed that plakoglobin could indeed suppress migration independent of its adhesive function (since keratinocytes expressing C-terminal-deleted plakoglobin were as adhesive to one another as wild-type plakoglobin expressing keratinocytes). Subsequent work using these plakoglobin null keratinocytes has suggested that plakoglobin affected individual cell motility by regulating the deposition of the extracellular matrix (ECM) protein fibronectin, actin cytoskeleton organization (which in turn regulates Src signaling), and RhoGTPases [98]. Collectively, these observations clearly demonstrate tumor/metastasis suppressor activity of plakoglobin independent of its role in cell-to-cell adhesion.

5. Plakoglobin Functions: Regulation of Gene Expression

When discussing roles for plakoglobin during tumorigenesis and metastasis, it is important to consider that while plakoglobin may function as both a regulator of cell-cell adhesion and an intracellular signaling molecule, it may also play a more active role in these processes through the regulation of gene expression. Evidence supporting the plakoglobin-mediated regulation of gene expression has started to emerge, and work from several groups, including ours, has suggested that plakoglobin can regulate the expression of genes involved in cell-cycle control, apoptosis, cell proliferation, and invasion.

Williamson et al. [99] have shown that plakoglobin acts as a repressor of the c-Myc gene. Using mouse keratinocytes and reporter assays, the authors of this study showed that plakoglobin suppressed c-Myc expression in a Lef-1-dependent manner, suggesting that when plakoglobin interacted with Lef-1, this complex was unable to promote gene expression. These findings confirmed previous results demonstrating the inefficiency of these complexes in binding

DNA [62–64, 100]. This study further showed that the plakoglobin-mediated suppression was similar in both wild-type and β-catenin null keratinocytes, demonstrating that plakoglobin could regulate gene expression independent of β-catenin. Finally, using chromatin immunoprecipitation with plakoglobin antibodies, the authors demonstrated that plakoglobin and Lef-1 associated with the c-Myc promoter in keratinocytes undergoing growth arrest, which implicated the downregulation of c-Myc gene expression as a possible reason for the suppression of cell growth by plakoglobin.

Plakoglobin-mediated regulation of gene expression has also been shown in renal carcinoma cells. Shtutman et al. [101] found that the exogenous expression of plakoglobin in cells lacking both β-catenin and plakoglobin resulted in the increased expression of the tumor suppressor gene PML, a nuclear protein that forms nuclear bodies and is involved in the regulation of p53 activity. Importantly, the increased PML levels due to plakoglobin expression were independent of β-catenin and Tcf, since β-catenin was not detected in the plakoglobin-expressing cells and the deletion of Tcf/Lef sites in the PML promoter did not affect the ability of plakoglobin to increase PML gene expression. Together, these observations suggest that plakoglobin may regulate gene expression independent of Tcf/Lef.

In β-catenin null mesothelioma and colon carcinoma cells, Wnt3a stimulation led to the nuclear accumulation of plakoglobin and induced the expression of the antiapoptotic gene Survivin [85]. Coimmunoprecipitation and chromatin immunoprecipitation showed that plakoglobin formed a transcriptional complex with both Tcf and the histone acetyltransferase CBP and that this complex was associated with the Survivin promoter [85]. While this study clearly demonstrated that plakoglobin was capable of regulating β-catenin target genes in a β-catenin null background, it is again of importance to emphasize that β-catenin null tumors are very rare and that the plakoglobin-mediated regulation of gene expression occurs mainly in the presence of cellular β-catenin.

As previously discussed, Todorović et al. [98] have shown that plakoglobin can regulate cell motility by regulating Fibronectin and Rho-dependent Src signaling. This study also demonstrated that plakoglobin expression resulted in increased levels of Fibronectin mRNA without increasing expression from the Fibronectin promoter. However, by using Actinomycin D to inhibit transcription, the authors were able to demonstrate that plakoglobin expression led to the increased stability of Fibronectin mRNA, suggesting that in addition to its role in regulating gene expression at the level of transcription, plakoglobin may also regulate gene expression posttranscriptionally. However, the mechanisms underlying this action remain unclear. Overall, these studies suggest that plakoglobin regulates gene expression at the transcriptional and potentially at posttranscriptional levels.

6. Plakoglobin Expression in Human Tumors

The initial characterization of *JUP*, the gene encoding plakoglobin, mapped the gene to chromosome 17q21, proximal to the *BRCA1* gene [102]. In this study, the authors also analyzed RNA isolated from ovarian and breast cancer tumors and showed that loss of heterozygosity in these tumors and low-frequency mutations in the plakoglobin gene predisposed patients to familial breast and ovarian cancer. Since then, several groups have observed the loss of plakoglobin expression in a wide range of tumors, with the majority of these studies examining plakoglobin in conjunction with other adhesive junctional proteins. These studies have demonstrated that loss of plakoglobin expression in conjunction with the lack of expression of other cell-cell adhesion proteins such as E-cadherin, α-catenin, β-catenin, desmoglein, or desmoplakin resulted in increased tumor formation and size and was correlated with increased tumor stage, poor patient survival, and increased metastasis in bladder, pituitary, oral, pharyngeal, skin, prostate, and NSCLC tumors [103–111]. However, several studies have found that decreased levels of plakoglobin alone also occur in various tumors.

The loss of plakoglobin expression has been observed in melanocytic and thyroid tumors [112, 113]. Cerrato et al. [113] found that nearly 90% of papillary and follicular tumors showed decreased or loss of membrane plakoglobin localization. Decreased expression of the plakoglobin gene was also observed in prostate tumors, where methylation of the plakoglobin gene is prevalent in localized prostate cancer when compared to benign prostatic hyperplasia, suggesting that loss of plakoglobin expression was an early step in prostate tumorigenesis [89]. In oropharynx squamous cell carcinomas, decreased plakoglobin expression as well as its abnormal cytoplasmic distribution was correlated with increased tumor size and poor clinical outcome [114].

In colon carcinomas, Lifschitz-Mercer et al. [115] showed that β-catenin accumulated in the nuclei of cells of primary and metastatic adenocarcinoma and adenoma lesions, while the levels of nuclear plakoglobin were decreased in these tumors, suggesting that nuclear plakoglobin did not promote tumorigenesis in the colon. In esophageal cancers, while decreased levels of E-cadherin and plakoglobin were associated with poor differentiation and decreased patient survival, reduced plakoglobin levels alone correlated with lymph node metastasis [116]. The finding that reduced plakoglobin levels alone correlated with increased metastasis was not limited to esophageal tumors. In renal carcinomas, decreased plakoglobin levels have been associated with metastasis, and patients with tumors expressing plakoglobin showed significantly higher survival rates than those that did not [117]. Aberrant or decreased plakoglobin levels have also been reported in Wilms' tumors and soft tissue sarcomas, where the decrease in plakoglobin was associated with increased risk of pulmonary metastasis [118, 119]. In endometrial tumors, the aberrant expression of plakoglobin was correlated with myometrial invasion [120], whereas medulloblastoma tumors expressing plakoglobin were nonmetastatic, with no evidence of subarachnoid or hematogenous metastasis [121]. Finally, reduced plakoglobin expression was also correlated with increased lymph node metastasis in oral squamous cell and bladder tumors [122, 123]. Collectively, these observations suggest that lack or decreased expression of plakoglobin due to genetic or

epigenetic causes in tumors of different origins is associated with poor clinical outcome and increased tumor formation and metastasis.

7. Growth/Metastasis Inhibitory Activities of Plakoglobin via Regulation of Gene Expression

We have developed two experimental model systems using squamous and breast carcinoma cell lines with no or very low plakoglobin expression and various degrees of transformation/invasiveness to specifically assess the growth/metastasis inhibitory activities of plakoglobin. Using a combination of molecular and cell biological approaches, including proteomics and transcriptome analysis, we compared the protein and mRNA profiles of plakoglobin-deficient and plakoglobin-expressing cell lines and their *in vitro* migration and invasiveness. These analyses led to the identification of several growth regulatory genes that were differentially expressed in plakoglobin-expressing transfectants compared to their plakoglobin-deficient parental cells.

Comparison of the proteomic profiles of plakoglobin null SCC9 cells and their plakoglobin-expressing transfectants allowed us to identify several tumor/metastasis regulating proteins, which were differentially expressed in plakoglobin-expressing transfectants (SCC9-PG-WT) relative to parental SCC9 cells. We performed RNA microarray experiments to determine whether changes in gene expression upon plakoglobin expression accompanied these changes in protein levels and compared the transcriptome profiles of SCC9 cells and SCC9-PG-WT transfectants. Furthermore, to determine whether the subcellular distribution of plakoglobin had an effect on gene expression, we also compared the RNA profiles of SCC9 and SCC9-PG-WT cells with those of SCC9 cells transfected either with cDNAs-encoding plakoglobin fused with a nuclear localization signal (NLS) to express plakoglobin exclusively in the nucleus (SCC9-PG-NLS), or cDNAs-encoding plakoglobin fused with a nuclear export signal (NES) to express plakoglobin exclusively in the cytoplasm (SCC9-PG-NES). From these experiments, we identified three subsets of genes that were differentially expressed based on plakoglobin expression and its subcellular distribution: those whose differential expression required exclusively cytoplasmic plakoglobin, those whose differential expression required nuclear plakoglobin, and those whose differential expression required the ability of plakoglobin to shuttle between the nucleus and the cytoplasm. Based on the results of these experiments and analysis of the expression patterns of plakoglobin-target genes in relation to plakoglobin subcellular distribution, we propose that plakoglobin can regulate gene expression by three concurrent mechanisms (Figure 3).

The first of these mechanisms involves the action of plakoglobin in the cytoplasm, where it would sequester a protein involved in the regulation of gene expression. In this case, plakoglobin would prevent an inhibitor of a tumor suppressor gene or a promoter of an oncogenic gene from entering the nucleus and affecting gene expression. Plakoglobin target genes whose expression patterns were similar in SCC9-PG-WT and SCC9-PG-NES cells and were opposite to SCC9-PG-NLS cells would be considered part of this group.

The second mechanism involves nuclear localized plakoglobin, which would directly associate with a nuclear factor and regulate gene expression. In this case, plakoglobin would interact with a transcriptional activator and promote gene expression, or, conversely, it would interact with a transcriptional repressor and silence gene expression. Plakoglobin target genes whose expression patterns were similar in SCC9-PG-WT and SCC9-PG-NLS cells and were opposite to SCC9-PG-NES cells would be considered part of this group.

The vast majority of plakoglobin target genes, however, belonged to the third group of genes: those whose differential expression depended on the ability of plakoglobin to shuttle between the nucleus and the cytoplasm. In this case, plakoglobin would interact with some cytoplasmic cofactor, translocate into the nucleus, and regulate gene expression. Plakoglobin target genes whose expression patterns were similar in SCC9-PG-NES and SCC9-PG-NLS cells and were opposite to SCC9-PG-WT cells would be considered part of this group.

Following these proteomics and microarray analyses, we began our initial characterization of the regulation of potential target genes by plakoglobin. We have recently shown that plakoglobin expression in SCC9 cells resulted in the increased expression of the metastasis suppressors Nm23-H1 and -H2, both at the mRNA and protein levels [124]. Nm23 was the first metastasis suppressor identified, as it is often downregulated in metastatic tumors and its expression in invasive cell lines resulted in decreased migration and invasion (for review, see [125, 126]). We have observed that plakoglobin interacted with Nm23-H1 and -H2 in squamous cell, mammary, renal, and colon epithelial cell lines with the colocalization of these two proteins at sites of cell-cell contact. We have also shown that these interactions occurred in both the cytoskeleton-associated and soluble pool of proteins, suggesting that these interactions have both adhesive and nonadhesive functions. Since plakoglobin was detected in the nucleus of plakoglobin-expressing SCC9 cells and since luciferase reporter assays have shown that β-catenin/Wnt signaling is not activated in SCC9 cells [65], these results together suggested that plakoglobin regulates gene expression in SCC9 cells independent of β-catenin. We are currently characterizing whether plakoglobin directly regulates Nm23 expression. Furthermore, we have also shown that plakoglobin interacts with the transcription factor p53 and regulates the expression of a number of p53 target genes (manuscript in preparation).

8. Concluding Remarks

Recent work has demonstrated that plakoglobin has novel roles in intracellular signaling and the regulation of gene expression, in addition to its previously well-established roles in cell-cell adhesion. Plakoglobin has emerged as a tumor/metastasis suppressor protein based on evidence from

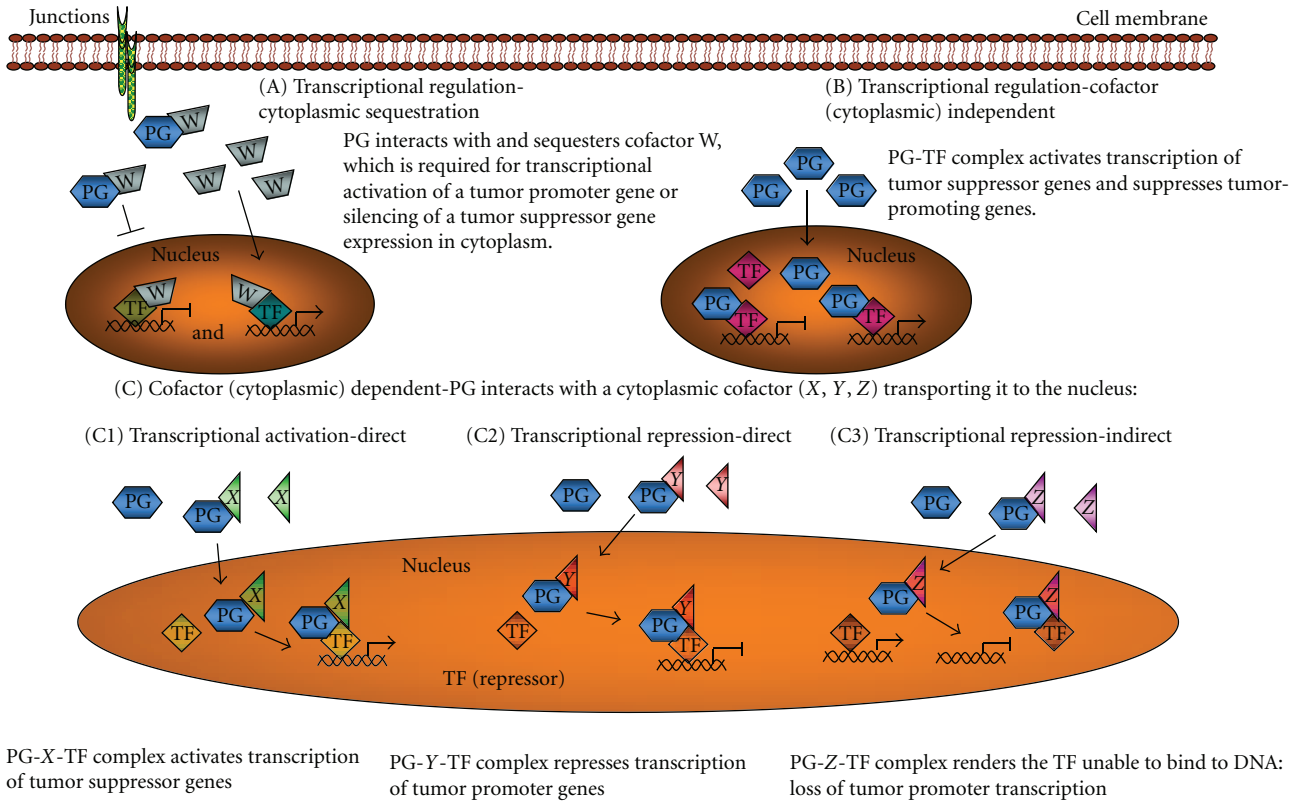

Figure 3: A potential model for regulation of gene expression by plakoglobin. Three concurrent mechanisms by which plakoglobin may regulate gene expression are proposed. (A) Cytoplasmic sequestration: plakoglobin sequesters a factor in the cytoplasm which, in the nucleus, suppresses the expression of a tumor suppressor gene or activates the expression of an oncogene. (B) Cytoplasmic cofactor independent: plakoglobin-transcription factor complexes promote the expression of tumor suppressor genes and repress the expression of oncogenes. (C) Cytoplasmic cofactor dependent: plakoglobin interacts with a cytoplasmic cofactor and this complex moves into the nucleus where it activates tumor suppressor gene expression or represses oncogenic gene expression. PG: plakoglobin; TF: transcription factor.

the great majority of the studies that have examined its signaling function. As more work focuses on the role of plakoglobin in tumorigenesis and metastasis, it is becoming clear that plakoglobin is a key, important player in these processes and consequently may be a useful therapeutic target in the treatment of cancer.

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Review Article

Prostaglandins in Cancer Cell Adhesion, Migration, and Invasion

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Prostaglandins exert a profound influence over the adhesive, migratory, and invasive behavior of cells during the development and progression of cancer. Cyclooxygenase-2 (COX-2) and microsomal prostaglandin E_2 synthase-1 (mPGES-1) are upregulated in inflammation and cancer. This results in the production of prostaglandin E_2 (PGE_2), which binds to and activates G-protein-coupled prostaglandin $E_{1–4}$ receptors ($EP_{1–4}$). Selectively targeting the COX-2/mPGES-1/PGE_2/$EP_{1–4}$ axis of the prostaglandin pathway can reduce the adhesion, migration, invasion, and angiogenesis. Once stimulated by prostaglandins, cadherin adhesive connections between epithelial or endothelial cells are lost. This enables cells to invade through the underlying basement membrane and extracellular matrix (ECM). Interactions with the ECM are mediated by cell surface integrins by "outside-in signaling" through Src and focal adhesion kinase (FAK) and/or "inside-out signaling" through talins and kindlins. Combining the use of COX-2/mPGES-1/PGE_2/$EP_{1–4}$ axis-targeted molecules with those targeting cell surface adhesion receptors or their downstream signaling molecules may enhance cancer therapy.

1. The Prostaglandin Pathway

Prostaglandins (PGs) and other eicosanoids are bioactive lipids that impact normal development, tissue homeostasis, inflammation, and cancer progression [1]. Prostaglandins are derived from the 20-carbon chain fatty acid, arachidonic acid (AA) stored in the plasma membrane of cells [2, 3]. As a storage mechanism, dietary AA is coupled to CoA molecules by acyl-coenzyme A (acyl-CoA) synthetases [4]. In turn, fatty acyltransferases utilize arachidonyl-CoA donor molecules to insert AA into membrane phospholipids [2, 3]. Membrane phospholipids generally retain AA until an appropriate stimulus catalyzes its release by phospholipase A2 [5–8] (Figure 1).

Once released, free AA serves a substrate for cyclooxygenases (COX) 1 or 2 (~72 kDa; Figure 1). Cyclooxygenases are mixed function oxidase enzymes that first peroxidate AA to form a hydroperoxy endoperoxide that links two oxygen molecules across carbons 9 and 11, prostaglandin G_2 (PGG_2). As the second coordinate enzymatic function, COXs reduce a hydroperoxy-group at carbon 15 of PGG_2 to form prostaglandin H_2 (PGH_2) [9, 10]. As a rate-limiting product in this pathway, PGH_2 serves as the substrate for a variety of PG synthases. These PG synthases include various isoforms of prostaglandin D_2 (PGD_2) synthases (PGDS) [11], prostaglandin E_2 (PGE_2) synthases (PGES) [12–16], and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) synthase (PGFS) [17]. PGH_2 can also be synthesized into prostacyclin (PGI_2) by its own separate synthase [18, 19] (PGIS) or thromboxane A_2 (TxA_2) by its synthase (TXS) [20]. In the case of inflammatory and carcinogenic activity, increased expression of COX-2 and microsomal PGE synthase-1 (mPGES-1) both occur to amplify the accumulation of PGE_2 in tumors [21–26]. Once synthesized, prostanoids are transported into the extracellular microenvironment by specific multidrug resistance associated proteins (MRPs). These MRP molecules contain 12-transmembrane spanning domains in the plasma membrane and two cytosolic ATP-binding/hydrolysis sites [27]. Among these export molecules, MRP4 is a 160 kDa protein that acts as the primary transporter for PGs. Once exported to the microenvironment, prostanoids bind to G-protein coupled receptors that contain 7 transmembrane spanning domains. These PG receptors include DP1, DP2, EP1-4, FP, IP, and TP that are classified according to their ligand specificity

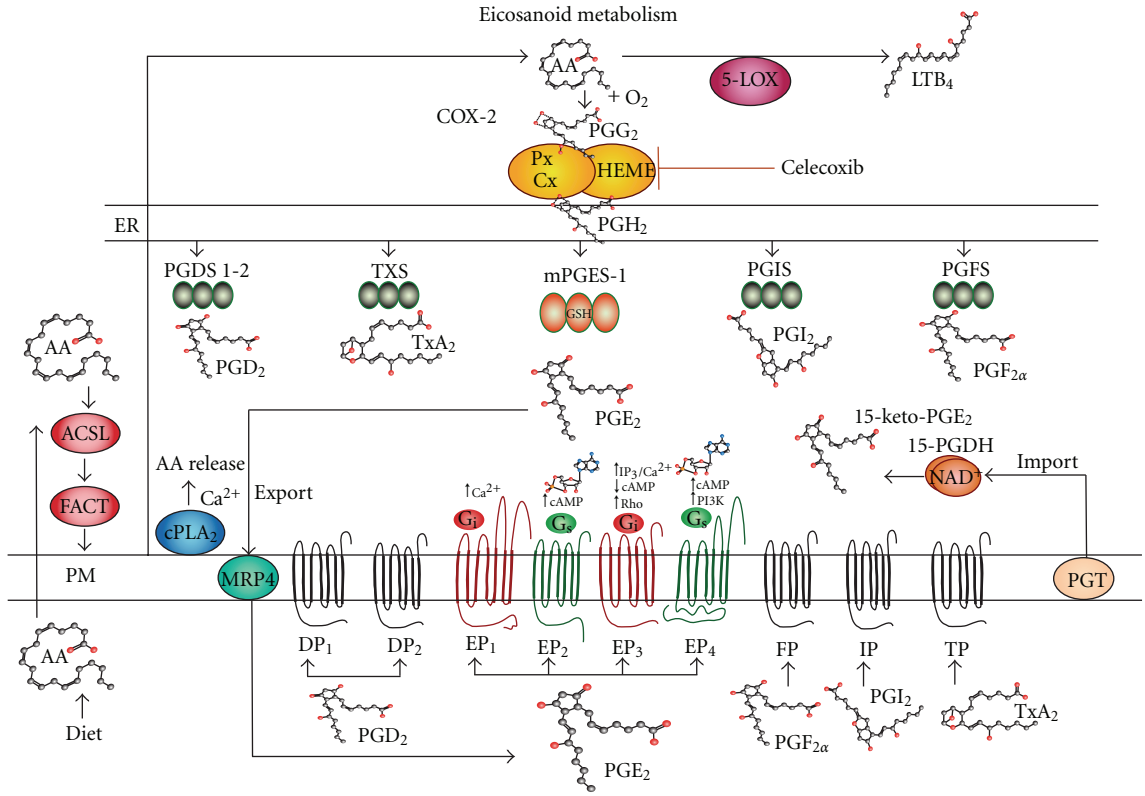

Figure 1: Eicosanoid metabolism. Arachidonic acid (AA) is an essential dietary fatty acid that is transported into cells and stored in membrane phospholipids. First AA is coupled to acyl-CoA by acyl-coenzyme A synthetases (ACLS). Fatty acyltransferases (FACT) then insert AA into membrane phospholipids. Cytoplasmic phospholipase A2 (cPLA2) releases AA from membrane phospholipids after agonist stimulation. In turn, free AA is converted to prostaglandin G_2 (PGG_2) and then prostaglandin H_2 (PGH_2) by cyclooxygenases (COXs). PGH_2 then becomes a substrate for a variety of PG synthases. These PG synthases are identified by the specific prostaglandin each one produces, namely, PGD_2 synthases (PGDSs), PGE_2 synthases (PGESs), ($PGF_{2\alpha}$) synthase (PGFS), PGI_2 synthase (PGIS), or TxA_2 synthase (TXS). Both COX-2 and microsomal PGE synthase-1 (mPGES-1) are elevated in tumors. Export involves multidrug resistance-associated protein 4 (MRP4). In the extracellular milieu, PGs bind to G-protein-coupled receptors identified as DP1, DP2, EP1-4, FP, IP, and TP. Among these, EP receptors interact with G-stimulatory (Gs) or G-inhibitory (Gi) proteins stimulating downstream signals such as cAMP, Ca^{2+}, inositol phosphates or IP3/Ca^{2+}, and Rho. Catabolism involves uptake by PG transporter (PGT) and inactivation by NAD+ dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH).

[28]. There are four EP receptors that require G-stimulatory (Gs) or G-inhibitory (Gi) proteins to initiate downstream signals such as cAMP, Ca^{2+}, and inositol phosphates [29]. More specifically, EP1 regulates Ca^{2+} flux; EP2 and EP4 both increase cAMP levels; whereas EP3 decreases cAMP, increases IP3/Ca^{2+}, and activates Rho. These signaling pathways frequently initiate transcription or crosstalk with other signal transduction pathways [30–32]. Prostaglandins can also interact with nuclear receptors. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that also bind PGs and complex with retinoic X receptors (RXRs) to initiate gene transcription [33, 34]. The catabolism of PG occurs as a two-step uptake and then inactivation process. PGs are taken up by a 12 transmembrane domain glycoprotein known as a PG transporter (PGT) [35–37]. After PGE_2 is transported across the plasma membrane, it is enzymatically catabolized by NAD+ dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) causing inactivation [36, 38, 39]. Two NAD+-15-PGDH protein monomers (29 kDa) form enzymatically active complexes by dimerization. Interactions with biologically active prostaglandins containing hydroxyl groups at carbon 15 are inactivated by conversion to 15-keto catabolites. The levels of both PGT and 15-PGDH are decreased in cancer leading to the accumulation of PGE_2 in tumor tissues [35, 36, 39, 40]. The accumulation of PGE_2 in the developing tumor microenvironment promotes tissue reorganization, angiogenesis, as well as cell adhesion, migration and invasion through the basement membrane barrier.

2. Prostaglandins and Cadherins: Making and Breaking Cell-Cell Contacts

Prostaglandins play an important role in wound healing and tissue reorganization [41–46]. The ordered structure of epithelial and endothelial tissues involves the cadherin family of molecules [47–51]. In many epithelial and vascular tissues, prostaglandins influence the formation and loss of

cell-cell contacts [52–56]. In vascular tissues for example, prostaglandins potentiate vascular endothelial VE-cadherin-dependent cell adhesion [57]. In the case of epithelial tissues, epithelial E-cadherins are structurally organized into adherens junctions that form extracellular Ca^{2+}-dependent transmembrane adhesion complexes between adjacent cells (Figure 2).

In the cytoplasm of epithelial cells, binding proteins mediate interactions between the E-cadherin cytoplasmic domain and the actin cytoskeleton that can trigger a variety of signaling processes [51, 58–60]. Dynamic analyses have revealed that α-catenin shuttles between cytoplasmic multiprotein complexes of β-catenin/E-cadherin or actin filaments [61]. β-catenin/E-cadherin interactions are regulated by IQGAPs that are actin-binding scaffold proteins [56–58]. IQGAPs interact with Rho GTPases and transmit extracellular signals that influence morphological and migratory cell behavior [62–64]. Alternate interactions through δ-catenin involve p190 and RhoA [65]. Additional adherens junctions stabilization pathways also exist. One of these pathways includes the involvement of Src and p140Cap. p140Cap regulates Src activation by C-terminal Src kinase (Csk) activity in epithelial-rich tissues that is phosphorylated after cell matrix adhesion [66–68]. Similarly, receptor protein tyrosine phosphatase mu (PTPμ) has a cell-adhesion molecule-like extracellular segment and a catalytically active intracellular segment involved in regulating cell-cell interactions [69, 70]. Nectins-afadin complexes also regulate cell-cell adhesion cooperatively with cadherins and integrins [71, 72]. Dynamic maintenance of cell-cell junctions in epithelial and endothelial tissues is critical to their functions as permeability or protective barriers and their continuous turnover as stress interfaces with the surrounding micro- or macro environment.

In order for epithelial cells to migrate, they must break their adhesive contacts with neighboring cells [56, 73]. The disassembly of cadherin containing adherens junctions involves internalization through endocytosis that result in the formation of phagosomes [51]. Internalization occurs by either caveolin-mediated endocytosis or clathrin-mediated coated pits [74–76]. Once cadherin-containing phagosomes are internalized, the extracellular domain resides inside the vesicles that form. At the same time, β-catenin and Src that are bound to the cytoplasmic domain of E-cadherin at the plasma membrane end up on the outside of these vesicles. Interactions of these vesicles with Ras-related protein A (RalA) drive cadherin recycling [77]. Interactions between E-cadherin with Ras-proximate-1/Ras-related protein 1 (Rap1)-GTPase, E3 ubiquitin ligase followed by ubiquitinization lead to proteosomal degradation [78–80]. Thus, the internalization and turnover of E-cadherin enables cells preparing to migrate with the ability to break their adhesive contacts between adjacent cells.

Breaking adhesive contacts occurs during tissue homeostasis, angiogenesis, and cancer progression in vascular or epithelial tissues and is a very rapid process based on live cell imaging [49, 81, 82]. In the case of epithelial tissues, their normal uniform structure typically becomes disorganized or dysplastic and then anaplastic during cancer progression. Disorganization in these tissues typically requires breaking cell-cell junctions maintained by cadherins such as E-cadherin [83]. In some cases this is mediated by prostaglandins. In squamous cell carcinoma, for example, chronically UVir-radiated SKH-1 mice sequentially lose E-cadherin-mediated cell-cell contacts as lesions progress from dysplasia to SCCs [53] (see Table 1). In these studies, the loss of E-cadherin levels was inversely associated with increased PGE_2 synthesis. Furthermore, the loss of E-cadherin involved the EP2 receptor and was reversed by indomethacin or potentiated by the EP2 receptor agonist butaprost [53].

Other epithelial tumors exhibit a similar loss of E-cadherin as COX-2/PGE_2 levels increase [84, 85]. This loss of E-cadherin is often accompanied by an elevation of vimentin that is a characteristic of cells becoming more migratory during epithelial-to-mesenchymal transition (EMT) [86]. This EMT involving COX-2 is observed in human colon cancers [87]. The loss of E-cadherin in conjunction with elevations in COX-2 occurs during the transformation of rat intestinal epithelial (RIE) cells [88] and during adenoma formation in $Apc^{Min\backslash+}$ that exhibit aberrant β-catenin signaling [89] or during gastrulation involving the Snail pathway in Zebra fish [90]. The COX-2 promoter contains a novel functional T-cell factor/lymphoid enhancer factor (TCF/LEF) response element that responds directly to Wnt/β-catenin signaling [91]. Regulation involving these pathways in some cases may be reversed. For example, caveolin-1-mediated suppression of COX-2 can occur via a β-catenin-Tcf/Lef-dependent transcriptional mechanism [92]. Overall, it is becoming clear that tissue homeostasis, reorganization, angiogenesis, and malignant transformation rely on very rapid dynamic making or breaking of cell-cell junctions centered on cadherin family of molecules. In most cases, epithelial tissues are strengthened by the synthesis and deposition of a basement membrane.

3. The Basement Membrane Barrier

Malignancies frequently develop from epithelial precancerous lesions that are initially confined to organ ducts or the epithelial strata of tissues. The pathologic conversion to cancerous lesions often involves malignant cells breaching or invading through the fibrous sheet-like barrier of the basement membrane (Figure 3) [93]. Prostaglandins are involved in the synthesis, homeostasis, turnover, and structural reorganization of the basement membrane [94, 95]. The basement membrane underlies the typical cellular epithelium or vascular endothelium and consists of two thin structural layers. The first layer consists of a basal lamina that is synthesized by epithelial or endothelial cells that differ in their respective characteristics [96]. The second layer is the reticular lamina made by fibroblasts, among other surrounding cells [97]. At the electron microscope level, the basal lamina is subdivided into a clear lamina lucida directly under the epithelial cells and a structurally opaque lamina densa [98, 99]. The lamina lucida contains protein and carbohydrate complexes at the cellular interface consisting of integrins, laminins (5, 6 and 10), and collagen XVII, as well as type IV collagen, laminin 1, and dystroglycans [97, 100, 101]. The lamina densa is a meshwork of type IV collagen fibers,

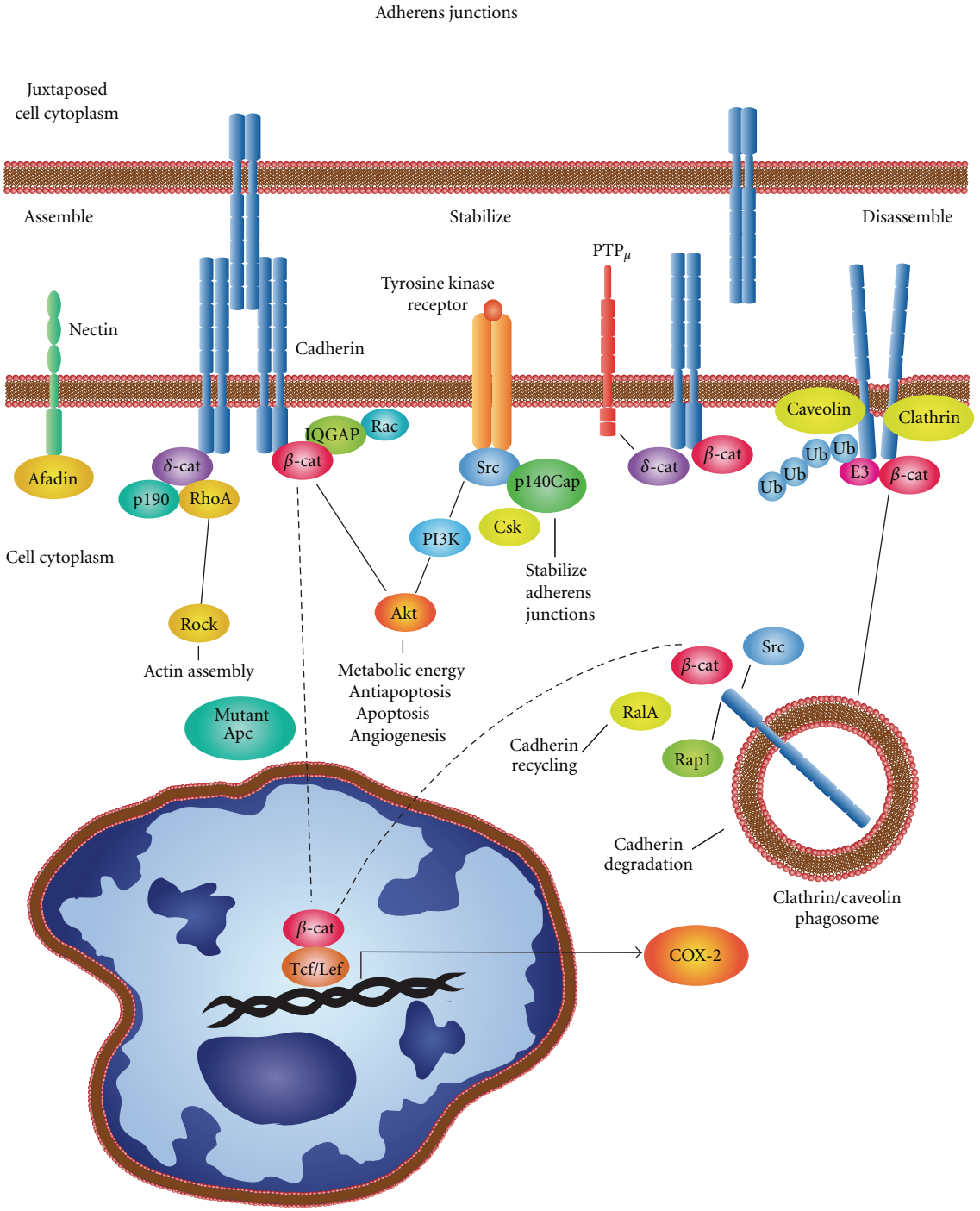

Figure 2: Dynamic adherens junctions. Prostaglandins influence the assembly, stabilization, and disassembly of cell-cell junctions. E-cadherins form Ca^{2+}-dependent transmembrane adhesion complexes between adjacent cells (Figure 2). Cytoplasmic regulatory proteins include α-catenin, β-catenin, IQGAPs scaffold proteins that interact with Rho GTPases to alter morphology and migration. Alternate interactions involve δ-catenin, p190, and RhoA influencing actin assembly. Together, Src and p140Cap influence C-terminal Src kinase (Csk) activity stabilizing cell-cell interactions as well as similar activity by receptor protein tyrosine phosphatase mu (PTPμ). Nectins-afadin complexes also cooperate with cadherins and integrins to regulate cell-cell adhesion. Disassembly of cadherin complexes involves either caveolin- or clathrin-mediated endocytosis and phagosome formation. Inside-out vesicles contain cadherin on the inside and β-catenin and Src exposed to the cytoplasm. When these vesicles interact with Ras-related protein A (RalA), cadherins are recycled. Whereas, interactions with Ras-proximate-1/Ras-related protein-1-(Rap1-)GTPase and E3 ubiquitin ligase followed by ubiquitinization result in proteosomal degradation that prepares cells for migration. The loss of E-cadherin in conjunction with elevations in COX-2 occurs during the transformation and adenoma formation in the presence of Apc mutations causing aberrant β-catenin signaling. Subsequent interactions with T-cell factor/lymphoid-enhancer-factor-(TCF/LEF-) can cause increases in COX-2 expression.

Table 1: Prostaglandins in cancer cell adhesion, migration, and invasion summary table.

Adhesive factor	Tissue	PG	Biological effect	Refs
Cadherins				
↓E-cadherin	RIE-S	↑PGE_2	COX-2-mediated PGE_2 production in rat intestinal epithelial cells (RIE) downregulates E-cadherin	[88]
↓E-cadherin	SCC	↑PGE_2	Downregulates E-cadherin through the EP2 receptor during squamous cell carcinoma (SCC) progression	[53]
↑E-cadherin	NSCLC	↓PGE_2	S-valproate and S-diclofenac increased E-cadherin but reduced vimentin and ZEB1	[84]
↓E-cadherin	TCC	↑PGE_2	Reciprocal correlation between cyclooxygenase-2 expression and E-cadherin in human bladder transitional cell carcinoma (TCC).	[85, 87]
↑E-cadherin	Melanoma	↑PGE_2	Decrease of TGFβ1-induced EMT properties in Madin-Darby canine kidney (MDCK) cells is associated with regaining E-cadherin expression	[257]
↑E-cadherin	MDCK	↑PGD_2	Decrease of TGFβ1-induced EMT properties in MDCK cells is associated with regaining E-cadherin expression	[258]
↓VE-cadherin	HLVE	↓PGI_2	Inhibition of PGI_2-mediated human lung vascular endothelial cell (HLVE) responses decreased VE-cadherin expression and increased eosinophil adhesion	[259]
Focal adhesions				
↑Actin bundles	HeLa	↑PGE_2	Examination of cyclooxygenase-dependent actin bundles in HeLa cells.	[144]
CREB activation	Raw264.7	↑COX2	Examination of Col-I on the COX-2 expression and the signaling pathways in macrophages.	[145]
↑Focal adhesions	Osteoblasts	↑COX2	Focal adhesion promotes fluid shear stress induction of COX-2 and PGE_2 release in osteoblasts	[146–148]
↑Focal adhesions	293-EBNA-HEK	↑PGF2α	Regulates Rho-mediated morphological changes	[150, 151]
Integrins				
$\alpha_2\beta_1$	Caco-2	↑LTD4/↑PGE_2	Increased adhesion to collagen I.	[177]
β_1	HT-29	↓PGE_2	Decreased adhesion and migration on extracellular matrix	[178]
α_3	Mammary TC	↓PGE_2	Decreased adhesion to laminin	[179]
$\alpha_5\beta_1$	HLC	↑PGE_2	Increased adhesion of human lung carcinoma (HLC) cells to fibronectin	[180]
αIIbβ_3	B16a melanoma	↑12-HETE	Increased adhesion to fibronectin, endothelial cells, and endothelial cell matrix	[182, 183]

entactin/nidogen-1, as well as perlecan, along with hydrous polysaccharide-rich gels of heparan sulfate proteoglycans. The reticular lamina contains collagens I, III, and V that form a heterogeneous network of fibers and a variety of proteoglycans [97]. Some basement membrane structures also contain pores that allow for the passage of cells [97]. The basement membrane is extensively remodeled during inflammatory responses [102, 103] or becomes disorganized in tumor vasculature [104] and in various cancers [105]. Thus there are a large variety of molecules encountered during invasion through the basement membrane that require the expression of many different cell surface adhesion receptors including integrins, cell surface proteoglycans, and tetraspanins.

4. Integrins

Mammalian integrins generate heterodimeric transmembrane glycoprotein adhesion receptor complexes consisting of α and β subunits (Figure 4) [106–109]. Alpha-numeric designations are applied to 18 known α subunits (α 1–11,D,E,L,M,V,W,X) and 8 β subunits (β 1–8) available to form pairs in this class of molecules. Each selective pairing recognizes a different ICAM, ligand, or protein substrate in the basement membrane or extracellular matrix [110, 111]. The α subunit dictates the ligand specificity by virtue of a seven-bladed β-propeller head domain connected to a leg support structure made of a thigh, a calf-1, a calf-2, a transmembrane, and a cytoplasmic domain [107, 112]. The β subunit interacts with the cell cytoskeleton and contains an N-terminal plexin-semaphorin-integrin (PSI) domain, a hybrid domain, a βI domain, four cysteine-rich epidermal growth factor (EGF) repeats, a transmembrane, and a cytoplasmic domain [107, 112]. In many cases, the N-terminal β-I domain of a β subunit inserts into the β-propeller domain of an α subunit (α1, α2, α10, α11, αL, αM, αX, and αD) to form a bulbous-binding headpiece complex [112]. The formation of integrin receptor complexes depends on divalent cation (i.e., Ca^{2+}, Mn^{2+}, Mg^{2+}) that bind to metal-ion-dependent adhesion site (MIDAS) motifs in the α subunits and adjacent to MIDAS (ADMIDAS) motifs in

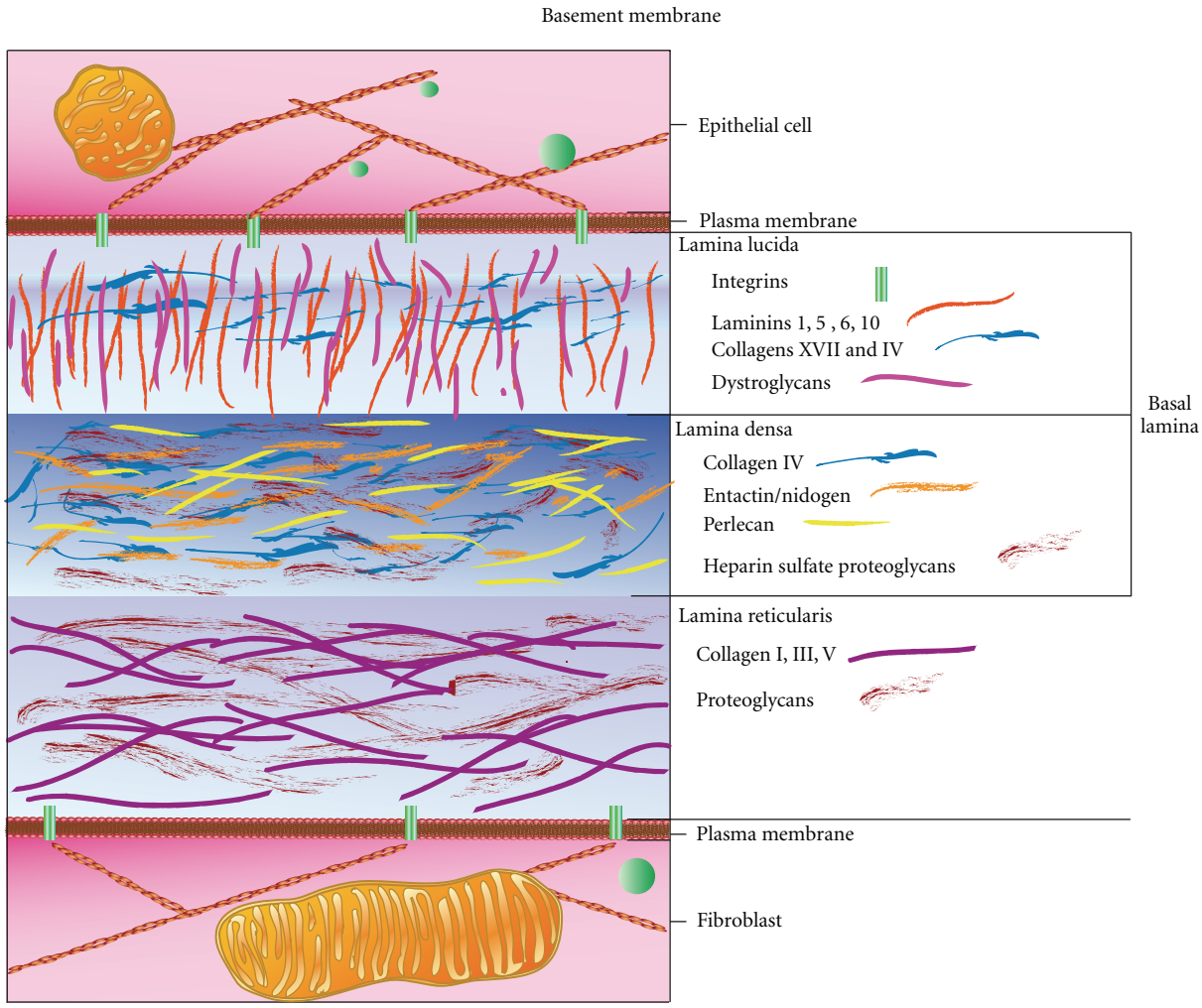

Figure 3: Basement membrane. The basement membrane underlies the typical cellular epithelium or vascular endothelium and consists of two thin structural layers. One layer is the basal lamina made by epithelial or endothelial cells. The second layer is the reticular lamina made by fibroblasts. Electron microscope data show that the basal lamina consists of a clear lamina lucida next to epithelial cells and an opaque lamina densa. The lamina lucida contains integrins, laminins (1, 5, 6 and 10), and collagen XVII, as well as type IV collagen, and dystroglycans. The lamina densa contains type IV collagen fibers, entactin/nidogen-1, perlecan, and heparan sulfate proteoglycans. The reticular lamina contains collagens I, III, and V and various proteoglycans. Invasion through the basement membrane requires the expression of many different cell surface adhesion receptors and matrix degrading enzymes.

β subunits found in the N-terminus of these receptors [107, 111–113]. Together the joined α and β subunit termini form an N-terminal headpiece [111].

Three conformation states exist for α and β subunit complexes [114–116]. The first conformation is usually unliganded with a closed headpiece and a bent receptor structure. In this case, the EGF domains of the β-subunit are juxtapositioned directly against the calf-1-calf-2 domains in the support leg of the α-subunit while the headpiece bends inward toward the plasma membrane [107, 111–113, 116]. Second, the integrin complex headpiece remains closed, but structural changes in the β-subunit EGF domains cause them to separate from the calf-1-calf-2 domains of the α-subunits and extend away from the plasma membrane [111, 114–116]. Third, altered conformation in the β6-α7 loops exposes the ligand-binding site while the β-subunit completely separates from the calf-1-calf-2 domains in the support leg of the α-subunit. These cooperative conformational changes in the heterodimer structures enable the full engagement of a specific integrin headpiece with its ligand [111, 114–116]. These conformational changes can occur during the regulation of "outside-in signaling" [117, 118] or alternatively "inside-out signaling" [112, 119].

5. Outside-In Signaling

Similar to conventional cell surface signal transducing receptors, integrins bind ligands and transmit information in an "outside-in signaling" (Figure 5) [111, 112]. "Outside-in signaling" behavior typically involves the engagement of integrins with the extracellular matrix or ICAM surface receptors [111, 118–121]. When external factors bind to

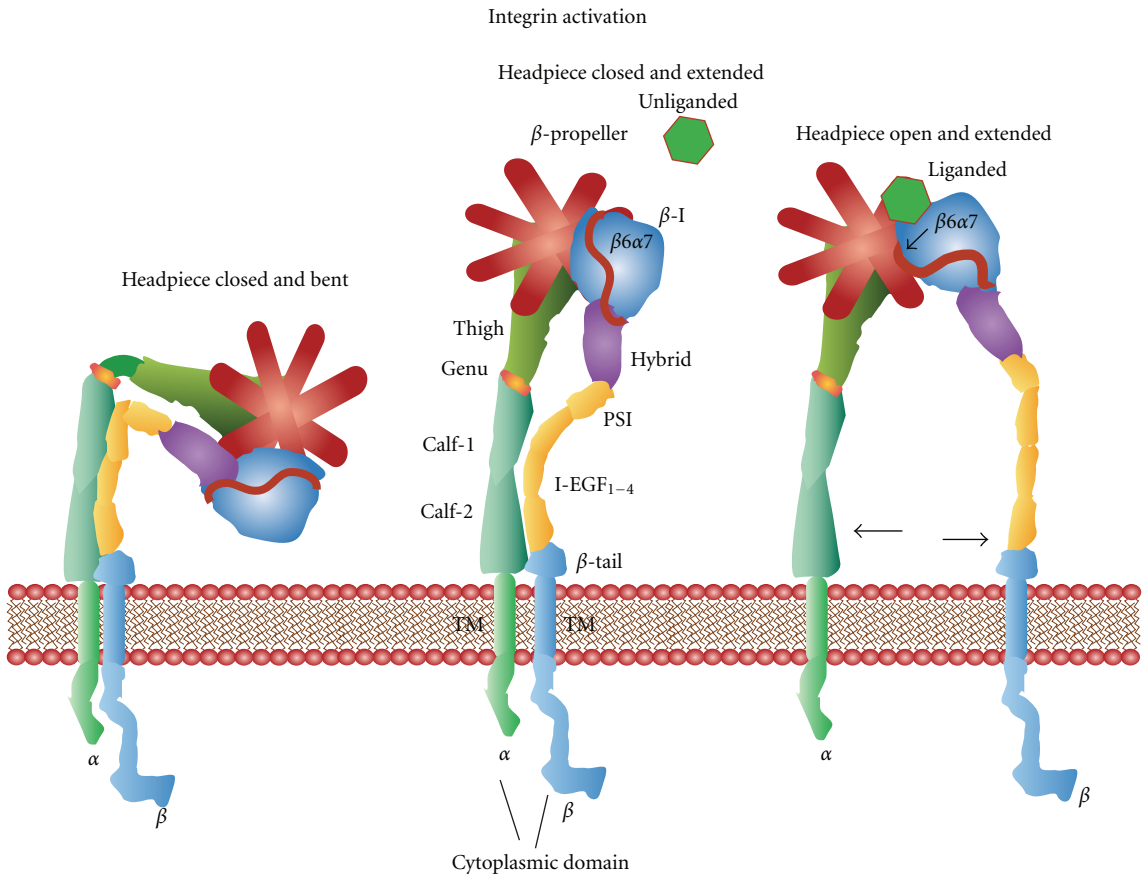

Figure 4: Integrins. Integrins are transmembrane glycoprotein adhesion receptor complexes consisting of α and β subunits. The α subunit contains a seven-bladed β-propeller head domain connected to a leg support structure made of a thigh, a calf-1, a calf-2, a transmembrane, and a cytoplasmic domain that mediates ligand specificity. The β subunit contains an N-terminal plexin-semaphorin-integrin (PSI) domain, a hybrid domain, a β-I domain, four cysteine-rich epidermal growth factor (EGF) repeats, a transmembrane, and a cytoplasmic domain that interacts with the cell cytoskeleton. The N-terminal β-I domain of a β subunit inserts into the β-propeller domain of an α subunit forming a headpiece complex. The formation of integrin receptor complexes depends on divalent cation (i.e., Ca^{2+}, Mn^{2+}, Mg^{2+}) that bind to metal-ion-dependent adhesion site (MIDAS) motifs in the α subunits and adjacent to MIDAS (ADMIDAS) motifs in β subunits. Three conformation states exist for α and β subunit complexes. (1) The unliganded conformation has a closed headpiece and a bent receptor structure with the EGF domains of the β-subunit touching the calf-1-calf-2 domains of the α-subunit. (2) The headpiece remains closed, but structural changes in the β-subunit EGF domains cause a separation from the calf-1-calf-2 domains of the α-subunits causing an extended structure. (3) Conformational changes in the β_6-α_7 loops expose the ligand-binding site along with a complete separation of the β-subunit from the calf-1-calf-2 domains in the α-subunit. These conformational changes engage the specific integrin headpiece with its ligand.

exposed ligand binding site on integrins this results in conformational changes described in the previous section. Most ECM proteins exhibit multivalent or recurrent molecular patterns, which trigger integrin clustering. As cells engage the repetitive patterns in the ECM, these events occur simultaneously thereby activating intracellular signals. The myriad of different extracellular signals that cells encounter in their microenvironment mediates cell polarity, cytoskeletal structure, adhesion, migration, invasion, gene expression, cell survival, and proliferation.

In the case of "outside-in signaling" initiated by ECM proteins, a single ligand-binding event can trigger integrin activation, but repetitive regularly spaced molecular patterns provide a more effective stimulus [122, 123]. This type of mechanoreception has been explored using nanopatterned molecular printing techniques that form regular cRGDfK patch spacings on a polyethylene glycol background matrix [122–125]. These adhesion-dependent sensory mechanisms lead to signal transduction inside the cell by the activating multiple pathways. Focal adhesions are often formed as a result of cell interactions with the ECM substrata, which initiate signal transduction via kinase cascades and other mechanisms.

6. Integrins and Focal Adhesions

Focal adhesions were first recognized in Rous sarcoma virus-transformed normal rat kidney cells using an antitumor serum specific for pp60src, as a speckled pattern of fluorescence on the ventral surface (Figure 5) [126]. Focal adhesion kinase (FAK) is a well-studied integrin-activated protein tyrosine kinase (PTK) [127, 128]. FAK was identified as a pp125

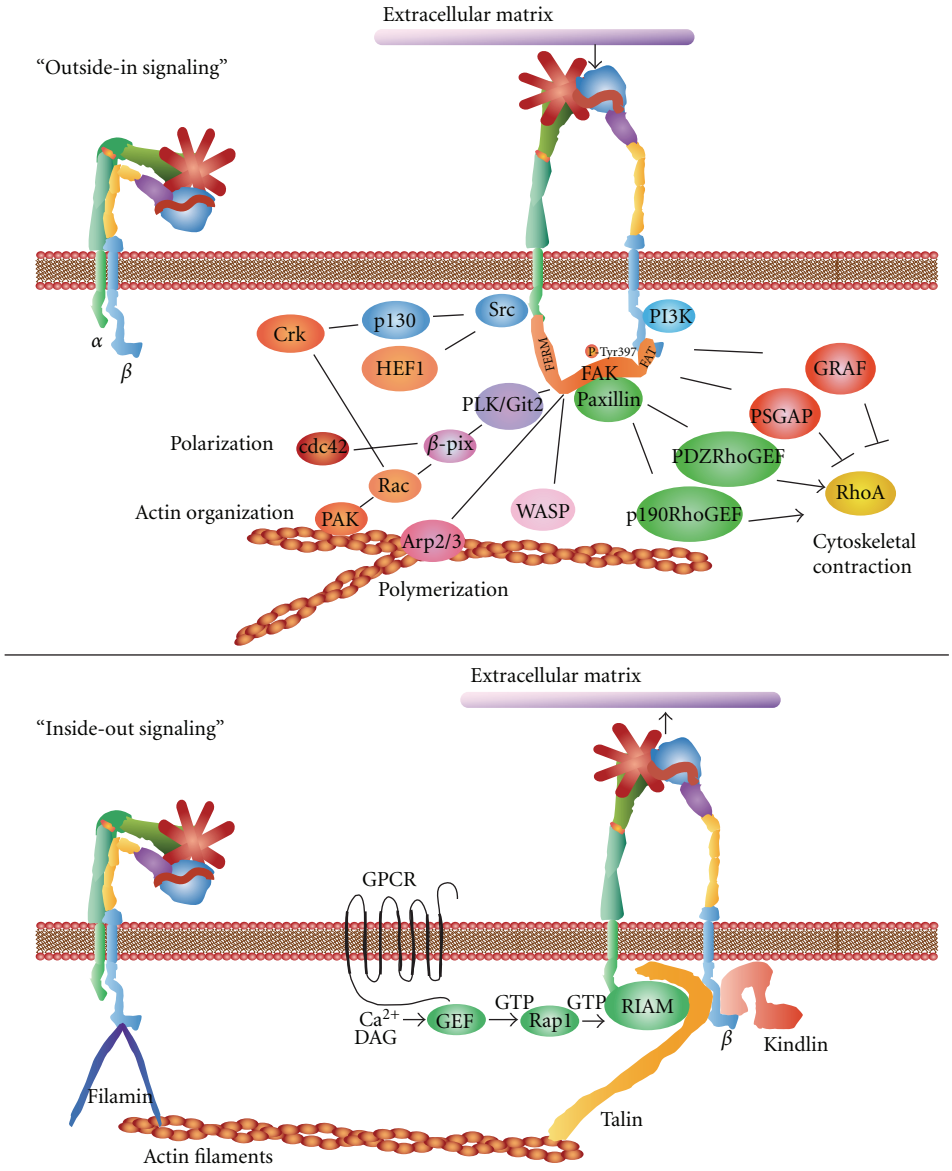

Figure 5: "Outside-in" and "Inside-out" signaling. The "outside-in" binding of ECM ligands to cell surface integrins stimulates conformational changes that activate focal adhesion kinase (FAK). FAK then is autophosphorylated on Tyrosine 397 near the catalytic domain, which binds Src. FAK contains a central kinase domain bordered by FERM (protein 4.1, ezrin, radixin, and moesin homology) domain at the N-terminus and a focal adhesion targeting (FAT) sequence at the C-terminus. Activated Src interacts with human enhancer of filamentation1 (HEF1) and p130 CRK-associated substrate (p130CAS) scaffold proteins that help to positively regulate Src-FAK-Crk interactions with Rac. FAK also activates (PKL/Git2)-β-Pix complexes and β-pix then serves either as an exchange factor for Cdc42 or a scaffold protein to promote signaling via Rac and p21-activated protein kinases (PAK). FAK also interacts with actin-related proteins (ARP2 and ARP3) which is regulated by the Wiskott-Aldrich Syndrome Protein (WASP). ARP2/ARP3 initiates the polymerization of new actin filaments. FAK also influences actin contraction and polarization through another GTPase protein, Rho. The regulation of Rho GTPase hydrolysis of GTP (active) to GDP (inactive) form occurs through the opposing activities of guanine nucleotide exchange factor (GEFs). GTPase regulator associated with FAK (GRAF) and p190RhoGAP blocks actin cytoskeleton changes. In contrast, PDZRhoGEF and p190RhoGEF both serve to activate Rho. "Outside-in signaling" transfers integrin-mediated external signals to the inside of cells."Inside-out signaling" depends on talin and kindlin. Both talin and kindlin contain FERM (4.1/ezrin/radixin/moesin) domains and a highly conserved C-terminal F3 domains. Talins bind β integrin, actin through the C-terminus, and also vinculin. Kindlins bind integrins, the cell membrane, and various actin adaptor proteins like migfilin, or integrin-linked kinase (ILK). Talin activation occurs through G-protein-coupled receptors that increases cytoplasmic Ca^{2+} and diacylglycerol. This activates GEF function in conjunction with Ras-proximate-1/Ras-related-protein-1- (Rap1-) GTPase. Rap1 then binds to Rap1-GTP-interacting adaptor molecule (RIAM). RIAM recruits talin to the membrane and the α and β integrin cytoplasmic domains. Kindlin interacts with β integrin cytoplasmic domain stabilizing the activated state of the integrin complex. "Inside-out signaling" strengthens adhesive contacts and the appropriate force necessary for integrin-mediated cell migration, invasion, ECM remodeling, and matrix assembly.

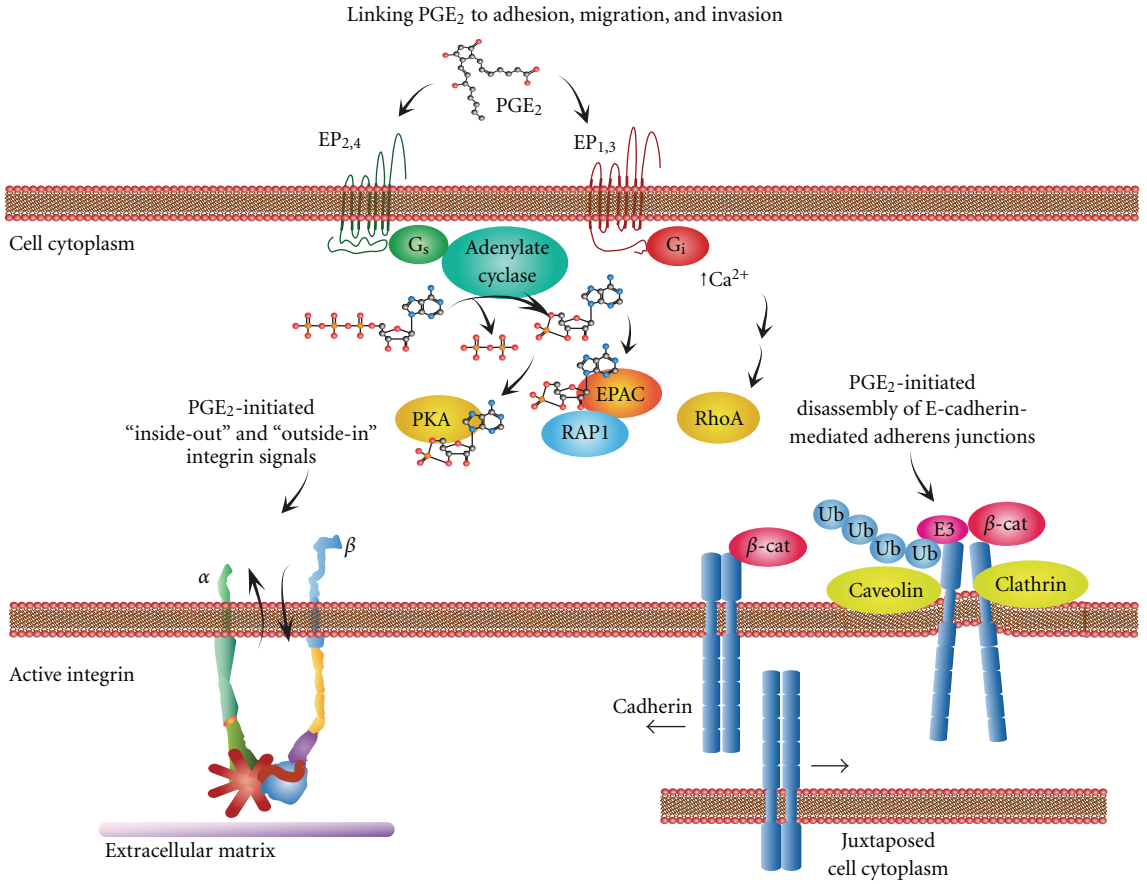

Figure 6: Linking PGE_2 to adhesion, migration, and invasion. Prostaglandin E_2 elicits profound changes in tumor cells that result in the disassociation of cadherin-mediated cell connections. This is accompanied by the establishment/turnover of integrin-mediated interactions with extracellular matrix during adhesion and subsequent migration and invasion. Stimulation of EP2 or 4 receptors leads to the activation of adenylate cyclase and results in the production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). The accumulation of cAMP in the cell cytoplasm activates protein kinase A (PKA) and the phosphorylation of downstream targets. This accumulation of cAMP can also activate exchange protein activated by cAMP (Epac). The activation of Epac may involve the interactions with Rap1 and subsequent downstream signals that influence adhesion, migration, and invasion. The activation of EP1 and EP3 leads to Ca^{2+} influx and the activation of Rho-mediated signal transduction that influences cadherin function during the disassociation of cadherin-based adhesive contacts or integrin interactions with the extracellular matrix contacts.

tyrosine-phosphoprotein in untransformed chicken embryo cells that increased in pp60v-src-transformed chicken embryo cells [129]. FAK is nonmembrane associated cytoplasmic protein that is autophosphorylated on Tyrosine 397 located at the juncture of the N-terminal and catalytic domains, which directs SH2-dependent binding of pp60src [130]. FAK contains a central kinase domain flanked by FERM (protein 4.1, ezrin, radixin, and moesin homology) domain at the N-terminus and a focal adhesion targeting (FAT) sequence at the C-terminus, which drives localization to focal adhesions [131, 132]. Upon interacting with the cyto-plasmic domains of integrins, autophosphorylated FAK interacts with numerous proteins recruited to focal adhesions. It can affect actin organization through the phosphorylation of paxillin [133, 134]. Paxillin phosphorylation by FAK on tyrosine residues 31 and 118 creates binding sites for the Src homology-2 (SH2) domains of adaptor proteins Crk, Csk, and Src [133]. Human enhancer of filamentation1 (HEF1) and p130 CRK-associated substrate (p130CAS) are scaffold proteins that help to positively regulate Src-FAK-Crk interactions [135, 136]. Paxillin can also interact with paxillin kinase linker- (PKL/Git2-) β-pix complexes [137]. β-pix functions as an exchange factor for Cdc42 or serves as a scaffold protein to promote signaling via the Rho family GTPase Rac and p21-activated protein kinases-(PAK) [137]. FAK-mediated regulation of Cdc42 and Rac activity asserts control over the extension of lamellipodia and cell migration as well as cellular polarization.

As another pathway influenced by FAK, interactions with actin-related proteins (ARP2 and ARP3) either occur directly or are regulated by the Wiskott-Aldrich Syndrome Protein (WASP) [132]. ARP2/ARP3 closely resembles the structure of monomeric actin. ARP2/ARP3 complexes serve as nucleation sites for new actin filaments [138]. When RP2/ARP3 complexes bind to the sides of preexisting actin filaments, they initiate the polymerization of new filaments

at a uniform 70° degree-angle during cytoskeletal rearrangements [138]. These molecular activities help organize and expand growing cytoskeletal meshworks of actin filaments.

FAK also influences actin contraction and polarization through another GTPase protein, Rho. The regulation of Rho GTPase hydrolysis of GTP (active) to GDP (inactive) form occurs through the opposing functions of GTPase-activating proteins (GAPs) or guanine nucleotide exchange factors (GEFs) [139]. GAPs make GTP a better substrate for nucleophilic attack thereby lowering the transition state energy for hydrolysis to GDP, inactivating Rho. In contrast, GEFs stimulate the release of GDP from Rho and accelerate the binding of GTP, thereby activating Rho. Among the Rho-inactivating GAP proteins is one that binds to the C-terminal domain of FAK, GTPase regulator associated with FAK (GRAF) to block actin cytoskeleton changes [140]. Another GAP protein, p190RhoGAP, can bind to complexes with p190RasGAP and FAK that alter the cytoskeleton [141]. In contrast, PDZRhoGEF and p190RhoGEF both serve to activate Rho. This activation promotes focal-adhesion turnover and their relocalization within the cell along with cell migration [142, 143].

7. Prostaglandins and Focal Adhesion Kinase

"Outside-in" stimulation by adhesion to ECM also stimulates PG pathway activity and FAK activity (Figure 5). When Hela or NIH3T3 cells are allowed to adhere to ECM, elevations in COX and PKA stimulate the formation of actin bundles that contain myosin II and associate with small focal adhesions and increase cell motility [144]. Similarly, stimulation of Raw264.7 cells with bovine type I collagen increased cyclic-AMP response element-binding protein (CREB) binding to DNA along with COX-2 expression that was reversed by inhibition of FAK [145]. Fluid shear stress stimulation of mechanoreceptors and RDGS mediated disruption of fibronectin adhesions-induced formation of focal adhesions and promoted the upregulation of COX-2 and PGE_2 release [146]. Similarly, mechanostimulation of osteoblasts activated FAK and PGE_2 release via integrin stimulation, which increased F-actin fiber formation, causing increased cell stiffness [147, 148]. Furthermore, HEF-1 adaptor proteins that positively regulate interactions with FAK are upregulated by PGE_2 and stimulate cancer cell migration [149].

Prostaglandins have a profound impact on FAK, immune cells, and cancer. This can occur by stimulation with a variety of PGs. For example, in 293-EBNA (Epstein-Barr nuclear antigen) cells stably expressing prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptors 1 or 2, stimulation with $PGF_{2\alpha}$ causes morphological and cytoskeletal changes [150]. The phosphorylation of FAK occurs in association with Rho-mediated morphological and cytoskeletal changes within two minutes, highlighting the rapidness of this process [150]. This FAK-mediated response to $PGF_{2\alpha}$ has also been observed in HEK293 cells [151] and endometrial adenocarcinoma cells [152]. Prostaglandin E_2 is also a strong stimulus for FAK activity. In hepatocellular carcinoma cells for example, PGE_2 increases the phosphorylation and synthesis of FAK in a dose-dependent manner [153]. Thus PG ligand binding to cognate GPCRs can also initiate "inside-out signaling".

8. Inside-Out Signaling

"Inside-out signaling" depends on a intracellular activators (Figure 5) [119]. These intracellular activators include proteins such as talin or kindlins [120, 154]. There are two talin isoforms and three kindlin isoforms identified thus far [154]. Both talin and kindlin contain FERM (4.1/ezrin/radixin/moesin) domains and a highly conserved C-terminal F3 domain [154]. Talins contain binding sites for several β integrin cytodomains, a highly conserved C-terminal actin-binding site and also VBS (vinculin-binding site) [119, 120]. Kindlins contain β integrin cytodomain-binding sites in their F3 domains, membrane-binding domains and a C-terminus that interacts with integrins, various actin adaptor proteins like migfilin, or integrin-linked kinase (ILK) [120, 154]. The activation process is thought to begin following stimulation of G-protein-coupled receptors that cause increases in cytoplasmic Ca^{2+} and diacylglycerol, followed by GEF activation in conjunction with Ras-proximate-1/Ras-related protein 1 (Rap1)-GTPase [119, 120]. Rap1 then binds to Rap1-GTP-interacting adaptor molecule (RIAM) [155]. RIAM is the believed to recruit talin to the membrane and the α and β integrin cytoplasmic domains [119]. Alternatively, talin interacts with PIPKIγ/PIP2 and then is cleaved by calpain [119]. Kindlin also interacts with the β integrin cytoplasmic domain stabilizing the activated state of the integrin complex [119]. "Inside-out signaling" strengthens integrin-mediated adhesion with extracellular ligands that transfers the appropriate force necessary for cell migration, invasion, ECM remodeling, and matrix assembly [119].

9. Prostaglandins, Integrins, and Angiogenesis

Prostaglandins are known to regulate cellular interactions with extracellular matrix and angiogenesis as early events in cancer progression [1] (Figure 6). The overexpression of COX-2 in rat intestinal epithelial cells was shown to increase adhesion to ECM and inhibit apoptosis which was reversed by sulindac sulfide (a COX inhibitor) [88]. COX-2 also plays a key role in endothelial cell migration and tube formation that relies on interactions with ECM during angiogenesis, which was reversed by NS398 (a COX-2 inhibitor) [156]. Prostaglandin E_2 plays an important role in stimulating the angiogenic behavior of endothelial cells [157–162]. By contrast, PGE_1 (alprostadil) inhibits angiogenesis *in vitro* and *in vivo* in the murine Matrigel plug assay [163]. Much of the migratory and invasive behavior of endothelial cells is regulated by signal transducing integrins that initiate changes in cellular shape, adhesion, and motility. For example, endothelial cell migration involves $\alpha_V\beta_3$ (vitronectin) and $\alpha_5\beta_1$ (fibronectin) integrin function, COX-2, the genesis of cAMP involving protein kinase A [164, 165]. This promotion of integrin $\alpha_V\beta_3$ integrin-mediated endothelial cell adhesion, spreading, migration, and angiogenesis appears to occur

through COX-2-prostaglandin-cAMP-PKA-dependent activation of the small GTPase Rac [165–167]. Others also confirmed the involvement of $\alpha_3\beta_1$ receptors [168]. Distinct integrins such as $\alpha_6\beta_1$ (laminin) or $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (collagen) receptors are also involved in the migration and invasion of endothelial cells during angiogenesis [169–175]. These studies highlight the role of PG-initiated responses by endothelial cells that involve integrins during the angiogenesis.

10. Prostaglandins, Integrins, and Tumor Cell Invasion

Tumor cells also migrate and invade through the basement membrane in response to stimulation by PGE_2 (Figure 6). For example, PGE_2 treatment of LS-174T human colorectal carcinoma cells leads to increased motility and changes in cell shape that involves stimulation of the prostaglandin EP4 receptor [176]. In another colon cancer cell line, CaCo2 cell adhesion to type I collagen via $\alpha_2\beta_1$ integrins was stimulated by PGE_2 and inhibited by COX-2 inhibitors [177]. Similarly, colon cancer cells expressing β_1 integrin levels along with COX-2 inhibition decreased adhesion and migration on ECM [178]. In another study using breast cancer cells, laminin receptor ($\alpha_3\beta_1$) binding to laminin-peptide PA-22 was reduced by PGE_2 receptor antagonist (LEO101) [179]. Similarly, the suppression of integrin $\alpha_3\beta_1$ in breast cancer cells reduced COX-2 gene expression and inhibited tumorigenesis and invasion [168]. In the case of lung cancer, FN stimulated cell proliferation through an $\alpha_5\beta_1$ (fibronectin) integrin-mediated process in conjunction with increases in COX-2 and PGE_2 biosynthesis that was blocked by NS-398 (a COX-2 inhibitor) [180]. The upregulation of COX-2 also induces tumor cell invasion in models of pancreatic cancer [181]. Other eicosanoids also influence integrin-mediated adhesion and invasion [182, 183]. Collectively, these studies highlight the importance of PGs during integrin-mediated adhesion, migration, and invasion through extracellular matrices by tumor cells.

11. Prostaglandins and CNN Proteins

Prostaglandins also regulate the production of matricellular proteins of the CCN family (CYR61/CTGF/NOV) that are emerging as major contributors to chronic inflammatory diseases and regulators of ECM [184]. CCN is an acronym that describes the first three protein family members identified out of six total: **C**YR61/CCN1 (cystein-rich 61;[185]), **C**TGF/CCN2 (connective tissue growth factor; [186]), and **N**OV/CCN3 (nephroblastoma overexpressed; [187]). The other family members consist of structurally conserved secreted multitasking Wnt-inducible secreted proteins (WISP-1/CCN4, WISP-2/CCN5, and WISP-3/CCN6) [188]. Each family member interacts with a specific subset of integrins and can be induced by PGE_2 depending on the cellular context [184]. In many instances, cell stimulation involving CCNs can alter the production of matrix metalloproteinases [184]. CCN proteins regulate cell adhesion, migration, proliferation, and inflammatory responses that are influenced by PGs [184].

12. Prostaglandins and Cell Surface Proteoglycans

Proteoglycans are very heavily glycosylated proteins on the surfaces of cells that heavily influence cell signal transduction and behavior [94, 189–194]. Proteoglycans exert profound control over various aspects of wound healing, angiogenesis, and cancer spreading [192, 195]. The fundamental proteoglycan unit contains a "core protein" and one or more covalently coupled glycosaminoglycans [190, 191]. Coupling occurs through a serine residue to a saccharide bridge found in the glycosaminoglycan. Glycosaminoglycan carbohydrate structures include chondroitin sulfate, dermatan sulfate, heparin sulfate, and keratin sulfate. Proteoglycans fall into two major categories based on size. Small proteoglycans range in molecular weight between 36 to 66 kDa and include decorin, biglycan, testican, fibromodulin, lumican, syndecan, and glypican. Large proteoglycans achieve molecular weights between 136 to 470 kDa and include versican, perlecan, neurocan, and aggrecan within this category. Proteoglycans cooperate dynamically with integrins and growth factors to local adhesion sites or signal complexes to integrate of both external and internal signals [196]. Together with prostaglandins, proteoglycans facilitate adhesion and migration and tubulogenesis by primary endothelial cells and promote phosphorylation of signaling molecules such as Akt and Src [94, 197]. Prostaglandins in concert with proteoglycans also promote the recruitment of stromal cells from the bone marrow to the developing tumor microenvironment [198, 199]. These include CXCL12, CXCR4, and S100A4 producing fibroblasts that involve signaling through a COX-2/PGE_2-EP3/EP4-dependent pathway [199]. Similarly, the combined effect of prostaglandins and proteoglycans regulates the transition from immature dendritic cells (iDCs) to mature DCs (mDCs) [198]. In breast cancer cells, prostaglandins and proteoglycans stimulate invasion across a basement membrane and induces synthesis of specific heparin-binding splice variants of vascular endothelial cell growth factor (VEGF) [200]. In like fashion, the malignant transformation of intestinal epithelial cells induces the production of VEGF that involves Ras pathway activation [160]. Among the proteoglycans, perlecan in particular plays an integral role in extracellular matrix deposition in response to PGE_2 [201]. Perlecan is also upregulated during tumor-associated angiogenesis [202], which can be inhibited by decreasing perlecan synthesis [203]. As a whole, proteoglycans work together with prostaglandins to regulate tumor growth and angiogenesis.

13. Prostaglandins and Tetraspanins

Tetraspanins form a family of 33 membrane proteins that contain 4 transmembrane-spanning domains [204]. They play important roles in cell adhesion, motility, invasion, immunity, and tumor progression [205–209]. Among these

tetraspanins, CD151, CD9, Tspan12, and KITENIN are most known for their role in cancer [205–208, 210]. Tetraspanin CD151 interacts with laminin-binding integrins $\alpha 6\beta 1$ and $\alpha 6\beta 4$ to regulate signal transduction activity during growth, migration, invasion, and metastasis [211, 212]. Tetraspanin CD9 in cooperation with cell-surface Ig superfamily proteins, EWI-2 and EWI-F acts to suppress tumorigenesis [213–215]. Tspan12 interacts with a disintegrin and metalloprotease 10 (ADAM10) to initiate protumorigenic functions [216, 217]. Also, KAI1 COOH-terminal interacting tetraspanin (KITENIN) contributes to tumor invasion and metastasis in human colorectal cancers [210] and gastric cancer [218]. In the case of interactions with prostaglandins, prostaglandin F_2 receptor-associated protein (FPRP) is frequently involved in binding to cancer cell tetraspanins [219, 220]. However, the direct regulation of this class of adhesion related molecules by prostaglandins or eicosanoids remains unknown.

14. Recent Advances in Prostaglandin and Adhesion-Based Cancer Therapy

Since (COX-2) is the rate-limiting enzyme in prostaglandin synthesis, it is an effective intervention point for inhibitors [221]. It is well documented that elevated COX-2 levels drive chronic inflammation and carcinogenesis [1]. Clinical and epidemiologic studies clearly demonstrate a significant benefit from inhibiting COX-2 in colon cancer [221, 222]. Unfortunately, COX-2 inhibition is associated with cardiovascular toxicity in a subpopulation of patients at high risk for cardiovascular disease [221, 222]. Nonetheless, this pathway remains an excellent target, based on very strong evidence that the upregulation of COX-2-mediated inflammatory mediators mediates many different cancers [1].

Selective COX-2 inhibition can also initiate a shunt of AA-based substrates to the 5-lipoxygnease (5-LOX) pathway [223], Based on these and other findings, a number of dual pathway inhibitors have been developed that appear to exhibit less toxicity [224–227]. Licofelone is a 5-LOX/COX inhibitor that was developed to treat inflammation and osteoarthritis [228, 229]. In osteoarthritis clinical trials, licofelone inhibits COX and 5-LOX and has low GI toxicity [230, 231]. In another osteoarthritis study, licofelone reduced osteoarthritis symptoms and less cartilage loss by MRI than naproxen [232]. Although developed and tested in osteoarthritis patients, cancer prevention is also an important target. In a lung carcinogenesis mouse model, for example, licofelone showed a dose-dependent inhibition of Cox-2 and 5-Lox and proliferating cell nuclear antigen (PCNA) staining in concert with an increase in apoptosis [233]. An overall reduction in GI toxicity in combination with enhanced anti-inflammatory activity makes these new inhibitors a promising class of compounds for the prevention and treatment of cancer.

Another approach is to specifically target specific points in the proinflammatory and procarcinogenic mPGES1-PGE_2-EP1-4 axis of the COX-2 pathway [221]. Inhibition of the inducible mPGES-1 has received significant attention [14, 15, 22, 23, 234]. In mouse models, EP(1) and EP(3) receptor antagonists ONO-8713 and ONO-AE3–240, but not the EP(4) antagonists ONO-AE3-208 and AH 23848, inhibited medulloblastoma tumor cell proliferation [235]. In $Apc^{Min\backslash +}$ models of colon carcinogenesis, by contrast, the genetic deletion of mPGES-1 significantly protected against azoxymethane-induced colon cancer [236]. In these studies genetic the deletion mPGES-1 reduced tumor multiplicity by ~80% and tumor load by 90% [236]. Also in a syngeneic mouse model of bone cancer, mPGES-1 enhances tumor growth and associated pain [237]. These studies emphasize the importance of mPGES-1 as a target for cancer prevention and therapy.

As a target further downstream, methods to decrease the accumulation of PGE_2 in tumors are also a potential target option. In this case, treatment may include enhancing the metabolic turnover of PGs by 15-PGDH. This may require upregulation by reversing histone deacetylase-mediated silencing of 15-PGDH [39]. These approaches are not as well developed as others but remain viable options for reducing prostaglandin-associated inflammation and cancer treatment.

As a target even further downstream, the development of EP selective receptor antagonists has seen extensive focus [238, 239]. In mouse models, ONO-AE3-208, an EP4 receptor antagonist significantly reduced metastasis [240]. Another EP4 antagonist is being tested as an inhibitor of migraine headache [241]. Selectively targeting the mPGES1-PGE_2-EP1-4 arm of this pathway will likely avoid cardiovascular and GI toxicity attributed to selective targeting of COX-2 alone. By combining targeting of the mPGES1-PGE_2-EP1-4 axis with selective adhesion-based therapy, it may be possible to significantly impact cancer prevention and therapy.

Adhesion-based therapy is generally targeted directly at surface receptors or the signal transduction pathways that mediate their activation [242]. In the case of direct integrin targeting, for example, Phase II clinical trials with cilengitide, a cyclicized arginine-glycine-aspartic acid-(RGD-)containing pentapeptide that acts as a $\alpha_V\beta 3$ and $\alpha_V\beta 5$ integrin antagonist, demonstrated clinical activity with limited side effects in glioblastoma patients [243, 244]. Based on these clinical results, the first Phase III clinical trial was initiated with an integrin antagonist [243, 244]. Similarly, $\alpha_5\beta_1$ integrins are also inhibited by the RGD amino acid sequence [245, 246], while $\alpha_4\beta_1$ are targeted by EILDV and REDV sequences [247]. In the case of cadherin targeting, ADH-1 is a cyclic pentapeptide that disrupts N-cadherin adhesion complexes that is being used to treat melanoma [248–250]. In Phase I studies ADH-1 used in combination with melphalan is well tolerated after isolated limb perfusion to treat regionally advanced melanoma. This approach using ADH-1 is believed to help overcome melanoma chemoresistance [250]. As we enter an era of personalized cancer therapy, using peptides to target specific adhesion receptors may be a viable adjuvant for selective targeting.

Targeting the signal transduction pathways downstream of adhesion receptor signaling involves a variety of molecular targets. These include the kinases, phosphatases GAP, GEF,

Rho family GTPases, adapter molecules, and scaffolding proteins among others. In the case of kinase targets, Src is a good candidate [251]. Src protein family members are useful because they serve as starting points for multiple signaling cascades involved in extracellular sensory activity [251]. This class of drugs includes the following: Bosutinib, AZD0530, and Dasatinib that target both cadherin/p120 catenin which affects adherens junctions [251]. Simultaneously, these compounds can affect integrin/FAK p130Cas, paxillin, and Rho, a downstream that affects interactions with ECM [251]. Preclinically for instance, AZD053 prevents phosphorylation of paxillin and FAK and suppresses metastasis *in vivo* [251].

Another effective kinase adhesion target is FAK [252, 253]. One of the most promising FAK inhibitors is PND-1186, which blocks FAK Tyr-397 phosphorylation [254–256]. *In vitro*, PND-1186 blocks FAK tyrosine phosphorylation while activating caspase-3 and initiating breast tumor cell apoptosis [255]. PND-1186 has also been tested *in vivo* and inhibits the growth of orthotopic breast carcinoma mouse models [254]. Targeting kinase molecules or the other intracellular signal pathway molecules may exhibit off-target effects that can be beneficial or cause unwanted side effects. Identifying patients with limited risk that will derive the most benefit from a given approach is essential to successful treatment.

In summary, targeting cell adhesion holds great promise for cancer therapy. As we learn more about individualizing cancer therapy, identifying patients that would receive the most benefit will help to direct targeting. For example, targeting specific adhesion pathways could be combined with inhibiting the mPGES1-PGE_2-EP1-4 axis in patients that also have elevated COX-2 in their tumors or elevated PGE_2 metabolites in their blood and/or urine. This approach may serve as an effective means of personalizing treatment or providing specifically targeted adjuvant therapy.

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Review Article

Anoikis Resistance: An Essential Prerequisite for Tumor Metastasis

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Metastasis is a multistep process including dissociation of cancer cells from primary sites, survival in the vascular system, and proliferation in distant target organs. As a barrier to metastasis, cells normally undergo an apoptotic process known as "anoikis," a form of cell death due to loss of contact with the extracellular matrix or neighboring cells. Cancer cells acquire anoikis resistance to survive after detachment from the primary sites and travel through the circulatory and lymphatic systems to disseminate throughout the body. Because recent technological advances enable us to detect rare circulating tumor cells, which are anoikis resistant, currently, anoikis resistance becomes a hot topic in cancer research. Detailed molecular and functional analyses of anoikis resistant cells may provide insight into the biology of cancer metastasis and identify novel therapeutic targets for prevention of cancer dissemination. This paper comprehensively describes recent investigations of the molecular and cellular mechanisms underlying anoikis and anoikis resistance in relation to intrinsic and extrinsic death signaling, epithelial-mesenchymal transition, growth factor receptors, energy metabolism, reactive oxygen species, membrane microdomains, and lipid rafts.

1. Introduction

Development, differentiation, and homeostasis are controlled by cell-cell interactions, cell-extracellular matrix (ECM) interactions, and soluble cues (hormones, cytokines, and growth factors) [1, 2]. Cell adhesion to ECM occurs through interactions between specific integrin receptors and ECM counterparts. These interactions cause the transduction of many different signals that regulate cellular functions, such as gene expression, differentiation, proliferation, and motility. Importantly, an appropriate adhesion to ECM components determines whether a cell is in the correct location and thus regulates cell survival and cell death. In 1994, Frisch and Francis noticed that loss of matrix attachment of epithelial cells resulted in apoptosis [3]. They referred to this form of programmed cell death that occurs upon detachment from the appropriate ECM as anoikis [4–6]. Because anoikis prevents detached epithelial cells from colonizing elsewhere, thereby inhibiting dysplastic cell growth or attachment to an inappropriate matrix, anoikis is a physiologically relevant process for tissue homeostasis and development. Dysregulation of anoikis, such as anoikis resistance, is a critical mechanism in tumor metastasis. Epithelial cancers initially arise as an organ-confined lesion, but eventually spread to distinct organs through the bloodstream, generating metastatic lesions that are responsible for most cancer-related lethality. The tumor cells that acquire anoikis resistance can survive after detachment from their primary site and while traveling through the vascular system until they colonize the distal organ [4–7]. In addition, anoikis resistance is also important for the peritoneal dissemination of gastric and ovarian cancer cells [6, 8]. This paper will focus on the current understanding of cellular and molecular mechanisms of anoikis resistance.

2. Adhesion and Cell Survival

For survival and proliferation, normal epithelial cells require adhesion to specific ECM components through cell surface receptors known as integrins. Integrins are heterodimers consisting of α- and β-subunits. There are at least 24 distinct integrin heterodimers assembled by the combination of 18

α-subunits and 8 β-subunits. Because specific integrin heterodimers preferentially bind to distinct ECM components, the repertoire of integrins on the cell surface guides where the cell adheres or migrates. Integrin expression patterns vary between normal tissue and tumors [1, 6, 9]. Although integrins $\alpha v\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 6$ are usually expressed at low levels, they are highly upregulated in some tumors, whereas some integrins, such as $\alpha 2\beta 1$, are decreased in tumor cells. Integrin ligation regulates not only cell adhesion and migration but also cell survival. Ligated integrins transduce survival signals, whereas unligated integrins can promote a proapoptotic cascade, thereby preventing cells from surviving in an inappropriate environment [2].

Integrins activate multiple signaling pathways that regulate cell motility and survival through interactions with cytoplasmic kinases, small G-proteins, and scaffolding proteins. Integrin ligation activates FAK, a nonreceptor tyrosine kinase, and activated FAK phosphorylates itself and other cellular proteins. FAK autophosphorylation at Y397 provides a binding site for SH2 domain-containing proteins such as Src family kinases and the p85 subunit of PI3K, which is important for integrin-mediated cell growth and migration [2, 6]. Integrin ligation activates the NF-κB and PI3K/Akt pathways and upregulates prosurvival proteins, Bcl-2 and FLIP, thereby enhancing cell survival [10]. Crosstalk between growth factor receptors and integrins activates Raf, which also promotes cell survival [6]. Although cell adhesion is critical for cell growth in normal tissues, adhesion-dependent control of cell growth is dysregulated in tumor cells, as anchorage-independent growth is prominently implicated in malignant transformation.

Recently, CUB domain-containing protein 1 (CDCP1), a transmembrane glycoprotein, has been linked to a noble pathway of anoikis resistance independent of Ras-MAPK and PI3K-Akt pathways in lung cancer and gastric cancer cell lines [11, 12]. Cell detachment induces CDCP1 phosphorylation by Src family kinases (SFKs) including Src, Fyn, and Yes. Upon SFK-mediated tyrosine phosphorylation of CDCP1, PKCδ forms a complex with CDCP1 and SFKs, is phosphorylated by SFKs and activated, leading to anoikis resistance [12]. Interestingly, CDCP1 phosphorylation by SFKs further activates SFKs and enhances metastasis in melanoma, indicating a "feed-forward loop" to maintain elevated activity of SFKs during tumor progression although the cause of the initial activation of SFKs is not clear [11, 13].

Resistance to detachment-induced cell death, or anoikis resistance, is emerging as a hallmark of metastatic malignancies, because it can ensure anchorage-independent growth and survival during tumor dissemination [4–6]. Numerous studies have suggested that stimulation of prosurvival signals and suppression of death signals are involved in anoikis resistance.

3. Prosurvival Signals and Apoptotic Signals in Anoikis Resistance

The role of the death receptor and the mitochondrial pathway in anoikis and anoikis resistance has been reviewed recently [5]. The death receptor (extrinsic) pathway is initiated by ligation of members of the TNF receptor superfamily, such as Fas and TNF-R1, causing the formation of the death-inducing signaling complex (DISC) as described in the Figure 1. Upon DISC formation, caspase-8 (FLICE) is activated and released into the cytoplasm where it cleaves caspase-3 and caspase-7. These activated effector caspases cleave cellular substrates, culminating cell death. FLIP (FLICE-inhibitory protein) has a higher affinity for the DISC than caspase-8 and is preferentially recruited to the DISC, thereby inhibiting caspase-8 recruitment and activation [5, 14, 15]. Anoikis employs this extrinsic pathway, at least partially. For example, matrix attachment protects cells from Fas-induced apoptosis, whereas matrix detachment sensitizes cells to Fas-mediated apoptosis. After cell detachment, FasL and Fas receptor are upregulated but FLIP, an endogenous antagonist of caspase-8, is downregulated, which leads to caspase-8 activation in human umbilical vein cells (HUVECs) [16, 17]. In addition, unligated integrin recruits caspase-8 to the membrane, where it becomes activated in a death receptor-independent manner, whereas integrin ligation disrupts this integrin-caspase complex and increases survival [18]. Caspase-8 activation triggers anoikis but FLIP overexpression, which inhibits caspase-8 activation and blocks anoikis in keratinocytes and HUVECs [16, 17]. Malignant cells avoid anoikis by overexpressing FLIP [5]. Anisomycin can sensitize cells to anoikis by decreasing FLIP protein levels and inhibits distal tumor formation in a mouse model of prostate cancer metastasis [19].

In the mitochondrial (intrinsic) pathway, proteins of the Bcl-2 family are key players and include anti-apoptotic proteins, such as Bcl-2, Bcl-xL, and Mcl-1, and proapoptotic proteins, such as the BH3-only proteins Bid, Bad, Noxa, Puma, and Bim, as well as Bax, Bak, and Bok [20]. In response to death signals, monomeric Bax or Bak translocate from the cytosol to the outer mitochondrial membrane (OMM) where they form oligomers, causing mitochondrial permeabilization and release of cytochrome c and Smac/DIABLO. Released cytochrome c triggers caspase-9 activation and consequently caspase-3 activation. Smac/DIABLO impedes the function of inhibitor of apoptosis proteins, IAP and XIAP. Bcl-2 exerts its antiapoptotic function by binding to pro-apoptotic proteins, Bax and Bad, preventing their oligomerization and thus maintaining mitochondrial membrane integrity. BH3-only proteins compete with Bcl-2 for binding with apoptotic activators, thus promoting apoptosis [21].

The mitochondrial (intrinsic) pathway is activated during anoikis. Bid translocates to the OMM following loss of adhesion with identical kinetics with Bax, which is required for anoikis of mammary epithelial cells [22]. Bim is sequestered by dynein cytoskeletal complexes when cells are attached to ECM. Cell detachment induces release of Bim from these complexes and causes its translocation to the mitochondria, where it interacts with Bcl-xL, neutralizing its prosurvival function [23, 24]. Loss of cell adhesion also causes the accumulation of cytoplasmic Bim via inhibiting its proteasomal degradation initiated by its phosphorylation through the ERK and PI3K/Akt pathways. Activated Bim promotes Bax-Bak oligomerization within the OMM and

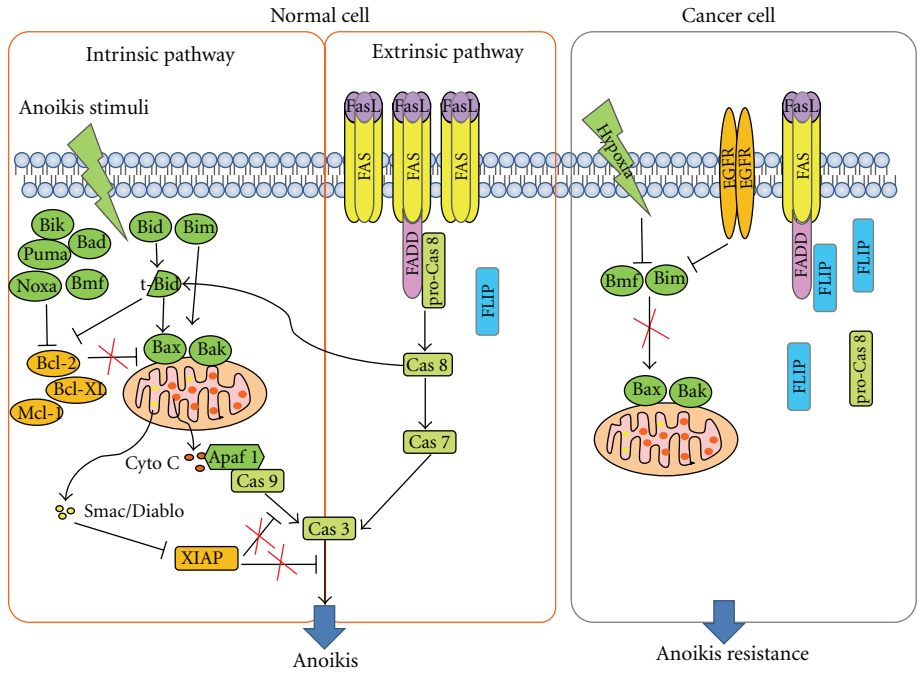

Figure 1: Schematic representation of intrinsic and exrtinsic pathways of anoikis. When cells are detached from ECM, normal cells induce anoikis through both intrinsic and exrtinsic pathways. Upon cell detachment, FAS and FasL are upregulated and FLIP is downregulated, leading to activation of Caspase 8, followed by activation of caspase-7 and caspase-3. Loss of cell adhesion also increases and activates proapoptotic Bcl-2 proteins (Bik, Puma, Bad, Noxa, Bmf, Bid, Bim, Bax, and Bak), which inactivate antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Mcl-1), and thus causing mitochondria membrane permeabilization through Bax/Bak oligomerization. Released cytochrome c from mitochondria activates caspase-9, subsequently caspase-3. Smac/DIABLO is released and inhibits XIAP, an inhibitor of apoptosis, leading to caspase-3 activation. Activaiton of these pathways leads to anoikis. However, increased FLIP expression in cancer cells inhibits extrinsic pathway and oncogene expression such as EGFR and hypoxia downregulate Bmf and Bim, resulting in inhibition of mitochondrial pathway in suspended cells. Accordingly, cancer cells acquire anoikis resistance.

induces intrinsic apoptosis pathway upon cell detachment [4, 24]. Bcl-2 modifying factor (BMF) is sequestered to myosin V motor complexes and, upon loss of cell attachment, is released. BMF then binds to antiapoptotic Bcl-2 to initiate anoikis in human intestinal epithelial cells [4, 25, 26]. Both Bim and BMF are associated with cytoskeletal structures and counteract the activity of antiapoptotic Bcl-2 when cell adhesion is disrupted. Therefore, suppression of Bim and BMF could confer anoikis resistance. In fact, overexpression of EGFR blocks both Bim expression and anoikis [27] and hypoxia decreases Bim and BMF expression and blocks anoikis in mammary epithelial cells [28].

4. EMT and Anoikis Resistance

During metastasis, epithelial cancer cells are released from their environment by breaking the basement membrane barrier. This process is involved in epithelial-mesenchymal transition (EMT). EMT is the biological process through which polarized epithelial cells undergo multiple biochemical changes, leading to a mesenchymal phenotype, such as enhanced migratory capacity, invasiveness, and resistance to apoptosis [29]. EMT is an essential process during development and is also induced during tissue repair and pathological processes, including inflammation and high-grade carcinomas in adults. EMT is characterized by loss of several epithelial proteins, including E-cadherin, β-catenin, and γ-catenin. It is also accompanied by increased expression of mesenchymal proteins, such as N-cadherin, vimentin, fibronectin, and smooth muscle actin. The significance of E-cadherin loss in metastasis has been shown in a variety of *in vitro* and *in vivo* models [30, 31] and loss of E-cadherin is the major hallmark of EMT. Loss of E-cadherin may be achieved through the upregulation of transcriptional repressors of E-cadherin, including E12/E47, Twist, and members of the Zeb and Snail protein families. Transforming growth factor-β (TGF-β), an inducer of EMT, upregulates these transcription factors, thereby promoting EMT and metastasis [32, 33].

EMT is not only a key event for epithelial-derived cells to acquire a motile and invasive phenotype but also an essential process for anoikis resistance [34]. EMT-promoting proteins are linked to anoikis resistance. Loss of E-cadherin induces anoikis resistance and promotes metastasis and N-cadherin expression also induces anoikis resistance [35]. Twist is the mediator of loss of E-cadherin-induced anoikis resistance [36, 37]. Conditional knockdown of E-cadherin and p53 in mammary epithelium induces mammary tumor initiation, metastasis, and anoikis resistance in the mouse model [37]. Moreover, the loss of E-cadherin induces Twist expression, indicating a feed-forward loop to maintain EMT [38]. Neurotrophic tyrosine kinase receptor B (TrkB) induces EMT,

and knockdown of Twist blocks TrkB-induced EMT, anoikis resistance, and tumor growth. Moreover, Snail was induced by Twist, and silencing of Snail impairs EMT and anoikis resistance [39]. Twist is upregulated in several malignancies and promotes EMT, and Zeb and Snail are often overexpressed in metastasizing tumors [36, 40]. Knockdown of Zeb1 induces E-cadherin expression and inhibits cell growth in anchorage-independent conditions [41]. Zeb1, acting downstream of Twist and Snail, is also required for TrkB-induced EMT, anoikis resistance, and metastasis [42]. The E-cadherin-interacting protein, ankyrin-G, mediates anoikis regulatory signals, and binds with neurotrophin receptor-interacting MAGE homolog (NRAGE). NRAGE represses the *p14ARF* gene and suppresses anoikis [43]. Recently, Shin et al. [44] reported that activation of EKR2 but not EKR1 is necessary for Ras-induced EMT in MCF-10A cells that are transformed by oncogenic Ras. ERK2 activation results in the Fra1 upregulation, which in turn triggers the accumulation of Zeb1/2, thereby inducing EMT and increasing migration, invasion, and survival. In addition, TGF-β induces EMT via isoform switching of FGF receptors, causing the cells to be more sensitive to FGF-2, which activates the MEK-ERK pathway to regulate complex formation of Zeb1 with transcription corepressor CtBP1 [45].

During the EMT process, E-cadherin expression is down-regulated whereas N-cadherin expression is up-regulated, referred to as a "cadherin switch" [46]. E/N-cadherin switch promotes cancer progression via TGF-β-induced EMT in extrahepatic cholangiocarcinoma [47]. N-cadherin expression appears to be more critical for tumor malignancy than E-cadherin. N-cadherin promotes cell motility and invasion via interactions with growth factor receptors such as FGF receptors and PDGF receptor. N-cadherin also promotes cell growth and survival by repressing apoptotic signals and numerous clinical studies have shown that aggressive human tumors express N-cadherin *in situ*, indicating a critical role for cadherin switch in human tumorigenesis [48]. Accordingly, both EMT and anoikis resistance are key processes for metastasis and they share common regulators, such as Twist, Snail, Zeb1, E-cadherin, and N-cadherin (Figure 2).

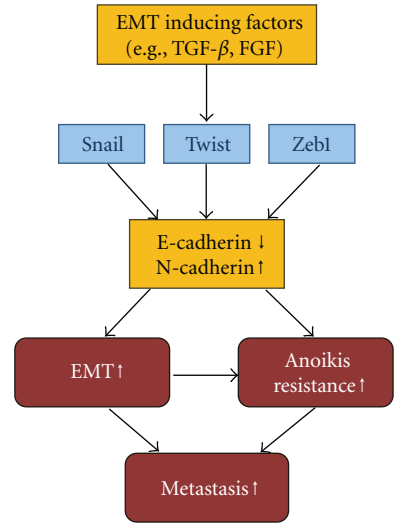

Figure 2: Schematic representation of EMT and anoikis resistance. EMT-inducing factors, such as TGF-β and FGF activate transcriptional factor, Twist, Snail and Zeb1. Activated these transcriptional factors repress E-cadherin (encoded CDH1 gene) expression and induce N-cadherin expression (Cadherin switch). Cadherin switch induces EMT and anoikis resistance, which are associated with tumor metastasis.

5. Growth Factor Receptors and Anoikis Resistance

Unregulated expression of growth factor receptors or components of their signaling pathways are associated with tumor malignancy due to their inhibition of cell death pathways and activation of cell survival pathways [49]. Abnormal regulation of growth factor receptors activates prosurvival signaling pathways, such as the PI3K/Akt, Ras/MAPK, NF-κB, and Rho-GTPase pathways [1, 49], leading to metastasis by inhibiting anoikis. This can be achieved through autocrine signaling of growth factors, including fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF). In addition, overexpression of growth factor receptors, such as EGF receptor, TrkB receptor, and HGF receptor, can suppress anoikis.

ErbB family members are tightly linked to tumor progression and malignancies through the activation of many different signaling pathways for cell proliferation, survival, and migration [49]. Upon detachment from ECM, EGFR (ErbB1) is downregulated and Bim is upregulated, which is a critical mechanism for anoikis. Overexpression of EGF receptor maintains ERK activation in suspended cells and blocks anoikis via suppression of Bim expression in MCF-10A cells, indicating that growth factor receptor expression uncouples anoikis from integrin regulation [27]. Moreover, ErbB2 overexpression blocks Bim expression and anoikis, via upregulation of α5 integrin and activation of Src in ECM-detached cells [50]. Loss of ECM attachment downregulates EGF receptor and β1 integrin both at the protein and mRNA levels. However, ErbB2 overexpression rescues both EGF receptor and β1 integrin protein via ERK and Sprouty, which stabilizes EGF receptor in ECM-detached cells [51]. Cell detachment causes ATP deficiency due to the decreased glucose transport but this deficiency can be rescued by ErbB2 overexpression, which stabilizes EGF receptor expression and thus PI3K activation [52]. TGF-α, a ligand for EGF receptor, prevents anoikis of intestinal epithelial cells by reversing the loss of Src activity and Bcl-xL expression induced by cell detachment [53]. Anchorage-independent Ewing sarcoma cells suppress anoikis through a pathway involving E-cadherin cell-cell adhesion, which leads to ErbB4 activation of the PI3K/Akt pathway [54]. The PI3K/Akt pathway plays a critical role in cell survival and PTEN, one of the most frequently mutated tumor suppressors in human cancer, negatively regulates the PI3K/Akt pathway [55]. PTEN also plays an important role in the anoikis induction through negative regulation of FAK. Overexpression of PTEN induces

anoikis via suppression of the phosphorylation of FAK and Akt in human bladder transitional carcinoma cells [56] and in U251 glioma cells [57]. Accordingly, loss of PTEN confers apoptotic resistance to cell rounding and matrix detachment in human mammary epithelial cells [58].

Insulin-like growth factor 1 (IGF-1) is a well-established cell survival factor that triggers Akt activation after loss of matrix contact [59]. IGF-1 receptor prevents anoikis in mouse embryo fibroblasts [60] and in the LNCaP human prostate epithelial cell line [59]. Disruption of IGF-1 receptor signaling decreased the number of circulating tumor cells in the blood of tumor-bearing mice and enhanced anoikis of LCC6 cells, a metastatic variant of MDA-MB-435 breast cancer cells [61]. Many studies have reported that PDGF receptor is also associated with metastasis of tumor [62–64]. PDGF receptor acts as the upstream tyrosine kinase for Src, a key contributor for anoikis resistance, in a human lung adenocarcinoma upon cell detachment [65]. The cooperation of autocrine PDGF-PDGFR signaling with oncogenic Ras strongly activates PI3K and is required for survival during EMT [66]. Transforming growth factor-β (TGF-β) and its receptor also play critical roles in tumor progression and metastasis. TGF-β1 coordinately and independently activates FAK and Akt kinase pathways through the early activation of SMAD3 and p38 MAPK, respectively, to confer an anoikis resistant phenotype to myofibroblasts [67]. In addition, in conjunction with EGF, TGF-β1 enhances migration, invasion, and anchorage-independent growth compared to that induced by EGF alone through the activation of MAPK and Akt [68]. Accordingly, the TGF-β inhibitor, LY2109761, suppresses metastasis of pancreatic cancer by inducing anoikis [69]. Conditional activation of FGF receptor causes anchorage-independent growth and EMT in a ribosomal S6 kinase-dependent manner in MCF-10A cells [70]. HGF and its receptor inhibits anoikis of pancreatic carcinoma cells through the PI3K pathway and of head and neck squamous carcinoma cells through activation of ERK and Akt signaling [71, 72]. Vascular endothelial growth factor A (VEGF-A) and its main signaling-receptor VEGFR2 (KDR) are expressed in primary ovarian tumors and autocrine VEGF-A/KDR loop protects ovarian carcinoma cells from anoikis [73].

6. Energy Metabolism, Autophagy, ROS, and Anoikis Resistance

Rapidly dividing tumor cells require rapid ATP generation, increased biosynthesis of biomolecules, and maintenance of an appropriate redox status to support cell division, despite the low oxygen (hypoxia) and nutrient levels within the tumor [74]. Tumor cells reprogram their metabolic pathways to meet these needs. The best-characterized metabolic phenotype in tumor cells is marked by the Warburg effect, which is a shift from ATP generation through oxidative phosphorylation to ATP generation through glycolysis, even under normal oxygen concentrations [75]. This aerobic glycolysis is regulated by PI3K/Akt [76, 77], hypoxia-inducible factor (HIF) [78–82], p53 [83–85], Myc [77, 86, 87], and AMP-activated protein kinase (AMPK)-liver kinase B1 (LKB1) [88–90] pathways. Alternatively, stress conditions, such as limited nutrients and hypoxia, activate autophagy to buffer metabolic stress during tumor growth [91].

An elegant model for the study of anoikis has been developed and provides insight into the mechanisms of detachment-induced apoptosis. Three dimensional culture of MCF-10A mammary epithelial cells form spheroid structures, termed acini, in which a layer of polarized epithelial cells surrounds a hollow lumen, resembling glandular epithelium *in vivo*. This lumen formation involves the clearance of central cells by selective anoikis of cells lacking ECM attachment [91]. When MCF-10A cells are detached from the ECM, glucose uptake is decreased, energy production is reduced [52]. Overexpression of ErbB2 has been linked to anoikis resistance. In line with this report, overexpression of ErbB2 rescues ATP deficiency by restoring glucose uptake through EGF receptor stabilization and PI3K activation, which is consistent with the studies that the PI3K/Akt pathway activates glucose uptake and protects cancer cells from starvation [52, 92]. Lack of glucose uptake in the detached cells blocks both glycolysis and the pentose phosphate pathway (PPP). In addition to macromolecular building blocks, PPP produces NADPH, a crucial cellular reducing agent, quenching the reactive oxygen species (ROS) produced during cell metabolism [74]. Accordingly, in the detached cells, ROS levels are increased and antioxidant treatment rescues low ATP levels by permitting fatty acid oxidation, leading to the filling of the luminal space in MCF-10A acini. An increase in oxidative damage blocks the consumption of fatty acids for energy production, and thus detached epithelial cells experience severe starvation and death. However, expression of the ErbB2 oncogene enables MCF-10A cells to overcome anoikis because there is enough ATP production with continuous glucose uptake.

Autophagy is a tightly regulated lysosomal self-eating process that is upregulated during cellular stress, such as deprivation of nutrients and growth factors. Autophagy produces nutrients and energy to enhance cell survival through the breakdown of cytosolic components. Basal levels of autophagy are required to maintain homeostasis by removing damaged proteins and organelles. Although autophagy is essential for cell survival, excessive autophagy results in programmed cell death. Autophagy is regulated by autophagy-related genes (ATGs), and the majority of pro-autophagic events converge on the mammalian target of rapamycin (mTOR) pathway [93]. Autophagy is known to suppress tumor formation by limiting chromosomal instability and promoting cellular senescence [94]. In contrast, protumorigenic functions for autophagy have been proposed and demonstrated. Autophagy is increased in cancer cells during many of the conditions directing metastasis, including hypoxia, metabolic stress, and cell detachment from ECM [93]. Silencing of autophagy regulators inhibits detachment-induced autophagy and enhances apoptosis, indicating that autophagy promotes epithelial cell survival during anoikis [91]. Recently, it was reported that oncogenic H-Ras expression induces autophagy upon cell detachment [95]. Interestingly, genetic deletion or RNAi-mediated knockdown of autophagy regulators decreases cell growth

in soft agar and reduces glycolysis, indicating that an intact autophagy pathway is required for adhesion-independent transformation and facilitates glycolysis through oncogenic H-Ras [95]. These studies suggest that anoikis resistance is associated with tolerance to bioenergetic stress due to oncogene expression.

The balance between oxidation and reduction plays a critical role in the cellular signaling pathways involved in cell growth and metastasis. Elevated oxidative stress is more frequently observed in many solid tumors and carcinoma cell lines than in normal cells. ROS are now recognized as key second messengers during growth factor and cytokine stimulation to elicit prosurvival signals. Upon integrin engagement, ROS are produced via Rac-1, and elevated ROS induce prosurvival signaling through Src activation. Activated Src phosphorylates EGF receptor in a ligand-independent manner, which activates the ERK and Akt pathways, leading to Bim degradation and suppression of anoikis. Src activation plays a critical role in anoikis resistance. Src is transiently activated upon cell detachment, which delays anoikis in intestinal epithelial cells [96]. In addition, in metastatic prostate cancer cells, ROS are constitutively produced due to sustained activation of 5-lopoxigenase (5-LOX). These amplified and persistent redox signals activate Src in the absence of adhesion, thereby sustaining ligand-independent EGFR activation. This pathway degrades pro-apoptotic protein Bim, thereby promoting anoikis resistance [97]. Elevated expression of angiopoietin-like 4 (ANGPTL4) is widespread in tumors and its role in anoikis resistance has been recently reported [98]. ANGPTL4 binds to $\beta 1$ and $\beta 5$ integrins directly, which stimulates NADPH oxidase (NOX)-dependent production of O_2^-. A high ratio of $O_2^- : H_2O_2$ activates Src, triggering the prosurvival PI3K/Akt and ERK pathways to confer anoikis resistance.

7. Membrane Microdomains and Anoikis Resistance

Another way to regulate anoikis is through modulation of membrane microdomains, including lipid rafts. This topic was recently reviewed [99]. There are two distinct types of lipid rafts: planar lipid rafts (noninvaginated rafts) and invaginated rafts known as caveolae. Caveolae are characterized by specific scaffolding proteins, called caveolins. Sphingolipids and cholesterol are the major lipid components of lipid rafts, and cholesterol is critical for the intact structure of lipid rafts. Because cell surface receptors, such as integrins and growth factor receptors, and some intracellular signaling molecules are enriched in lipid rafts, lipid rafts are regarded as the sites of assembly and initiation of signaling pathways [100, 101].

Caveolin-1, a structural protein for caveolae, acts as a scaffolding protein via its binding to signaling molecules such as EGF receptor, Src family members, MAPK, PKC, endothelial nitric oxide synthase (eNOS), and G-protein α subunits through its scaffolding domain [101]. This binding is known to negatively regulate these molecules. Initially, caveolin-1 was regarded as a tumor suppressor because it is downregulated in transformed cells and re-expression of caveolin-1 inhibits colony formation and induces apoptosis in transformed cells and breast cancer cells [102]. However, caveolin-1 levels are elevated in prostate cancer and lung adenocarcinomas, and the elevated caveolin-1 levels are associated with increased metastatic capacity and poor prognosis [103, 104], indicative of an oncogenic role for caveolin-1. Several studies have demonstrated that caveolin-1is associated with anchorage-independent growth. Caveolin-1 expression inhibits anoikis by inhibiting p53 activation and activating IGF receptor-mediated ERK and Akt signaling pathways upon cell detachment [105]. In human lung carcinoma H460 cells, caveolin-1 is downregulated during cell detachment through a mechanism involving ubiquitin-mediated proteasomal degradation. Interestingly, nitric oxide (NO) prevents downregulation of caveolin-1 by ubiquitination and this event suppresses anoikis [106]. Alternatively, increased levels of hydrogen peroxide in detached cells prevent caveolin-1 degradation and stabilize it, thereby inhibiting anoikis [107]. Overall, caveolin-1 appears to have dual functions depending on the cell type: as a tumor suppressor by inhibiting anchorage-independent cell growth and as a promoter of tumor metastasis by preventing anoikis.

Lipid rafts are associated with integrin signaling. Thus, modulation of lipid rafts is linked to adhesion-dependent cell survival. Cell detachment triggers internalization of lipid rafts, and inhibition of lipid raft internalization maintains Rac1 membrane targeting and downstream effector activation in suspended cells [108]. This study indicates that integrins regulate lipid raft localization, thereby controlling anchorage-dependent cell growth. In line with this notion, disruption of lipid rafts by cholesterol depletion results in FAK down-regulation, cell detachment, lipid raft internalization, and anoikis-like cell death in A431 human cervical cancer cells [109, 110]. Lipid raft disruption induces tyrosine phosphorylation of caveolin-1 through Src activation, which could be involved in lipid raft internalization. Caveolin-1 is tyrosine phosphorylated by Src and EGF-receptor signaling [99, 111], and this phosphorylation has been shown to be involved in caveolae internalization [112]. Under lipid raft disrupting stress, HIF-1α is induced via EGF receptor activation, which delays anoikis. This is consistent with the finding that knockdown of HIF-1α accelerates anoikis [113]. Aloe-emodin is an anthraquinone derivative that alters lipid rafts by decreasing sphingolipid and cholesterol in the lipid raft fraction. It inhibits tumor cell adhesion through disruption of the lipid raft-associated integrin signaling pathways, such as FAK recruitment to $\beta 1$ integrin [114] and it sensitizes anoikis in gastric carcinoma cells and lung cancer H460 cells [115, 116]. Akt activation is important for cell survival and anoikis resistance [4]. Intact lipid rafts are critical for PI3K/Akt signaling because they facilitate Akt recruitment and activation upon phosphatidylinositol-3,4,5-triphosphate accumulation in the membrane [117]. Lipid rafts disruption by cholesterol depletion results in both lipid raft internalization and Akt inactivation even in the presence of EGF stimulation. Cholesterol repletion reverses these effects [109, 110], indicating that lipid raft localization in the plasma membrane is important for Akt activation.

It is possible that metastatic tumor cells possess a mechanism to regulate lipid raft localization to escape anoikis, which remains to be investigated further.

8. Conclusion

Normal epithelial cells require adhesion to the appropriate ECM for survival and proliferation, and loss of this adhesion induces a type of cell death known as anoikis. Anoikis is important for normal development and tissue homeostasis because it prevents detached cells from reattaching to an inappropriate ECM and growing dysplastically. Anoikis resistance, or survival in the absence of attachment to ECM, is a prerequisite for the development of tumor metastases, the major cause of cancer mortality. Currently, anoikis dysregulation or resistance has evoked special attention in the cancer research fields because circulating tumor cells in the blood stream are resistant to anoikis, which allows the cancer cells to disseminate from the primary tumor site to a distinct lesion. Anoikis is regulated by many different signaling pathways depending on the cell type and expressed oncogenes. Both the intrinsic and extrinsic death pathways are employed in anoikis. Expression of oncogenes, such as ErbB family members, is enable cells to avoid anoikis by inhibiting apoptotic pathways. In this paper, we included current data on the mechanisms of anoikis in relation to alterations of energy metabolism, autophagy, ROS, and lipid rafts, which are emerging now as a major factor in the regulation of anoikis and remain to be explored further. A better understanding of the molecular mechanisms involved in anoikis resistance would assist in the development of anticancer drugs to eradicate circulating tumor cells and prevent tumor metastasis.

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Review Article

Integrins and Their Extracellular Matrix Ligands in Lymphangiogenesis and Lymph Node Metastasis

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In the 1970s, the late Judah Folkman postulated that tumors grow proportionately to their blood supply and that tumor angiogenesis removed this limitation promoting growth and metastasis. Work over the past 40 years, varying from molecular examination to clinical trials, verified this hypothesis and identified a host of therapeutic targets to limit tumor angiogenesis, including the integrin family of extracellular matrix receptors. However, the propensity for some tumors to spread through lymphatics suggests that lymphangiogenesis plays a similarly important role. Lymphangiogenesis inhibitors reduce lymph node metastasis, the leading indicator of poor prognosis, whereas inducing lymphangiogenesis promotes lymph node metastasis even in cancers not prone to lymphatic dissemination. Recent works highlight a role for integrins in lymphangiogenesis and suggest that integrin inhibitors may serve as therapeutic targets to limit lymphangiogenesis and lymph node metastasis. This review discusses the current literature on integrin-matrix interactions in lymphatic vessel development and lymphangiogenesis and highlights our current knowledge on how specific integrins regulate tumor lymphangiogenesis.

1. Introduction to the Lymphatic Circulation

Blood vessels supply tissues with nutrients and oxygen, remove waste products, and provide a mechanism for leukocyte homing. Capillary exchange is vital for this process. As blood pressure causes fluid extravasation in the arterial side of the capillary bed, colloid osmotic pressure drives resorption of the fluid on the venous side. However, ~10% of this fluid is retained in the tissue accumulating as interstitial fluid [1]. During inflammation and tumorigenesis, this accumulation of interstitial fluid is augmented due to enhanced permeability of the capillaries resulting in tissue edema [2].

The lymphatic system regulates the transfer of interstitial fluid and cells from the tissue back into the circulation [1]. Nearly all vascularized tissues contain lymphatics with the exception of the bone marrow, retina, and brain [3, 4]. Disrupting lymphatic vessel function, due to either primary (genetic) or secondary (infectious, vessel damage) mechanisms, causes chronic tissue edema. In addition to fluid transport, the lymphatic circulation plays a vital role in the inflammatory response. Antigen-presenting cells such as macrophages and dendritic cells encounter antigen at sites of local tissue inflammation. Endothelial cells in the lymphatic capillaries produce CCL21 [5], a chemokine that stimulates antigen-presenting cells to migrate into the lymphatic capillaries [6]. Targeting to the lymphatics and subsequently to the lymph nodes allows antigen-presenting cells to interact with T cells and B cells, a key step in adaptive immunity [7].

While similar in composition, lymphatic vessels and blood vessels show some striking differences. Unlike the continuous vascular circulation, the lymphatic vessels are divided into two distinct lymphatic trees (Figure 1(a)). Lymphatic vessels in the head, thorax, and right arm drain into the right lymphatic trunk and empty into the right subclavian vein. The lymphatics in the lower limbs, abdomen, and left arm drain into the thoracic duct and empty into the left subclavian vein [8]. Lymphatic capillaries are closed ended tubes that lack a normal subendothelial basement membrane and show no smooth muscle cell or pericyte coverage [9, 10]. The material collected by these lymphatic capillaries, termed lymph, is driven into the collecting lymphatic vessels by

interstitial pressure. Collecting vessels resemble venous vessels in that both have a subendothelial basement membrane, smooth muscle cells, and bileaflet valves which prevent fluid backflow (Figure 1(b)). Intrinsic contractility of lymphatic smooth muscle and skeletal muscle contractions propel the lymph forward where it is eventually returned to the venous circulation via the thoracic ducts [10]. Cell-cell junctions of lymphatic endothelial cells (LECs) are discontinuous and "button-like" allowing for a high degree of permeability in these vessels [11, 12]. Elevated interstitial pressure creates tension on LEC anchoring filaments enhancing LEC permeability and interstitial fluid uptake (Figure 1(c)).

2. Lymphangiogenesis in Cancer

Metastatic tumor spread is responsible for more than 90% of cancer mortality [32] and tumor access to blood and lymphatic vessels drives systemic metastasis. For multiple types of cancer, including melanoma and carcinoma of the breast, cervix, colon, and prostate, lymph node metastasis represents the first step in tumor dissemination [10, 33, 34]. For this reason, the presence of lymph node metastasis is a key determinant in tumor staging and the leading indicator of poor prognosis [35, 36]. Lymphatic vessel density (LVD), the product of both preexisting lymphatic vessels and new vessels arising from lymphangiogenesis, correlates with lymph node metastasis in a number of cancer models [37]. As such, cancers arising in regions possessing an already high LVD (e.g., tonsillar, tongue, head, and neck cancer) may not require lymphangiogenesis for subsequent lymph node metastasis [38]. In addition to vessel density, the location of the lymphatic vessels may be critical as intratumoral vessels have been reported as non-functional based on high intratumoral interstitial fluid pressures which collapse lymphatics [39]. These results suggest peritumoral lymphatics may serve as the primary site of lymphatic entry for metastatic cells.

2.1. VEGF-C and Tumor Lymphangiogenesis. Multiple growth factors modulate lymphangiogenesis, including hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), angiopoietin-1, endothelin-1, and members of the vascular endothelial cell growth factor (VEGF) family [40–48]. Several VEGF isoforms mediate tumor angiogenesis and VEGF/VEGF-receptor interactions have been targeted to modulate angiogenic responses [49]; the inhibitory anti-VEGF antibody *Avastin* was the first angiogenesis inhibitor to enter the market in 2004. The VEGF-A/VEGF-R2 interaction drives blood vessel angiogenesis, whereas lymphatic endothelial cells also express VEGF-R3 which shows higher affinity for VEGF-D and fully processed VEGF-C isoforms [41, 50]. Overexpression of VEGF-C or VEGF-D in mouse xenograft models enhances both lymphangiogenesis and lymph node metastasis [10, 51], and VEGF-C expression in human cancer correlates with enhanced lymphangiogenesis, lymph node metastasis, and poor prognosis (Figure 2(a)). Induction of skin carcinogenesis in transgenic mice overexpressing VEGF-C did not affect primary tumor size, but enhanced tumor metastasis to lymph nodes and the lung [52, 53]. Interestingly, VEGF-C overexpression enhanced lymph node metastasis even in xenografts from tumors that do not typically metastasize to lymph nodes [33, 51]. Perhaps most importantly, inhibitors of VEGF-C/VEGF-R3 signaling, including siRNA and soluble VEGF-R3, reduce lymphangiogenesis, lymph node metastasis, and enhance survival in mouse tumor models [54–56].

2.2. Lymphangiogenesis Inhibitors. Angiogenesis regulation involves the balance of proangiogenic and antiangiogenic factors. While many stimuli are known to activate lymphangiogenesis, less data exists describing the presence of endogenous lymphangiogenesis inhibitors (as has been described for angiogenesis). Mice deficient for the extracellular matrix protein thrombospondin-1 (TSP1), the first described endogenous inhibitor of angiogenesis [57], show exacerbated corneal lymphangiogenesis suggesting TSP1 may similarly inhibit lymphangiogenesis [58]. However, TSP1 overexpression does not show a similar antilymphangiogenic effect in skin carcinogenesis models presumably due to the absence of TSP1's antiangiogenic CD36 receptor in lymphatic endothelial cells [59]. Despite this, TSP1 may exert antilymphangiogenic activity indirectly by altering the levels of other lymphangiogenesis effectors. Consistent with a mostly indirect effect, the TSP1-activated growth factor TGFβ actively suppresses lymphangiogenesis [60, 61], and TSP1-mediated CD36 ligation on corneal macrophages suppressed VEGF-C and VEGF-D expression [58]. Vasohibin and the collagen XVIII fragments endostatin and neostatin 7 reduce both angiogenesis and lymphangiogenesis [62–64], suggesting that these inhibitors target pathways common to both angiogenesis and lymphangiogenesis. Interestingly, a splice variant of VEGF-R2 encoding for a soluble form of the receptor did not affect tumor angiogenesis but blocked lymphangiogenesis presumably due to the ability of soluble VEGF-R2 to bind VEGF-C [65].

2.3. Cancer Cell Chemotaxis toward Lymphatic Chemokines. Cancer cells often enter lymphatics at the level of the lymphatic capillaries. This process is aided by the LECs themselves, which secrete chemokines such as CCL21 that induce chemotaxis in antigen-presenting cells and some cancer cells [5]. VEGF-C expressed by tumor cells and monocytes in the tumor stroma stimulates LEC production of CCL21, and CCL21 in turn activates its receptor CCR7 in cancer cells (Figure 2(b)) [66]. Xenografts of CCR7 expressing melanoma cells were found to grow towards regions of implanted LECs. Interestingly, only metastatic malignant melanoma cells express CCR7, while their nonmalignant counterparts do not [67, 68]. Similarly, breast cancer cells showing lymph node metastasis also show enhanced CCR7 expression [69], and breast cancer cell xenografts showed lymph node metastasis when CCR7 was expressed [70].

In addition to the CCL21/CCR7 axis, lymphatic endothelial cells also express SDF-1 which promotes metastasis to lymph nodes in several cancer cells that express the SDF-1 receptor CXCR4 (For a full review see [71]). PDGF-D

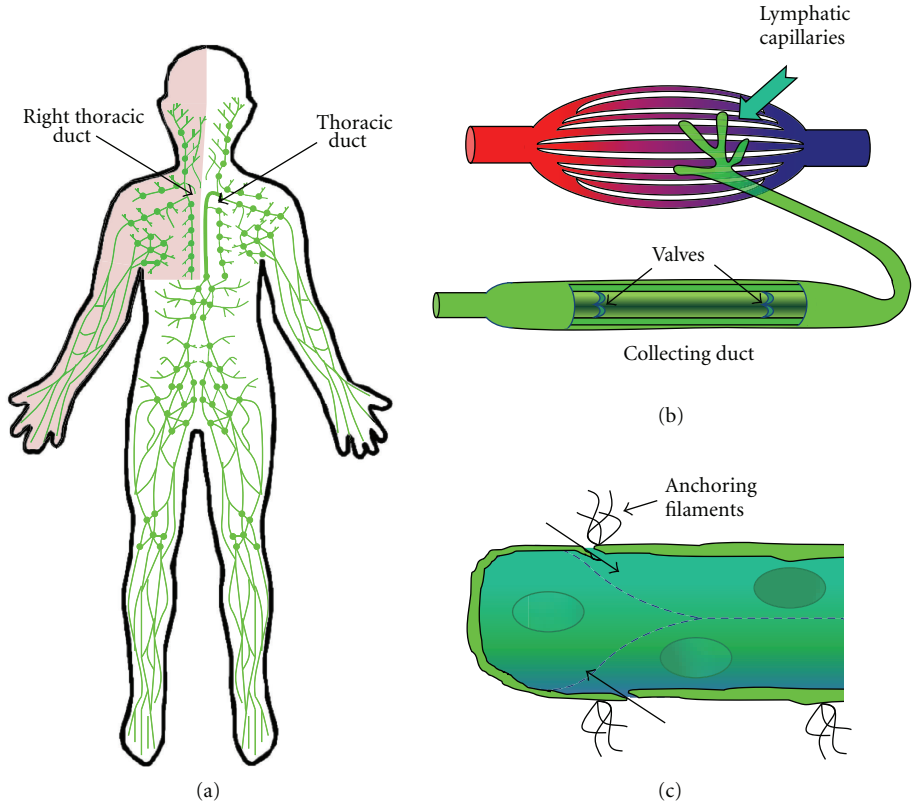

Figure 1: Lymphatic system structure. (a) The lymphatic system is separated into two distinct sets of tubules. Lymphatic vessels drain various areas of the body passing the material through a series of lymph nodes before returning the material to the venous circulation through the thoracic ducts. (b) Lymphatic capillaries drain interstitial fluid that accumulates during capillary exchange. The protein and cell-rich fluid termed lymph is then transported into vein-like valved collecting tubules. (c) Anchoring filaments couple lymphatic capillary endothelial cells to the surrounding matrix. Forces applied through these anchoring filaments enhance lymphatic permeability to promote tissue drainage.

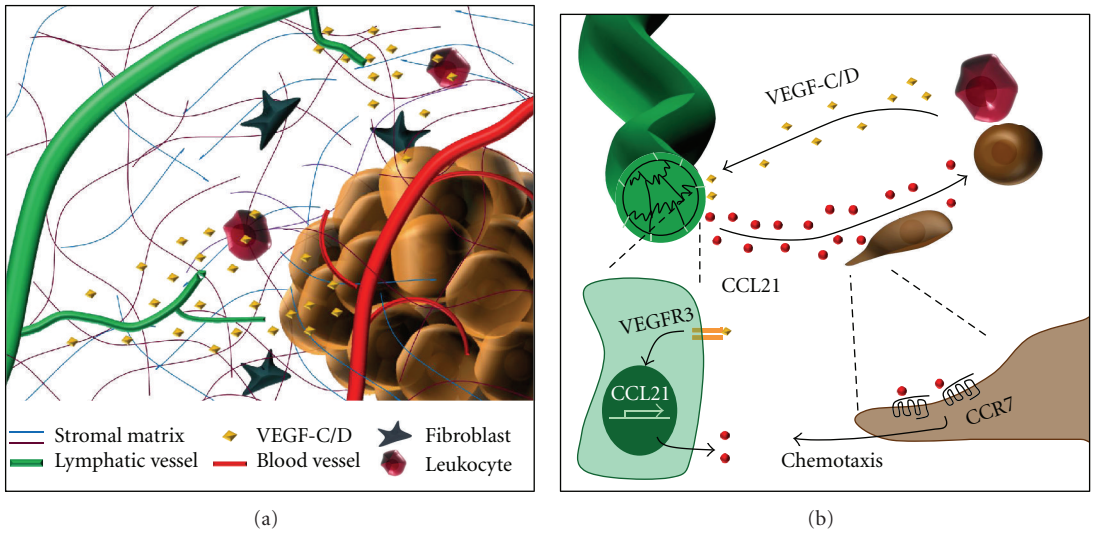

Figure 2: Local paracrine signaling controls lymphangiogenesis and lymph node metastasis. (a) Release of growth factors such as VEGF-C and VEGF-D by tumor and stromal cells promotes lymphatic endothelial cell sprouting, invasion, and capillary tube formation. (b) VEGF-C stimulates lymphatic endothelial cells to produce the chemokine CCL21. Expression of the CCL21 receptor on leukocytes and some tumor cells stimulates chemotaxis toward the lymphatic vessel promoting lymphatic dissemination.

overexpression stimulated CXCR4 expression in breast carcinoma xenografts and promoted lymph node metastasis [72]. Furthermore, a polymorphism in SDF-1α (G801A) which results in elevated SDF-1 expression was associated with lymph node metastasis and shorter survival time in patients with colorectal cancer [73].

3. The Integrin Family of Receptors and Their Extracellular Matrix Ligands

The extracellular matrix (ECM) is a highly organized complex of collagens, proteoglycans, glycoproteins, and growth factors capable of creating varying degrees of tissue tensile strength, from mucosal linings to bones. Laminins and collagen IV form a thin sheet-like matrix termed basement membrane that separates epithelial and endothelial cells from underlying connective tissue [74]. Fibrillar collagens (e.g., collagen I, collagen III) make up the bulk of the body's connective tissue and play a major role in regulating tissue tensile strength due to their capacity to be cross-linked into fibers. Although not normally involved in maintaining tissue structure, provisional and matricellular matrix proteins are rapidly deposited during tissue remodeling responses coordinating cell migration and proliferation to heal injured tissue. Provisional matrix proteins (e.g., fibronectin, fibrinogen, vitronectin) present in the bloodstream leak into wounded areas and provide an adhesive scaffold for the recruitment of cells [75, 76]. Matricellular matrix proteins (e.g., thrombospondin, tenascin-C, SPARC, osteopontin) generally play a minimal role in tissue structure but instead regulate the cell's interaction with structural matrix proteins and modulate cell function [77, 78]. Interactions with ECM proteins affect nearly every aspect of cellular physiology, from cell proliferation and migration, to gene expression and differentiation [79]. Specific cell-matrix interactions are critical for the survival of many cell types, and loss of this adhesion dependence is a classic hallmark of neoplastic change [80]. Furthermore, ECM proteins are secreted and organized by the cells in the local environment, suggesting that cells and their matrices exist in a state of "dynamic reciprocity" as each one serves to regulate the other [81].

3.1. Matrix Composition in the Tumor Stroma. During tumorigenesis, the expanding tumor stimulates the production of local supportive tissue termed the tumor "stroma" which is composed of proliferating fibroblasts, leukocytes, blood and lymphatic vessels, and ECM proteins. Mounting evidence suggests that the local tumor microenvironment plays a critical role in cancer progression from a collection of transformed cells to a clinically relevant disease [82]. During stromal matrix formation, cancer cells and stromal fibroblasts show enhanced deposition of fibrillar collagens (e.g., collagen I and III), provisional matrix proteins (e.g., fibronectin), and matricellular proteins (e.g., tenascin-C, osteopontin) [83, 84]. While the stromal matrix plays established roles in angiogenesis and tumor metastasis [85], mounting evidence suggests that the stromal matrix regulates tumor lymphangiogenesis as well.

3.2. The Integrin Family of Matrix Receptors. Interactions between ECM components and the integrin family of matrix receptors serves to anchor cells to the underlying matrices, mechanically couple the actin cytoskeleton to the external environment, and activate a broad spectrum of integrin-specific signaling pathways. The integrin family exists as heterodimers of unique α and β subunits; mammals express 18 α and 8 β subunits forming 24 distinct $\alpha\beta$ integrin dimers (Figure 3(a)). Integrin expression patterns depend on the specific cell type and vary with environmental context [86, 87]. Leukocyte homing responses typically involve a separate subset of integrins (αLβ2, αMβ2, αXβ2, αDβ2) that interact with counter-receptors on the endothelial cell surface such as ICAM-1 and VCAM-1. Distinct collagen-binding integrins (α1β1, α2β1, α10β1, α11β1) and laminin-binding integrins (α3β1, α6β1, α6β4) serve to anchor cells to the basement membrane and interstitial matrices. Provisional matrix proteins often contain RGD sequences that mediate interactions with α5β1, αvβ3, and αvβ5 among others. While the affinity for an RGD sequence is a common theme for provisional matrix binding integrins, there are exceptions to this trend such as the related integrins α4β1 and α9β1. The α4β1/α9β1 integrin subfamily interacts with both components of the provisional matrix (e.g., fibronectin CS-1 and EDA domains, tenascin-C, osteopontin) and vascular ligands involved in leukocyte homing (VCAM-1).

3.3. Integrin Signaling. As cells contact the ECM, the integrin extracellular domains bind to their ligands anchoring the cell to the matrix and altering the integrin cytoplasmic domain structure (Figure 3(b)). Structural proteins such as talin and vinculin serve as bridges between the integrin cytoplasmic tail and the actin cytoskeleton [88]. Although the integrin cytoplasmic domain lacks intrinsic enzymatic activity, the structural alteration assumed following integrin ligation stimulates interactions with intracellular signaling proteins [79, 89]. Integrin cytoplasmic domains differ considerably between individual integrin subunits allowing for integrin-specific signaling responses, although some motifs are common [79, 90]. Ligated integrins recruit several nonreceptor tyrosine kinases, including focal adhesion kinase (FAK), integrin-linked kinase (ILK), and Src-family kinases, among others [79, 90]. Integrin adhesion regulates signaling through the Rho family of small GTPases including Rac, cdc42, and Rho resulting in cytoskeletal changes associated with cell migration [91]. In addition, integrin signaling activates multiple signaling pathways that affect gene expression patterns, such as the MAP kinases (ERK, JNK, and p38) and the transcription factors c-fos, c-jun, and NF-κB [79].

4. Cell Matrix Interactions in Lymphangiogenesis

4.1. Extracellular Matrix of the Tumor Stroma and Lymphangiogenesis. Although abundant in the tumor stroma, collagen's role in tumor lymphangiogenesis remains unclear. A recently identified protein termed collagen and calcium-binding EGF domain-1 (CCBE1) is essential for developmental lymphangiogenesis in both zebrafish and mouse

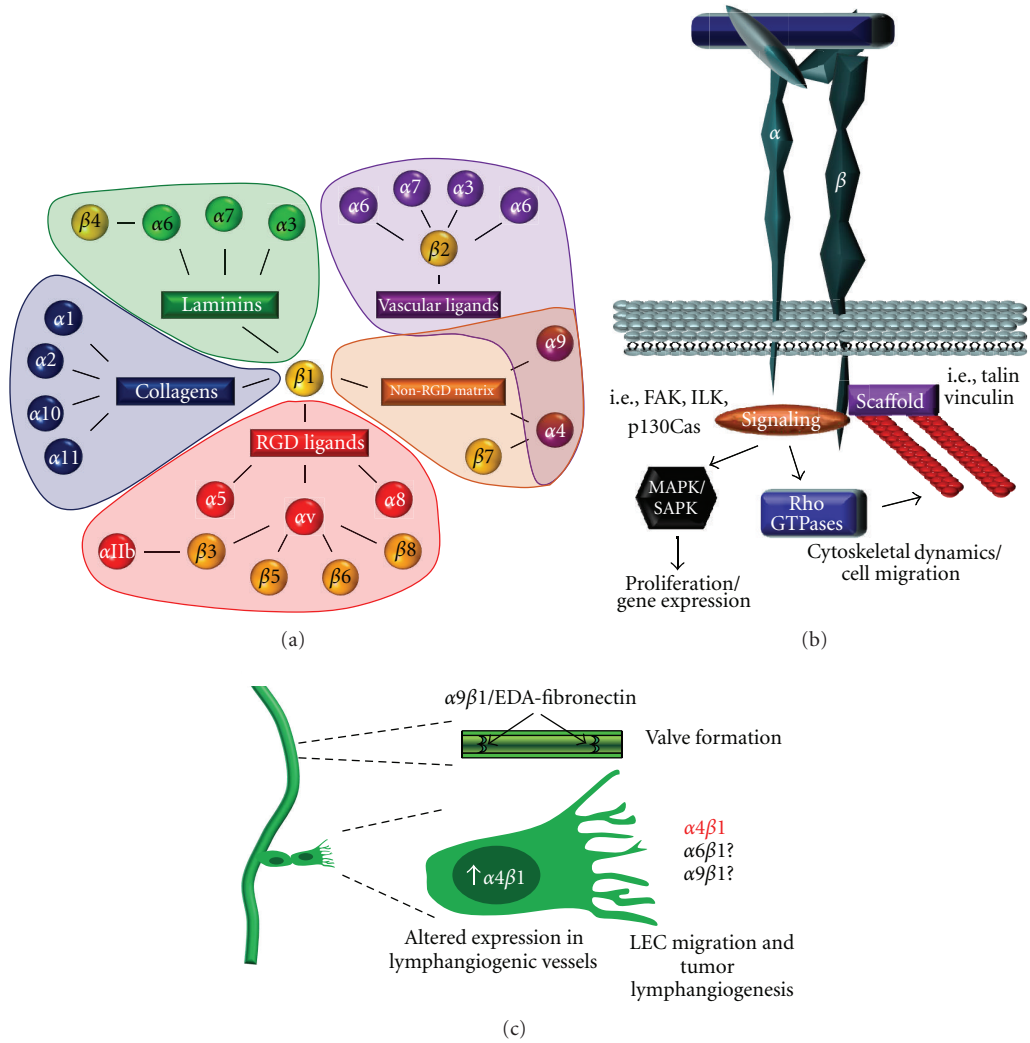

Figure 3: The integrin family of matrix receptors in lymphangiogenesis. (a) Integrin subunits divided by their binding partners (connecting lines) and ligand-binding preferences (shaded areas). (b) Structure of integrin adhesions. Integrins link the extracellular matrix to the intracellular actin cytoskeleton through structural adaptor proteins. Recruitment of signaling proteins activates pathways that regulate gene expression (e.g., MAP kinases) and cytoskeletal reorganization (Rho GTPases). (c) Expression of the integrin $\alpha 9\beta 1$ and its EDA-fibronectin ligand are required for proper lymphatic valve development. While $\alpha 6\beta 1$ and $\alpha 9\beta 1$ are implicated in tumor angiogenesis, only $\alpha 4\beta 1$ has been shown to be upregulated in lymphangiogenic vessels, to mediate LEC migration and tube formation in culture, and to be required for tumor-associated lymphangiogenesis.

models [92, 93]. Although little is currently known about CCBE1, it appears to bind to collagen and vitronectin in the extracellular matrix, and a lack of CCBE1 expression prevents the budding of new lymphatic endothelial cells from the cardinal vein [93]. However, collagen's role in mediating the lymphangiogenic effects of CCBE1 has yet to be explored.

While multiple provisional matrix and matricellular proteins are implicated in tumor lymphangiogenesis, the most convincing data for matrix-dependent lymphangiogenesis involves the provisional matrix protein fibronectin. The fibronectin gene can undergo alternative splicing to include three additional sites: the connecting segment-1 (CS-1), extra domain A (EDA), and extra domain B (EDB) [94]. Fibronectin in the tumor stroma often contains the CS-1 and EDA domains [95, 96]. Blocking antibodies against the EDA site reduce LEC expression of Prox1 and F-actin, key regulators of lymphangiogenesis [95]. In contrast to the CS-1 and EDA domains, the EDB site in fibronectin has not yet been implicated in the lymphangiogenic process. In addition to fibronectin, tenascin-C and osteopontin expression in the tumor stroma is associated with enhanced lymph node metastasis [97, 98], and LECs upregulate tenascin-C expression during lymphangiogenesis [99]. Taken together, these data show that the ECM composition in the tumor stroma is a critical regulator of both lymphangiogenesis and lymph node metastasis.

4.2. Interactions with Anchoring Filaments in Lymphatic Development. Lymphatic capillary endothelial cells share some similarities with vascular endothelial cells with the exception

Table 1: Integrins in lymphangiogenesis.

LEC integrins	Matrix ligands	Data implicating integrin in lymphangiogenesis	Current inhibitors FDA approved or in clinical trials
$\alpha1\beta1$, $\alpha2\beta1$	Collagens	Overexpressed in LEC treated with VEGF-A [13]; blocking antibodies reduce lymphangiogenesis in wound healing [13]; corneal inflammation models [14]	None
$\alpha5\beta1$	Fibronectin	Expressed in sprouting LECs [15]; small molecule inhibitors reduce lymphangiogenesis in cornea [15]; lung inflammation models [16]	Volociximab PF-04605412 JSM6427 [17–19]
$\alpha v\beta3$, $\alpha v\beta5$	Fibronectin (RGD), Osteopontin, Vitronectin, Fibrinogen Fibrillin	Minimal expression in LECs [20]; no role in lymphangiogenesis described to date [20, 21]	Cilengitide, CNTO95 EMD525797 IMGN388 [17–19]
$\alpha9\beta1$	Fibronectin (EDA), Osteopontin, Tenascin-C, VEGF-A/C/D	Knockout mice die postnatally due to defective lymphatic valve development (lung chylothorax) [22–24]; binds directly to VEGF-A/C/D and blocking antibodies inhibit LEC migration [25, 26]; endostatin ($\alpha5\beta1$ and $\alpha9\beta1$ inhibitor) blocks lymphangiogenesis in cancer models [27, 28]	None
$\alpha4\beta1$	Fibronectin (CS1), Osteopontin, Emillin-1	Not required for developmental lymphangiogenesis [29]; expressed in tumor-associated lymphangiogenic vessels and in proliferating LECs [20, 29]; blocking antibodies prevent VEGF-C-induced LEC migration [20, 29]; knockout and dominant negatives block tumor lymphangiogenesis [29]	Natalizumab Vedolizumab ELND002 [17–19]
$\alpha6\beta1$	Laminin, Netrin-4	Mediates LEC adhesion and migration to prolymphangiogenic factor Netrin-4 [30, 31]; colocalizes with netrin-4 in lymphangiogenic vessels associated with breast tumor xenografts [30]	None

of the absence of a continuous basement membrane and surrounding pericytes. Instead of adhering to the basement membrane, capillary LECs are attached to anchoring filaments composed of fibrillin and emilin-1 which anchor the lymphatic capillaries to the surrounding collagen filaments in the interstitial matrix (Figure 1(c)) [100, 101]. This allows for coupling of interstitial fluid pressure changes to the LEC cytoskeleton such that increased interstitial pressure increases permeability of the lymphatic capillaries to enhance drainage of interstitial fluid. Lymphatic vessels express multiple integrins including $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, $\alpha6\beta1$, $\alpha v\beta3$, and $\alpha9\beta1$ (Table 1) [13, 20, 30, 42, 102–104], and LECs appear to utilize multiple integrins to interact with these anchoring filaments. Fibrillin stimulates LEC adhesion through the RGD-binding integrins $\alpha5\beta1$ and $\alpha v\beta3$ [105]. However, the importance of $\alpha5\beta1$ and $\alpha v\beta3$ to lymphatic endothelium is questionable, since mice deficient for both $\alpha5$ and αv integrin subunits in endothelial cells show no apparent developmental defects in lymphangiogenesis or lymphatic function [21]. Mutations in fibrillin genes are associated with Marfan's syndrome, and fibrillin knockout mice recapitulate this phenotype [106]. However, no lymphatic phenotype has been described to date associated with either Marfan's syndrome or fibrillin knockout mice. In contrast, mice deficient for the anchoring filament protein emilin-1 show reduced numbers of anchoring filaments [107], as well as hyperplastic and dysfunctional lymphatic vessels. The integrin $\alpha4\beta1$, classically associated with leukocyte homing to regions of inflammation, is the only known receptor for emilin-1 to date [108]. However, again, no defects in developmental lymphangiogenesis were described in mice lacking endothelial $\alpha4$ integrins or expressing a dominant negative form of $\alpha4$ (Y991A) deficient in talin and paxillin binding [29].

4.3. Integrin $\alpha9\beta1$ in Lymphatic Development. In contrast to $\alpha4\beta1$, $\alpha5\beta1$, and $\alpha v\beta3$ integrins, the expression of $\alpha9\beta1$ integrins in LECs is crucial to the process of developmental lymphangiogenesis. The lymphatic network arises by the initial segregation of a discrete endothelial cell population from the cardinal vein [4]. This early transition from venous endothelium to lymphatic endothelium is driven by the homeobox transcription factor Prox1. Prox1 is required for sprouting and migration of LECs toward lymphatic growth factors, for example, VEGF-C and -D [109]. In mouse embryos, Prox1 drives the expression of VEGFR3 and $\alpha9$ integrin in the newly forming LECs [110]. Mice deficient for $\alpha9$ integrin die postnatally due to lung chylothorax, an accumulation of lymph in the pleural cavity [22, 23]. Interestingly, a missense mutation in the human

$\alpha 9$ integrin gene is associated with congenital chylothorax in human fetuses [24]. The extracellular matrix ligand for $\alpha 9\beta 1$ during lymphatic development remains unclear, since multiple matrix proteins can interact with $\alpha 9\beta 1$, including tenascin-C, the EDA domain of fibronectin, and osteopontin (Figure 3(c)). However, fibronectin appears to be the dominant ligand for $\alpha 9\beta 1$-dependent lymphatic valve formation. EDA-positive fibronectin deposition occurs early during lymphatic valve formation in an $\alpha 9\beta 1$ integrin-dependent manner [22]. Mice deficient in either $\alpha 9\beta 1$ knockout or EDA-positive fibronectin show similar defects in lymphatic valve formation [22, 111]. Taken together, these data illustrate a major role for $\alpha 9\beta 1$ integrin and its matrix ligand EDA-positive fibronectin in lymphatic development.

4.4. Integrins in Inflammatory Lymphangiogenesis. Multiple integrins have been implicated in pathological lymphangiogenesis. However, these studies are often limited to a single model system providing little insight into their relevance to tumor lymphangiogenesis. For example, VEGF-A stimulates expression of the collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in LECs [13], and blockade of these integrins using antibodies prevents lymphangiogenesis in both wound healing and corneal inflammation models [13, 14]. However, the role of these integrins in tumor lymphangiogenesis has not yet been addressed. The provisional matrix binding integrins $\alpha 5\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ mediate tumor angiogenesis, and inhibitors to these integrins are currently being tested in clinical trials [17–19]. However, the role these integrins play in tumor lymphangiogenesis is less clear. The fibronectin-binding integrin $\alpha 5\beta 1$ shows enhanced expression in lymphatic sprouts [15], and fibronectin can induce LEC proliferation in culture [103]. Blocking the $\alpha 5\beta 1$ integrin with small molecule inhibitors JSM6427 and JSM8757 significantly blunts lymphangiogenesis in corneal inflammation and airway inflammation models [15, 16]. Despite these findings, $\alpha 5\beta 1$ does not appear to be involved in tumor lymphangiogenesis [20]. Furthermore, $\alpha v\beta 3$ and $\alpha v\beta 5$ show only minimal expression in LECs and do not appear to be involved in lymphangiogenic responses [20].

4.5. $\alpha 9\beta 1$ and $\alpha 4\beta 1$ Integrins Mediate Tumor Lymphangiogenesis. Because $\alpha 9\beta 1$ integrin has an established role in developmental lymphangiogenesis, it likely also participates in tumor angiogenesis as well, and several lines of evidence support this. VEGF-C and VEGF-D are key mediators of tumor lymphangiogenesis and $\alpha 9\beta 1$ binds to the EYP sequence in VEGF-A, C, and D to promote endothelial and tumor cell migration [25, 26]. Consistent with this, $\alpha 9\beta 1$ blocking antibodies were shown to suppress VEGF-C-induced chemotaxis in LECs [26]. The angiogenic suppressor endostatin also reduces lymphangiogenesis in colorectal and skin squamous cell carcinomas [27, 28] and inhibits lymph node metastasis [27]. Interestingly, endostatin was recently shown to block interactions between $\alpha 9\beta 1$ and the EDA domain of fibronectin [28]. However, endostatin can also inhibit fibronectin interactions with $\alpha 5\beta 1$ [112], suggesting that endostatin's effects may not be solely mediated by $\alpha 9\beta 1$. As such, no studies to date have definitively proven that $\alpha 9\beta 1$ plays a functional role in tumor lymphangiogenesis *in vivo*.

While quiescent lymphatic endothelial cells weakly express $\alpha 4\beta 1$, lymphatic vessels associated with variety of human and murine tumors show enhanced $\alpha 4\beta 1$ expression. The lymphangiogenic/angiogenic growth factors VEGF-A, VEGF-C, and bFGF all induce $\alpha 4\beta 1$ expression in lymphangiogenic vessels, whereas proliferating LECs *in vitro* show high levels of $\alpha 4\beta 1$ expression [20, 29]. Both $\alpha 4\beta 1$-blocking antibodies and recombinant soluble VCAM-1 suppress lymphangiogenesis induced in VEGF-A or VEGF-C infused matrigel plugs and lead to elevated LEC apoptosis [29]. Blocking antibodies to $\alpha 4\beta 1$, but not $\alpha 5\beta 1$, $\alpha v\beta 3$, or $\alpha v\beta 5$, suppressed VEGF-C-induced LEC migration, matrigel invasion, and tube formation (Figure 3(c)). Endothelial-specific $\alpha 4$ integrin knockout mice showed significantly reduced lymphangiogenesis in VEGF-C infused matrigel plugs. Mutating the Y991A in the $\alpha 4$-cytoplasmic tail disrupts paxillin binding and inhibits leukocyte homing [113]. LECs isolated from $\alpha 4$ Y991A transgenic mice show reduced migration to VEGF-C, and VEGF-C-induced lymphangiogenesis was reduced in $\alpha 4$ Y991A transgenic mice [29]. Treatment with $\alpha 4\beta 1$ blocking antibodies reduced lymphangiogenesis and lymph node metastasis in implanted Lewis lung carcinoma and B16 melanoma tumors. However, bone marrow transplant experiments using wild-type and $\alpha 4$ Y991A knock-in mice suggested that $\alpha 4\beta 1$ inhibition in either recipient or donor cells reduces lymphangiogenesis. Therefore, $\alpha 4\beta 1$ inhibitors may interfere with lymphangiogenesis by both inhibiting LEC migration and preventing homing of proangiogenic leukocytes [29].

Recent evidence suggests the laminin-binding integrin $\alpha 6\beta 1$ may play a role in tumor lymphangiogenesis as well. The netrin family of axonal guidance molecules are secreted laminin-like proteins implicated in angiogenesis and tumor metastasis [114]. Lymphatic vessels associated with breast tumors express netrin-4, and LECs show enhanced proliferation, migration, and tube formation in response to netrin-4 (Figure 3(c)) [31]. Overexpression of netrin-4 increases LVD in mouse skin, and breast cancer xenografts overexpressing netrin-4 show enhanced LVD and metastasis [31]. Netrin-4 binding to $\alpha 6\beta 1$ cooperatively enhances binding between $\alpha 6\beta 1$ and laminin, suggesting netrin-4 directly modulates $\alpha 6\beta 1$ activation [30]. Inhibition of $\alpha 6\beta 1$ blocks LEC migration on netrin-4, and $\alpha 6\beta 1$ colocalizes with netrin-4 in lymphatic vessels during embryogenesis, in adult intestine, and in breast tumor xenografts [30]. However, a direct causal role of $\alpha 6\beta 1$ signaling in netrin-4-associated lymphangiogenesis has yet to be determined.

5. Clinical Perspective: Targeting Lymphangiogenesis with Integrin Inhibitors

Several integrin inhibitors have made their way into the clinic, and a new wave of integrin inhibitors are advancing through clinical trials (Table 1). Current integrin inhibitors

fall into three categories: therapeutic antibodies, ligand-mimetic peptides, and small molecule antagonists [17–19]. To date, the only FDA-approved integrin inhibitors have targeted the integrin $\alpha 4$ (natalizumab) and platelet integrin $\alpha IIb\beta 3$ (abciximab, eptifibatide, tirofiban) [18]. Most of the inhibitors currently in clinical trials target the RGD-binding integrins $\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha IIb\beta 3$ which do not appear to significantly modulate lymphatic function [21]. The $\alpha v\beta 3/\alpha v\beta 5$ inhibitor cilengitide is the agent closest to approval, with Phase III clinical trials for glioblastoma ongoing. There are no current clinical trials specifically testing the efficacy of integrin inhibitors in lymphangiogenesis and lymph node metastasis. Still, lymphangiogenesis itself is only specifically targeted by a handful of trials, and these tend to focus on the role of growth factor signaling in lymphangiogenesis. While $\alpha 9$ is closely associated with lymphatic development and LEC migration, the best data for integrin involvement in lymphangiogenesis involves the integrin $\alpha 4\beta 1$ [20, 29], and an inhibitor of the $\alpha 4$-integrin natalizumab (Tysabri) has been approved for the treatment of chronic inflammatory diseases such as multiple sclerosis and Crohn's disease since 2004 [115]. While this approval was quickly recalled following multiple cases of progressive multifocal leukoencephalopathy in a subset of patients, the benefits of natalizumab for multiple sclerosis patients were found to outweigh the potential risks and the drug was again approved for use in the USA in 2006 [116].

Targeting the $\alpha 4\beta 1$ and $\alpha 9\beta 1$ integrins for therapeutic reduction in lymphangiogenesis would be predicted to reduce inflammation in the tumor, as both $\alpha 4\beta 1$ and $\alpha 9\beta 1$ are known to regulate leukocyte homing responses. However, the benefits of this potential off target effect are unclear since inflammation plays a complex role in tumor formation, progression, and metastasis [117, 118]. The tumor stroma contains both tumor-associated macrophages and lymphocytes. Tumor-associated macrophages, generally alternatively activated M2 macrophages, produce a variety of cytokines and growth factors that promote tumor growth and reduce apoptosis [119]. Additionally, tumor-associated macrophages promote tumor metastasis by enhancing ECM degradation in the tumor stroma, promoting angiogenesis, and stimulating endothelial adhesion molecule expression to allow extravasation [117, 120]. As such, the presence of a high number of tumor-associated macrophages is associated with poor prognosis in multiple cancers [121]. Therefore, integrin inhibitors that could restrict both inflammation and lymphangiogenesis/lymph node metastasis may prove beneficial. Consistent with this, integrin $\alpha 4\beta 1$ antagonists suppress macrophage colonization of tumors and subsequent tumor angiogenesis [122]. Alternatively, adaptive immunity plays a well-accepted role in immunosurveillance that is thought to limit the growth of some tumors, including many of those prone to lymph node metastasis such as colon cancer and melanoma [118, 123]. In this case, inhibiting $\alpha 4\beta 1$ and $\alpha 9\beta 1$ might be expected to propagate tumor formation. Therefore, differences in tumor type and stage, immunogenicity, and tendency for lymph node metastasis are likely to influence when and how $\alpha 4\beta 1$ and $\alpha 9\beta 1$ integrin inhibitors can be used in cancer therapy.

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Review Article

Cancer Cell Adhesion and Metastasis: Selectins, Integrins, and the Inhibitory Potential of Heparins

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Cell adhesion molecules play a significant role in cancer progression and metastasis. Cell-cell interactions of cancer cells with endothelium determine the metastatic spread. In addition, direct tumor cell interactions with platelets, leukocytes, and soluble components significantly contribute to cancer cell adhesion, extravasation, and the establishment of metastatic lesions. Clinical evidence indicates that heparin, commonly used for treatment of thromboembolic events in cancer patients, is beneficial for their survival. Preclinical studies confirm that heparin possesses antimetastatic activities that lead to attenuation of metastasis in various animal models. Heparin contains several biological activities that may affect several steps in metastatic cascade. Here we focus on the role of cellular adhesion receptors in the metastatic cascade and discuss evidence for heparin as an inhibitor of cell adhesion. While P- and L-selectin facilitation of cellular contacts during hematogenous metastasis is being accepted as a potential target of heparin, here we propose that heparin may also interfere with integrin activity and thereby affect cancer progression. This review summarizes recent findings about potential mechanisms of tumor cell interactions in the vasculature and antimetastatic activities of heparin.

1. Introduction

Metastasis is facilitated by cell-cell interactions between tumor cells and the endothelium in distant tissues. Tumor cells in circulation interact also with platelets and leukocytes that further contribute to tumor cell adhesion, extravasation, and the establishment of metastatic lesions. Hematogenous cancer metastasis is a multistep cascade encompassing process, starting with local invasion of tumor cells at primary tumors, survival in systemic circulation, extravasation in secondary sites, and ending with establishment of growing metastatic lesions. The metastatic capacity of tumor cells correlates with their ability to exit from the blood circulation, to colonize distant organs, and to grow in distant organs [1]. The organ-specific character of metastasis has been already observed by S. Paget more than a century ago, and the "seed and soil" hypothesis postulates specific interactions of tumor cells with the "friendly" environment of distant organs that enables the establishment of metastasis and subsequent growth [2]. While cell-cell interactions facilitating tumor cell adhesion in the vasculature of specific organs are essential steps in the metastatic cascade, inhibition of these interactions represent a therapeutically useful target for attenuation of metastasis (Figure 1). Two major cell adhesion molecule families, selectins and integrins, have been identified to participate in metastasis [3–6]. In addition to direct targeting of selectin and integrins, recent evidence suggests that heparin possesses selectin (P- and L-selectin) and also integrin inhibitory activity [7–9]. Heparin and low-molecular-weight (LMW) heparin are commonly used anticoagulants for treatment of cancer-associated thromboembolism in cancer patients [10, 11]. The exact mechanism of cell-cell interactions involved in cancer progression remains to be fully elucidated; however, the potential of heparins to interfere with this process warrants further investigations.

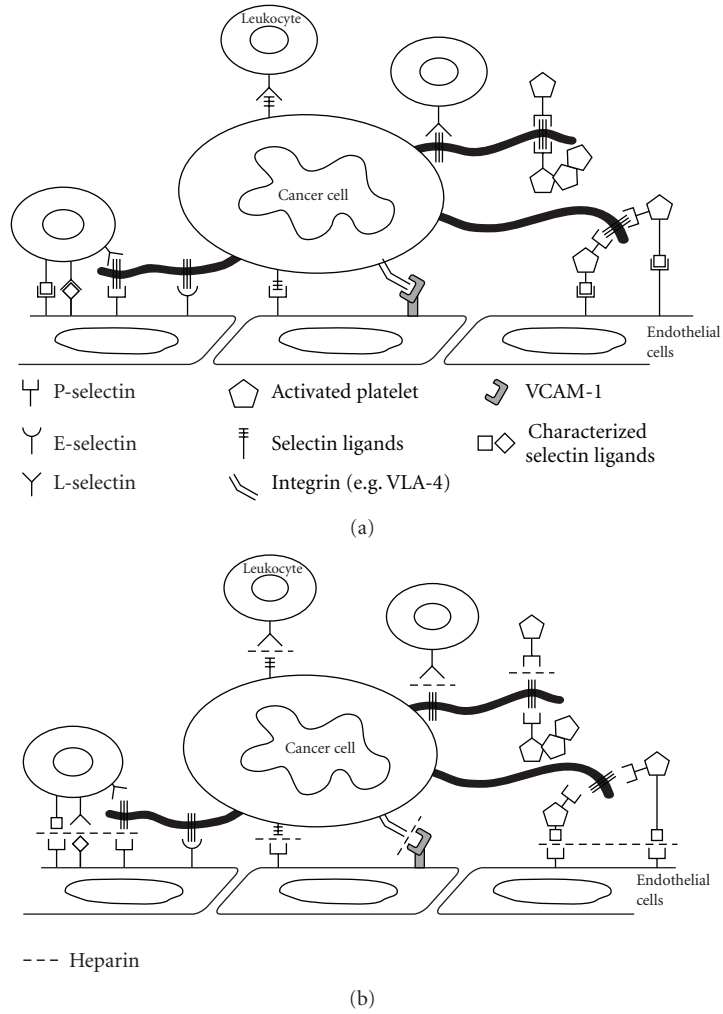

Figure 1: Selectins and integrins contribute to metastatic spread. (a) Schematic presentation of selectin- and integrin-mediated cancer cell interactions with several blood constituents (e.g., platelets, leukocytes, and endothelial cells) during hematogenous metastasis. (b) Heparin application in mouse models blocks both P- and L-selectin-mediated; and VLA4-mediated interactions of cancer cells within blood circulation thereby affecting metastasis.

2. Cell Adhesion as a Determinant of Metastasis

Within blood vessels, circulating tumor cells ultimately interact with the endothelium that might lead to tumor cell arrest and extravasation (Figure 1). Studies of tumor cell-endothelial contact formations have been based on parallels to the leukocyte-endothelial cell interactions during inflammation. Although the mechanism of tumor cell adhesion certainly differs from leukocyte recruitment to inflammatory sites, the cell adhesion molecules involved in the contact formation with endothelium are potentially the same [3, 5, 6]. Indeed, there is accumulating evidence for the role of selectins and integrins in cancer progression of various cancer types, including colon and lung carcinomas and melanomas [5, 6]. While selectin-mediated tumor cells arrest and adhesion contribute to metastasis, integrin-mediated interaction from both tumor cells and the surrounding environment further contribute to cancer progression.

3. Selectins

Selectins are vascular cell adhesion molecules involved in adhesive interactions of leukocytes and platelets and endothelium within the blood circulation. There are three members of the selectin family: P-, E-, and L-selectin. P-selectin is present in the storage granules of platelets (α-granules) and endothelial cells (Weibel-Palade bodies), thus enabling rapid translocation on cell surfaces upon activation [12]. On the contrary, endothelial expression of E-selectin requires *de novo* transcription, leading to expression on activated endothelial cell surfaces several hours after stimulation [12]. L-selectin is constitutively expressed on cell surfaces of almost all leukocyte subpopulations. The physiological functions of selectins are well described in processes of inflammation, immune response, wound repair, and hemostasis [13]. The role of selectins in these processes has been elucidated in mouse deficient in individual selectins.

Whereas L-selectin mediates fast rolling of leukocytes on endothelium, P- and E-selectins support rolling at lower velocities [13]. The initial steps in leukocyte tethering and rolling on endothelium are supported by rapid and reversible interactions of selectins with their carbohydrate ligands.

The majority of selectin ligands consist of distinct glycan structures carrying the terminal core tetrasaccharide structure sialyl Lewis$^{x/a}$ (sLe^x/sLe^a) on a protein backbone [14, 15]. Selectins binds to various classes of molecules (mucins, sulfated glycolipids, glycosaminoglycans), and most of these molecules were shown to be functional selectin ligands *in vivo* [12]. Efficient selectin binding to carbohydrates usually requires a proper glycoprotein scaffold that presents several selectin ligands in clusters, thereby increasing the avidity of the interaction.

3.1. Selectins as Facilitators of Metastasis. Hematogenous metastasis is the common route for cancer spread of epithelial cancers—carcinomas. In the normal physiological state, epithelial cells line the lumen of hollow organs and are covered by mucins that are either cell surface attached or building soluble layers covering the epithelium. Mucins are high-molecular-weight molecules with a large proportion of O-linked glycans attached to a protein backbone. During malignant transformation cell surface glycans undergo dramatic changes [16]. The major alterations of glycans on tumor cells are associated with enhanced expression of sLe^x or its isomer sLe^a, Tn and sialyl-Tn antigen structures [16, 17]. Enhanced expression of sLe^x and sLe^a structures is frequently associated with progression and poor prognosis in various cancers including colon, gastric, lung, renal, and breast cancers, melanomas, and others (reviewed in [18]). Several laboratories have shown that at least one selectin (P-, L-, or E-) is capable to bind to any human carcinoma tested (e.g., [19, 20]), which emphasize the potential of selectins to mediate contacts with tumor cells within vasculature. The fact that, during the hematogenous phase of metastasis, selectin ligand-carrying tumor cells may encounter selectins, present on blood constituents (leukocytes, platelet, and endothelium) in the circulation, supported the notion of selectin involvement in metastatic progression [3, 5]. This hypothesis has been evaluated by several laboratories, and recent findings using mouse models deficient in one or more selectins confirmed the involvement of P-, L-, and also E-selectin in metastasis [7, 21–24].

3.2. P-Selectin and Metastasis. The rapid expression of P-selectin on cell surfaces of endothelial cells and platelets upon activation makes P-selectin a likely candidate involved in the metastatic process. There is accumulating evidence that formation of platelet-tumor cell thrombi helps evading host responses, thereby contributing to metastasis [7, 25–27]. In the absence of platelet-tumor cell interactions, tumor cells are cleared by NK cells [26, 28]. Minimal platelet-tumor cell microthrombi has been detected in the absence of P-selectin, leading to reduced tumor cell adhesion in the lungs of mice and subsequently attenuation of metastasis [7, 22, 29]. Furthermore, removal of cell surface mucins from tumor cells prior to intravenous injection resulted in reduction of metastasis [7]. Bone marrow reconstitution experiments in P-selectin-deficient mice have shown that endothelial P-selectin, in addition to platelet P-selectin, contributes to metastasis [29].

Patients with metastatic carcinoma cancer are at high risk for thromboembolic events, a finding initially described by Trousseau [30]. The association between mucinous carcinoma and Trousseau's syndrome led to the hypothesis that mucin might directly induce thrombi formation [31]. Intravenous injection of purified carcinoma mucin led to thrombi formation that was dependent both on the presence of P- and L-selectin. Recently, the molecular mechanism of mucin-induced initiation of tumor cell-platelet complexes has been described [32]. Microthrombi formation induced by carcinoma mucins was found to be dependent on L-selectin and PSGL-1 expression on neutrophils that induced cathepsin G release thereby triggering platelet activation and P-selectin expression.

3.3. L-Selectin and Metastasis. Participation of leukocytes in platelet-tumor cell emboli is well described, yet the role of leukocytes in the process of metastatic initiation remains under investigation. The establishment of a metastatic niche is based on the initial recruitment of bone marrow-derived cells to distant sites in organs where metastatic cells tend to seed [33, 34]. In general, contribution of leukocytes to metastasis largely depends on spatial and temporal situation that is defined by the microenvironment and tumor cells [34–36]. Whether L-selectin mediates leukocyte recruitment to metastatic sites has been tested in L-selectin-deficient mice [21, 37]. The absence of L-selectin led to significant attenuation of metastasis, indicating that L-selectin actively contributes to leukocyte recruitment and formation of a metastatic niche [5, 38]. Reduced numbers of CD11b-positive leukocytes has been detected at early time points after tumor cell injection that correlated with reduced tumor cell survival in the lungs [37]. Recent evidence indicates that selectin-mediated interactions through cooption of inflammatory pathways contribute to formation of a permissive microenvironment for metastasis [38]. Tumor cell-mediated activation of the adjacent endothelium upon vascular arrest resulted in NF-κB activation and expression of E-selectin, thereby contributing to metastasis.

3.4. E-Selectin and Metastasis. E-selectin has been investigated as a mediator of metastasis at sites where arrest of tumor cells in the microvasculature has been observed [39, 40]. E-selectin expressed on activated endothelial cells has been detected during metastatic colonization of the liver [39, 41]. Inhibition of E-selectin or downregulation of E-selectin expression resulted in attenuation of experimental liver metastasis. In contrast, transgenic overexpression of E-selectin in the liver redirected metastasis to this organ, thereby confirming the role of E-selectin in this process. Interestingly, experimental metastasis of human colon carcinoma cells lines was not affected by the absence of E-selectin [24]. However, spontaneous breast metastasis to the lung was

reduced in E-selectin-deficient mice, indicating E-selectin involvement during lung metastasis [42]. Organ-specific differences may contribute to the colonization process and different requirements for selectin-mediated interactions may be dependent on the primary tumor and the metastatic organ.

While selectins were identified as potential facilitators of metastasis, they have not been explored as pharmacological targets for treating cancer progression.

4. Biology of Integrins

Integrins are large and complex transmembrane glycoproteins. The structure of integrins consists of two distinct chains, α- and β-subunit, which form a non-covalent heterodimer [43, 44]. In mammals, 18 α- and 8 β-integrins have been characterized that combine to form 24 unique canonical α/β receptors identified so far. Integrins mediate cell adhesion and directly bind components of the extracellular matrix (ECM), such as fibronectin, vitronectin, laminin, or collagen, thereby providing anchorage for cell motility and invasion. Since specific integrins can bind to different ligands and identical ligand can be shared by different integrins, this redundancy underscores the general importance of integrins in cell communication.

Integrins are mediators of a bidirectional signaling where intracellular signals induce alterations in the conformation, thus ligand-binding properties (inside—out signaling). Since integrins are linked to cytoskeletal structures (e.g., α-actinin, talin, and vinculin) ligation of extracellular ligands can influence intracellular processes (outside—in signaling) through activation of kinases, GTPases of the Ras/Rho signaling pathways [44, 45]. The convergence between the cytoskeleton and ECM components is mediated via a cluster formation of integrins and their downstream signaling molecules, focal adhesion kinase (FAK) or Src family kinases, which affect the cellular shape and migratory properties of cells [46]. In addition to the well-established role of integrins during migration and invasion, integrins also regulate cell proliferation, survival and angiogenesis, all processes actively investigated during cancer progression.

5. Integrins during Cancer Metastasis

The ubiquitous presence of integrins on tumor cells, blood components, vasculature, and stromal cells suggest that integrins might essentially contribute to different steps in the metastatic cascade. As many human tumors originate from epithelial cells, integrins expressed on epithelial cells are generally present also in tumor cells. Studies correlating integrin expression levels with the pathological outcomes, such as metastasis or patient survival, have identified several integrins that might be involved in cancer progression [6]. Tumor cell expression of $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 5\beta 1$, $\alpha 6\beta 4$ correlated with metastatic progression in melanoma, breast carcinoma, prostate and pancreatic and lung cancer [6].

During hematogenous phase of metastasis tumor cell platelet interaction are mediated either by P-selectin (see above) or through platelet integrin αIIb$\beta 3$. Inhibition of αIIb$\beta 3$ integrin or P-selectin by function-blocking antibodies significantly reduced platelet-tumor cell interaction and tumor cell adhesion on activated endothelium [47–49]. Accordingly, attenuation of metastasis has been observed.

Tumor cell expression of $\alpha v\beta 3$ integrin together with its capacity to bind several ECM components, including fibronectin, vitronectin, and osteopontin, has been regarded as critical factor for affecting the site of metastasis. In this respect, fibrinogen was identified as a bridging factor between αIIb$\beta 3$ integrins on platelet and $\alpha v\beta 3$ on tumor cells [50, 51]. This interaction facilitates tumor cell arrest in the vasculature and metastasis to various tissues, including bone marrow and lungs.

The contribution of integrins to angiogenesis and thereby tumor progression and metastasis has recently been reviewed [52]. Tumor-associated vessels express $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins that were not detected in quiescent vasculature [53]. The binding of these vascular integrins to ECM components in the tumor microenvironment contributes to invasion and migration of endothelial cells. Therefore, targeting of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins with antibodies or peptide- (RGD-) derived structures has been investigated as a promising antiangiogenic approach.

The establishment of metastatic niche is dependent on the recruitment of bone marrow-derived cells [33, 34]. Homing of circulating progenitor cells to tumors was shown to require $\alpha 4\beta 1$ integrins [54]. Expression of integrin $\alpha 4\beta 1$ (VLA-4) on bone marrow-derived cells mediates binding to VCAM and cellular fibronectin, which are present at sites of endothelial remodeling.

Initial studies on the role of integrins during metastasis, specifically investigating primary endothelial contacts, were focused on melanoma metastasis [55–57]. Metastatic dissemination of murine melanoma B16F10 cells has been blocked by peptide displaying the RGD integrin-binding sequence of fibronectin [55]. The targeted integrin involved in the vascular arrest of melanoma cells has later been identified to be VLA-4 [56, 57]. Experimental pulmonary metastasis of melanoma cell lines, B16 and A 375 M, was confirmed to be mediated by VLA-4. The specific VLA-4-mediated interaction with VCAM-1 on endothelium is required for melanoma cell adhesion and endothelial transmigration [58]. Recently, VLA-4 mediated melanoma adhesion to VCAM-1 on activated endothelium was shown to support extravasation under the shear flow also in the absence of selectin ligands [59], indicating the potential of VLA-4 to serve as an adhesion molecule during metastatic spread of cancer.

Besides the role of VLA-4 in melanoma metastasis, $\beta 1$ integrins strongly contribute to metastasis of other tumor types, for example, lymphomas [60]. Silencing of $\beta 1$ integrins in the highly metastatic Esb murine T-lymphoma cell line, and thus the loss of VLA-4 and VLA-6 binding, strongly reduced the metastatic dissemination to the lungs and spleen. Furthermore, a change in metastatic pattern with prevalence for skeletal muscle invasion has been observed [60]. To investigate the role of $\beta 1$ integrins during tumor growth and metastasis, a fibroblastoid cell line with disrupted $\beta 1$ integrin

gene has been generated [61]. Overexpression of β1 integrin significantly correlated with metastatic spread of these cells to the lung and liver, when compared with parental cells with disrupted β1 integrin gene [61]. Taken together, integrins are becoming attractive therapeutic targets for therapeutic strategies focused not only on tumor development but also on metastasis.

Since heparins effectively block both P-, L-selectins and VLA-4 integrin-mediated tumor cell adhesion, heparin and heparin derivatives have been tested in a number of animal models for their potential to attenuate metastasis (reviewed in [9]).

6. Heparin and Cancer: Clinical Evidence

Heparin is commonly used for the prevention or treatment of venous thromboembolism in cancer patients. In addition to its antithrombotic activity, cancer patients treated with heparins showed an improved survival in a number of retrospective and prospective studies (reviewed in [62, 63]). A recent review on antithrombotic therapy using heparins concluded that, despite the heterogeneity of completed clinical studies, heparin treatment of cancer patients with better prognosis is beneficial for patients primarily due to prolonged survival [10]. Based on this conclusion, together with observations from animal models, heparin appears to directly affect cancer progression associated with metastatic spread.

7. Heparin Attenuates Metastasis through Inhibition of Selectins

Heparin is a complex mixture of natural glycosaminoglycans based on repeating disaccharide units containing glucosamine and glucuronic/iduronic acid residue with a high degree of sulfation [64]. Heparin and LMWH were tested in many different animal models for their potential to inhibit cancer progression primarily using experimental metastasis models (reviewed in [65, 66]). Despite large variation in heparin preparations, doses applied, time of application, and different tumor models, attenuation of metastasis has been observed almost in all independent studies when heparin was applied around the time of tumor cell injection. Together with the limited effect on tumor growth [67, 68], these findings indicate that heparin affects processes when tumor cells are still in the blood circulation. Because of the very nature of heparin, several other biological activities in addition to the anticoagulant activity have been detected such as binding to cell adhesion molecules (P- and L-selectin, VLA-4 integrin), enzymes (heparanase), growth factors, and cytokines [69–73]. Chemically modified heparins were prepared and tested for various biological activities [71]. Modified heparins containing mostly P-selectin inhibitory activity were shown to efficiently attenuate metastasis almost to the same levels as observed in P-selectin-deficient mice [71]. Since heparin injection in P-selectin-deficient mice resulted in no further attenuation of metastasis, these findings indicated that heparin affects metastasis by inhibition of P-selectin [7, 29, 71]. Interestingly, a single dose of heparin prior to tumor cell injection further attenuated metastasis in L-selectin-deficient mice, indicating that L-selectin involvement in this process is subsequent to P-selectin [21]. Further evidence for sequential involvement of P- and L-selectin in metastasis was confirmed by observation that L-selectin contributes to metastasis first several hours after tumor cell injection [37]. Heparin injection 6–12 hours after the tumor cell challenge further reduced metastasis in P-selectin-deficient mice confirming the potential of heparin to inhibit also L-selectin-mediated interactions. Taken together, these findings indicate that inhibition of P- and L-selectin attenuates metastasis and heparins appear to be efficient inhibitors of selectin mediated interactions *in vivo*.

8. Heparin Binding and Inhibition of Integrin Functions

Heparin as a potential inhibitor of integrin-mediated cell-cell interaction has been evaluated only in few studies. The αMβ2 (Mac-1) integrin on hematopoietic progenitor cells was shown to mediate adhesion to stromal compartment through binding to heparin and heparan sulfate [74, 75]. Another study described the leukocyte integrin αXβ2 to bind sulfated heparin in a low micromolar range [76]. These studies suggest that heparin can interfere with leukocyte binding and recruitment to the endothelium.

Zhang et al. reported that platelet integrin αIIbβ3 can efficiently be blocked by heparin and non-anticoagulant heparin derivatives [77]. Platelet interaction with melanoma cells A375 or B16F10 was strongly reduced *in vitro* and metastasis *in vivo*. These findings indicate that heparin inhibition of αIIbβ3 integrins is additional to P-selectin inhibition of platelet binding (as mentioned above) with relevance for those tumors cells with low expression levels of P-selectin ligands.

Heparin inhibition of integrin-mediated melanoma adhesion to endothelium has been reported only recently [73]. Expression of integrin α4β1 (VLA-4) by B16F10 melanoma cells mediated their adhesion to endothelial cells through binding to VCAM-1. Heparin was shown to inhibit the VLA-4 mediated melanoma binding to VCAM-1 substrates under dynamic conditions. A follow-up study using human melanoma MV3 cells confirmed heparin binding to VLA-4 with binding affinities in the low micromolar range [78]. Structural analysis of heparin indicated a size dependency of integrin binding, since short heparin fragment or the pentasaccharide (Fondaparinux) was not able to bind VLA-4 [8]. Further analysis revealed that also the sulfation density is critical for VLA-4 recognition [79].

Altogether, these studies indicate that heparin can affect important steps in the metastatic cascade by inhibition of integrins. The relevance of this contribution depends on tumor cell types carrying integrin and/or selectin ligands. Melanoma cells with their high expression levels of VLA-4 appear especially relevant for this consideration. However, the efficiency to inhibit metastasis *in vivo* remains to be analyzed.

9. Tumor Cell Seeding and the Establishment of Metastatic Niche

Beside direct heparin inhibition of adhesion receptor functions, heparin can affect the activities of cellular proteoglycans related to cell adhesion. This possibility will shortly be introduced below with respect to potential antimetastatic approaches or novel targets.

Chemokines are chemotactic cytokines that induce direct migration of leukocytes to sites of inflammation or cancer progression [80]. Beside the well-described role of chemokines during inflammation, seeding of tumor cells to distant tissues was shown to be facilitated by chemokines.

There is compelling evidences that chemokine receptors, for example, CXCR4, mediate breast cancer metastasis [81]. Breast cancer cells expressing CXCR4 in circulation effectively enter the bone marrow niche due to enhanced expression of CXCL12 in this environment [82]. Targeted metastasis to the bone marrow or other sites with high expression of CXCL12 has been described in a number of cancers including breast, colon, and prostate [80]. Chemokines bind to glycosaminoglycans chains of proteoglycans presented on surfaces of epithelial and vascular endothelial cells or on extracellular matrix molecules. Cell migration is dependent on chemokine gradient presented by chemokines at specific sites [83]. Recently, syndecan-1 and syndecan-4 proteoglycans were shown to be required for chemokine- (CCL5-) induced hematoma migration and invasion [84]. Similarly, CCL2-induced human hepatoma cell migration and invasion has been blocked by anti-syndecan-1 and -4 antibodies, but also when hepatoma cells were pretreated with heparitinases that remove glycosaminoglycans from cell surfaces [85]. Interestingly, a recent study reported on the ability of LMW heparin to bind SDF-1 (CXCL-12) in a sub-micromolar range [86]. Since proper chemokine presentation by endogenous proteoglycans is a prerequisite for successful metastasis, heparin treatment might "remove" chemokine as a decoy and thereby reduce tumor cell adhesion and recruitment to the metastatic sites. Chemokine binding to its chemokine receptor could directly activate integrin binding function by inducing the conformational change of the integrins [44]. Whether heparin binding to chemokines indeed attenuates metastasis remains to be explored.

Fibronectin, an ECM protein, possesses specific and partly overlapping binding sites for the integrin VLA-4, VLA-5, and heparin [87]. The anchorage of cells to heparin-binding domains of fibronectin or other ECM components is mainly linked to syndecans. Several studies point to the critical role of the syndecan extracellular domains in tumor cell adhesion and invasion behavior where syndecan-4 acts as a "coreceptor" with integrin VLA-5, but not VLA-4 [43, 88]. Syndecan-4 binds to the extracellular matrix and is also connected to the actin cytoskeleton via interaction with structural and signaling proteins such as FAK, syndesmos, and paxillin. This represents both a mechanical and signaling link to cell surface integrin VLA-5 required for focal adhesion and stress fiber formation in cells adherent to fibronectin [89, 90]. Thus, the potential heparin antimetastatic activity can be based on interference either directly with the integrin-fibronectin binding or indirectly via competing for the proteoglycan binding of syndecan-4 to fibronectin. However, a direct interference of heparin with this pathway in cell migration remains to be defined.

Integrins were also shown to bind cystein-rich protein 61—Cyr61 [91]. Cyr61 was first identified as a growth factor-inducible immediate early gene and belongs to the CCN family of matricellular proteins (CCN1). Elevated levels of Cyr61 have been correlated with increased breast adenocarcinoma, endometrial tumors, pancreatic cancer, or glioma malignancy [92–95]. Cyr61 has been reported to mediate numerous cellular processes, such as cell adhesion, cell survival, proliferation, enhancement of growth factor-induced DNA synthesis, and angiogenesis [96]. These effects result, at least in part, through a direct binding of Cyr61 to the extracellular regions of the integrin $\alpha v\beta 3$ thus activating these adhesion and signaling receptors [97]. However, Cyr61 has also a binding ability to $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha M\beta 2$ [91]. Cyr61 overexpression in gastric cancer cell line AGS was shown to increase peritoneal dissemination through increased $\alpha 2\beta 1$ integrin activity [92]. In contrast, Cyr61 silencing in PC-3 and DU-145 prostate cancer cells strongly inhibited proliferation [98]. Though, Cyr61 also significantly enhanced TRAIL-induced apoptosis through interaction with integrins $\alpha v\beta 3$ and $\alpha 6\beta 4$ [98]. Therefore, inhibition of Cyr61 activity appears as a promising therapeutic approach to inhibit tumor cell growth, migration, and adhesion. The Cyr61 molecule has two discrete heparin binding sites which contribute to binding to cell surface on heparan sulfate proteoglycans on Syndecan-4 [91]. Consequently, heparin could indirectly influence integrin functions by depleting released Cyr61. Although, previous heparin treatment in a number of animal models might have affected also Cyr61 activity, further analysis is required to elucidate the relevance of this signaling pathway for antiadhesive approaches of heparin applications.

10. Conclusions

Accumulating evidence from several preclinical models confirms that tumor cell interactions through selectins and integrins actively contribute to the metastatic spread of tumor cells. However, the current cancer therapies are focused only on targeting of tumor cells while no specific therapy for inhibition of metastatic spread is available. Clinical findings suggest that heparin and LMW heparin possess anticancer activities leading to survival benefits for cancer patients in the early stage of the disease. Although the identification of underlying molecular mechanisms is still ongoing, several preclinical studies confirmed the dominant contribution of selectins to metastasis and their role as primary targets of heparin.

In addition, integrins were shown to contribute to cancer progression. Since heparin binds to particular integrins implicated in metastasis, targeting integrins opens additional ways for interference with metastatic progression in addition to inhibition of angiogenesis, cell migration, or establishment of the premetastatic niche.

The elucidation of the orchestrated functions of selectin and integrins and possibly other adhesion molecules during metastatic cascade requires further studies. Clearly further clarification of heparin interactions with selectins and integrins is required, yet the abundant clinical experience with heparin and LMW heparin proposes its evaluation as a potential antimetastatic treatment.

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Review Article

Turnover of Focal Adhesions and Cancer Cell Migration

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Cells are usually surrounded by the extracellular matrix (ECM), and adhesion of the cells to the ECM is a key step in their migration through tissues. Integrins are important receptors for the ECM and form structures called focal adhesions (FAs). Formation and disassembly of FAs are regulated dynamically during cell migration. Adhesion to the ECM has been studied mainly using cells cultured on an ECM-coated substratum, where the rate of cell migration is determined by the turnover of FAs. However, the molecular events underlying the disassembly of FAs are less well understood. We have recently identified both a new regulator of this disassembly process and its interaction partners. Here, we summarize our understanding of FA disassembly by focusing on the proteins implicated in this process.

1. Introduction

Adhesion of cells to the ECM is key to the regulation of cellular morphology, migration, proliferation, survival, and differentiation [1, 2]. These functions are indispensable during development and for maintenance of tissue architecture and the induction of tissue repair. Integrins are the predominant receptors that mediate cell adhesion to components of the ECM [3–8]. Integrins are expressed on the cell surface as heterodimers comprised of noncovalently associated α- and β-subunits. Both subunits are type I transmembrane proteins containing both a large extracellular domain responsible for binding to ECM ligands and a cytoplasmic portion (CP) that recruits multiple intracellular proteins. Eighteen different α- and 8 β-subunits have been characterized in mammals, and 24 distinct integrin heterodimers have so far been identified [5, 9, 10]. Each integrin recognizes a distinct ECM ligand. As such, the repertoire of integrins expressed on the surface of a particular cell acts as a sensor of the ECM environment [5].

Attachment of cells to ECM components induces clustering of integrins on the cell surface. The cytoplasmic portions of the clustered integrins then act as a platform for the recruitment of cellular proteins such as adaptor/scaffold and signaling proteins to the inner surface of the plasma membrane, where they form structures called focal adhesions (FAs) (Figure 1) [11–13]. The adaptor/scaffold proteins in FAs, such as talin, paxillin, tensin, p130Cas, and α-actinin, provide strong linkages to the actin cytoskeleton and, thereby, connect cells firmly to the ECM [14–18]. This linkage enables the generation of the tension necessary to alter cell morphology and the traction force necessary to move the cell body during migration. In addition, multiple signaling proteins, including kinases or phosphatases, are also recruited to FAs where they transmit ECM-derived signals to cellular pathways controlling proliferation, survival and migration [19–23]. In particular, two well-characterized tyrosine kinases, focal adhesion kinase (FAK) and Src, play central roles in integrin-mediated signaling cascades [20, 24, 25]. Since integrins have no intrinsic enzymatic activity, these tyrosine kinases transmit signals from FAs to the cellular machinery by phosphorylating multiple integrin-associated proteins [25–30]. Thus, both FAK and Src act as molecular switches that trigger a variety of cellular responses via FA complexes. There are many excellent reviews discussing how integrin-mediated signals regulate cellular behavior [20, 25, 31, 32].

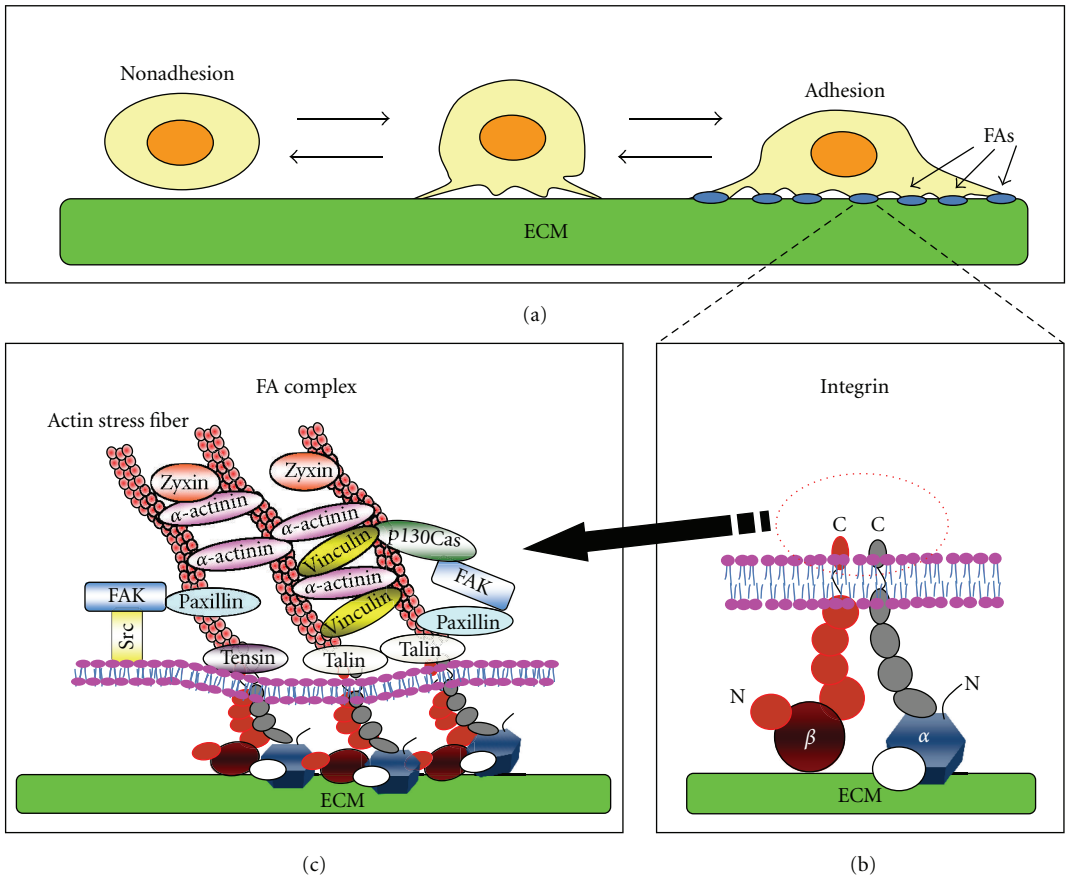

Figure 1: Integrin-mediated cell adhesion to the ECM. (a) Suspended cells adhere to the surface of ECM via integrins. Some of the nascent adhesion contacts grow and form mature focal adhesions (FAs). (b) Integrins function as a heterodimer composed of α- and β-chains. (c) The cytoplasmic portions of integrins recruit multiple cellular proteins and form cross-linked platforms to regulate both the actin cytoskeleton and signal transduction.

The process of cell adhesion to the ECM has been studied by seeding cells onto an ECM-coated substratum in culture [33, 34]. These analyses contributed to the elucidation of the process of the initial attachment of cells to the ECM and the formation of integrin-mediated cell adhesion structures. However, cells must also detach from the ECM during migration, and the mechanism and regulation of the disassembly of cell adhesion structures is less well studied. In contrast to most review articles discussing cell adhesion, we focus here on our understanding of the turnover of FA complexes during cell migration.

2. Adhesion of Cells through the Formation of Focal Adhesion Structures

Cells adhere to the ECM via integrins and form FA complexes as discussed elsewhere [7]. Numerous proteins are involved in integrin-mediated cell adhesion, and these proteins are collectively referred to as the adhesome [35–38]. Among the latter, talin is a key regulator of the initial step of FA assembly [39–41]. Talin contains two unique domain structures, the head and rod domains [42–45]. The head domain mediates binding to the CP of the β-subunit of integrin, whereas the rod domain contains multiple binding sites for adhesome proteins, including one for the CP of β-integrin, two sites for actin, and multiple sites for vinculin. In addition, talin forms a dimer through its carboxy-terminal helix and thus serves as a core platform to expand intracellular structural frameworks mediated by protein-protein interactions. The binding of talin to integrin stabilizes the ligand-induced clustering of the latter at an initial step of FA formation by mediating crosslinking of integrins with filamentous actin (F-actin) and F-actin-binding proteins such as vinculin and α-actinin (Figure 3(a)) [14, 46–48]. This initial structure, called the nascent FA, is immature and often short lived [6]. However, some of the nascent FAs grow and form mature FAs that require actin-based tension regulated by the Rho small GTPase and its effector ROCK [6].

3. Regulation of Focal Adhesion Complexes during Cell Migration

Stimulation of the formation of FA complexes enhances the adhesion of cells to the ECM, giving rise to cells with a spread morphology (Figure 2(i)). In contrast, destabilization of FAs reduces adhesion to the ECM and gives rise to spherical

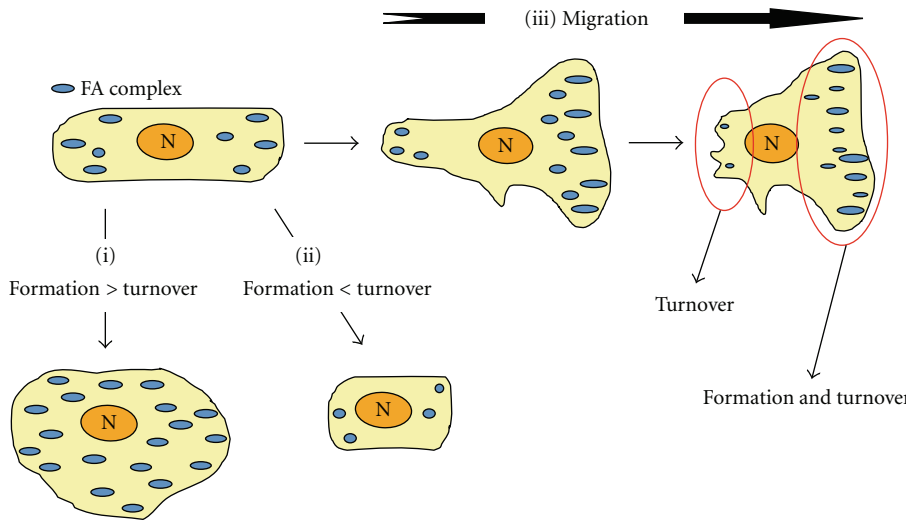

Figure 2: The formation and turnover of FAs during cell migration. The formation and turnover of FAs is crucial for cell adhesion to the ECM. A higher ratio of formation relative to turnover leads to stable adhesion (i). On the other hand, a higher ratio of turnover relative to formation leads to unstable adhesion (ii). During cell migration, both rapid formation and turnover of FAs are required at the leading edge of cell migration, whereas turnover of FAs is predominant at the rear (iii).

nonadherent cells (Figure 2(ii)). During cell migration on a substratum, FAs grasp the ECM so as to generate the forces necessary to pull the cell body forward. Subsequently, cells must release from the ECM, so as to continue cell movement. As such, directional migration of the cell requires continuous, coordinated formation and turnover of FAs at the leading edge of the cell body and release of this attachment at the rear (Figure 2(iii)) [49, 50]. Clustering of integrins is the initial step of cell adhesion and is stabilized to form FAs by linking to actin stress fibers in a process regulated by Rho/ROCK [6, 51, 52]. By contrast, extension of microtubules to FAs triggers their disassembly and induces the subsequent internalization of integrins from the cell surface [53–56]. Therefore, the assembly and disassembly of FAs are regulated by different mechanisms. Although the fate of the internalized integrins has not yet been established, several studies have reported the transport of internalized integrins from the rear to the leading edge of the cell body via intracellular vesicle trafficking [57–59]. This recycling of integrins may contribute to directional cell migration.

4. Factors Involved in the Disassembly of FAs

The molecular events leading to FA disassembly are not yet well understood although some fragmentary knowledge has recently accumulated [54, 60, 61]. Most importantly, it has been established that microtubules (MTs) play a crucial role in inducing FA disassembly [54]. MTs extend to FAs and trigger the disassembly process. During the final stage, the internalization of integrins is mediated by dynamin, a GTPase that regulates endocytosis, and FAK is involved in the recruitment of dynamin into FAs (summarized in Figure 3(b)).

In the following sections, we summarize the proteins involved in disassembly and link their involvement in this process so as to generate a more coordinated model of disassembly based on recent findings. Various disassembly factors and their domain structures are schematically illustrated in Figure 4.

4.1. Microtubules. The importance of MTs for FA disassembly has been demonstrated using nocodazole, which disrupts polymerized MTs in cells adherent to the ECM [54, 56]. Exposure of cells to nocodazole stabilizes FA structures by preventing their disassembly and thereby enhances adhesion of cells to the ECM. The removal of the drug from the culture media initiates disassembly of FAs in a synchronous manner and recovery of MT structures [53, 54, 62]. Thus, the use of this drug allows us to analyze the FA disassembly process independently from FA formation. Tyrosine phosphorylation of proteins within FAs increases following exposure to nocodazole and decreases rapidly after its removal. Extension of MTs to FAs has been observed by live imaging microscopy, and targeting of MTs to FAs appears to trigger FA disassembly [54]. Since the MT motor protein, kinesin-1, has been implicated in regulating MT-induced FA disassembly [55], MTs may deliver disassembly factors to FAs in a kinesin-1-dependent fashion.

As extension of MTs to FAs triggers release of cell adhesion and promotes cell migration, it is of interest how targeting of MTs to FAs is regulated during the induction of cell motility. Indeed, Rho family GTPases regulates the capture and stabilization of extended MTs to the cell cortex via their downstream effectors, and MTs in turn have been shown to affect the activity of Rho GTPases [63]. Although it is not precisely clear how MTs target FAs, actin filaments presumably play a role.

4.2. Kinesin-1. Kinesin-1 is a member of the kinesin superfamily of motor proteins and is also known as conventional

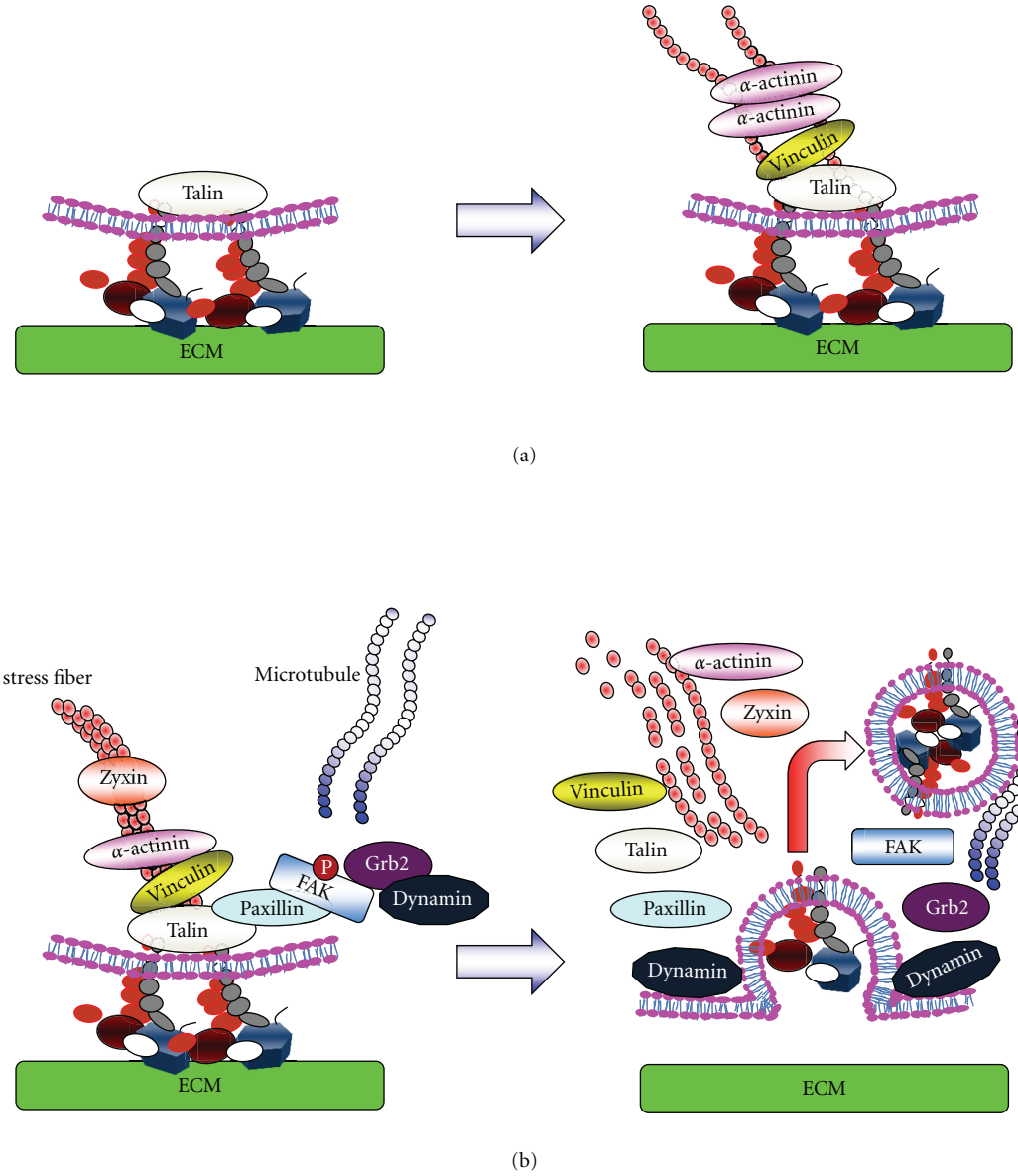

Figure 3: Formation and turnover of FAs. (a) The process of the formation of FAs. Attachment of cells to the ECM induces clustering of integrins at the attachment sites. Clustered integrins recruit cytoplasmic adaptor proteins such as talin to the cytoplasmic portion of the integrins. Actin-binding proteins such as vinculin and α-actinin then bind to talin and connect the ECM structure to the cytoskeleton via integrin. (b) The process of FA turnover. FAK phosphorylated at Tyr^{397} plays a role in recruiting the endocytosis regulator dynamin into FAs via interaction with the adaptor protein Grb2. The extension of MTs initiates the internalization of integrins in a dynamin-dependent manner. During the process of integrin endocytosis, rapid dephosphorylation of FAK at Tyr^{397} is observed.

kinesin [64, 65]. Kinesin-1 plays a crucial role in protein trafficking along polymerized MTs to the direction of plus end of latter. The inhibition of kinesin-1 in *Xenopus* fibroblasts, using either a specific antibody or forced expression of a dominant-negative mutant, leads to stabilization of FAs accompanied by an increase in their size and a reduction in their number, as was seen in cells exposed to nocodazole [55]. These findings suggest that kinesin-1 activity is necessary for the turnover of FAs. Although nocodazole inhibits the polymerization of MTs, inhibition of kinesin-1 activity affects neither the targeting of MTs to FAs nor the polymerization dynamics of MTs [55]. This suggests that FA disassembly factors are conveyed along MTs in a kinesin-1-dependent manner.

4.3. Focal Adhesion Kinase. FAK is involved in both maturation and turnover of FAs [20, 66]. However, FAK deficiency has a greater effect upon disassembly than upon formation of FAs, giving rise to a reduced rate of FA turnover leading to an increase in the level of steady-state FAs [60, 66]. FAK contains an N-terminal FERM (protein 4.1, ezrin, radixin, and moesin

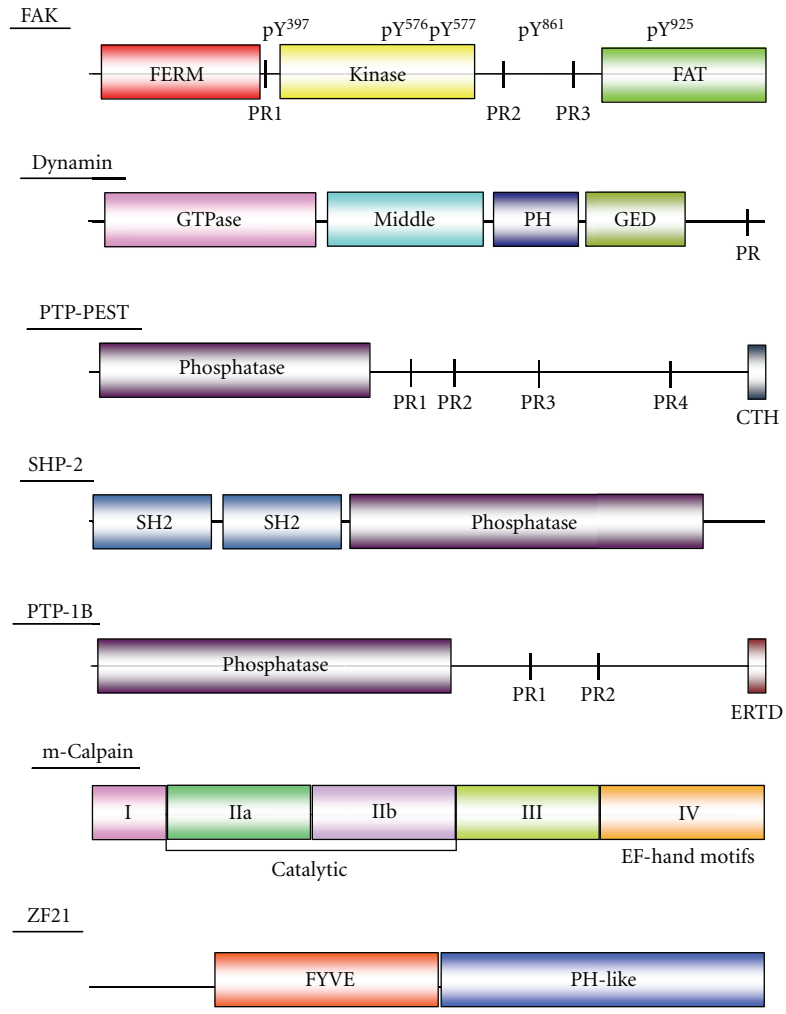

Figure 4: Domain structures of FA disassembly factors. FAK: FERM (protein 4.1, ezrin, radixin, and moesin homology), PR (proline-rich motif), FAT (focal adhesion targeting), pY (phosphorylated tyrosine), Dynamin: PH (pleckstrin homology), GED (GTPase effector domain), PTP-PEST: PR (proline-rich motif), SHP-2: SH2 (src homology 2 domain), PTP-1B: PR (proline-rich motif), ERTD (endoplasmic reticulum-targeting domain), m-Calpain: I (possible autoinhibitory region), IIa and IIb (protease domain), III (putative phospholipid-binding sites), and IV (the region containing 4 EF-hand motifs), ZF21: FYVE (Fab1, YOTB, Vac1, and EEA1), PH-like (pleckstrin homology-like).

homology) domain, a central kinase domain, and a COOH-terminal focal adhesion-targeting (FAT) domain as illustrated in Figure 4. The FERM domain is found in many proteins and mediates protein-protein interactions [67, 68]. The FAK FERM domain has been shown to bind the CP of integrin $\beta 1$ and growth factor receptors [69, 70]. Recent structure analysis of the FERM domain has indicated that it binds the catalytic cleft of the kinase domain [71]. This intramolecular interaction prevents autophosphorylation of Tyr^{397}, which is a prerequisite for the successive phosphorylation of FAK by Src. Autophosphorylation of FAK at Tyr^{397} is elevated in highly motile and invasive cancer cells [72, 73]. Src binds to phosphorylated Tyr^{397} and further phosphorylates multiple tyrosine residues within FAK, including Tyr^{576} and Tyr^{577} within the kinase domain, Tyr^{861} located between the kinase and FAT domain, and Tyr^{925} within the FAT domain [20, 25]. Phosphorylation within the kinase domain is crucial for full kinase activity. Phosphorylated Tyr^{861} mediates the interaction of FAK with talin and paxillin [20, 25]. Phosphorylation at Tyr^{925} is necessary for the interaction of FAK with Grb2 [20, 25]. Binding of Grb2 to FAK helps recruit dynamin to FAs [54]. This ternary complex is responsible for the internalization of integrins and thereby induces turnover of FAs. However, the role of FAK during FA disassembly is not so simple. Whereas $pTyr^{397}$ FAK is required for recruitment of dynamin, its dephosphorylation is induced after extension of MTs to FAs, and this is a prerequisite step for the successive disassembly of FAs [54, 62]. Thus, FAK is a central regulator of the formation and disassembly of FAs, and for the transmission of integrin-mediated signals. Nevertheless, FAK deficiency has little effect upon FA formation but has nevertheless been shown to stabilize FAs. The roles of FAK during FA formation might be performed by other redundant kinases or factors recruited to FAs.

4.4. Dynamin. Dynamin is a GTPase that was identified as an MT-binding protein [74]. Three independent dynamin genes have been identified. Dynamin I is expressed specifically in neurons, and Dynamin III is expressed exclusively in testis, lung, and brain, whereas Dynamin II is expressed ubiquitously [74]. The domain structure common to the dynamins is shown in Figure 4. Dynamin is required for the internalization of integrins during MT-dependent FA turnover [54]. The carboxyl terminus of dynamin contains a proline-rich (PR) motif, which is indispensable for assembly of a ternary complex with FAK and Grb2 [54]. The PR motif of dynamin also interacts with MTs. Dynamins recruited to the inner surface of the cells membrane assemble in a ring around FAs [54] and initiates the internalization of integrins when the FAs are sufficiently disassembled. FAK deficiency markedly reduces the accumulation of dynamin around FAs [54]. Interaction of the tubulin polymer with dynamin markedly increases the GTPase activity of the latter, although the physiological significance of this is unclear [75].

4.5. Phosphatases. A specific set of protein tyrosine phosphatases mediates dephosphorylation of FAK at Tyr^{397} after the extension of MTs to FAs [62]. These include PTP-PEST, SHP-2, and PTP-1B. However, it is not clear whether FA disassembly requires concerted action of all three phosphatases or whether the action of a single phosphatase is sufficient, depending on the cellular context.

PTP-PEST is known to regulate cell adhesion and migration (Figure 4) [76]. As Zheng et al. have reported, PTP-PEST dephosphorylates FAK at Tyr^{397} upon activation by an oncogenic Ras-induced signal [77, 78]. Ras induces the activation of ERK via the Fgd1-Cdc42-PAK1-MEK1 cascade ultimately resulting in interaction between FAK and PTP-PEST. Activated ERK phosphorylates FAK at Ser^{910}, and the phosphorylated Ser^{910} and the adjacent Pro^{911} residue serves as a binding site for peptidyl-prolyl *cis/trans* isomerase (PIN1). PIN1 stimulates the binding of FAK to PTP-PEST, in a fashion dependent upon the isomerase activity of PIN1, although the exact role of the isomerase activity is not clear. PTP-PEST then dephosphorylates $pTyr^{397}$ [79]. Intriguingly, substitution of FAK Tyr^{397} by Phe promotes metastasis of v-H-Ras-transformed rat fibroblasts.

SHP-2 can also dephosphorylate FAK at Tyr^{397} [80]. SHP-2 contains two SH2 domains at its N-terminus (Figure 4), and the N-terminal most of the two acts as an intramolecular inhibitor of the phosphatase activity [81]. This inhibition can be released by Gab2, a pleckstrin homology (PH) domain-containing docking protein. Gab2 binds the N-terminal SH2 domain and exposes the phosphatase domain of SHP-2 by releasing the intramolecular inhibition [81]. Deficiency of SHP-2 in cultured cells increases the number of FAs and impairs cell migration [82]. These findings are reminiscent of the phenotype of FAK-deficient cells. However, there is no clear evidence that SHP-2 localizes to FAs during their turnover. SHP-2 might be recruited to FAs by interacting with the phosphorylated tyrosines of Gab2 via its two SH2 domains.

There are several substrates for PTP-1B in FAs, including FAK, Src, and α-actinin [83, 84]. PTP-1B is a complicated regulator of FAK. It directly mediates dephosphorylation of $pTyr^{397}$ [84] but can also promote phosphorylation of the same tyrosine residue by Src [83]. As Zhang reported, α-actinin plays a key role in the dual functions of PTP-1B [84]. α-Actinin phosphorylated at Tyr^{12} promotes dissociation of Src bound to FAK at $pTyr^{397}$. This allows PTP-1B to dephosphorylate the exposed $pTyr^{397}$. On the other hand, PTP-1B can dephosphorylate α-actinin $pTyr^{12}$ so as to increase the α-actinin-free Src pool that is then available to phosphorylate FAK. At the same time, PTP-1B can activate Src by dephosphorylating Src $pTyr^{527}$, which mediates intramolecular inhibition of Src activity. Overall, dephosphorylation of FAK by PTP-1B enhances subsequent phosphorylation of FAK by Src. These functions of PTP-1B may play roles in the dynamic turnover of FAs during dynamic cell attachment rather than simply by promoting detachment.

4.6. m-Calpain. m-Calpain, also known as Calpain-2, is a member of the calpain family of intracellular calcium-dependent proteases [85]. It comprises five functionally and structurally distinct domains. Domain I is a possible autoinhibitory region, and it is cleaved off by autolysis. Domain II is a catalytic domain composed of two split subdomains (IIa and IIb) linked by a loop called the catalytic cleft. Domain III is a putative regulatory region of the protease activity, and it contains phospholipid-binding sites. Domain IV contains four EF-hand motifs that are necessary for binding calcium.

m-Calpain has been shown to regulate the turnover of FAs by cleaving multiple FA-related proteins such as talin, FAK, and paxillin [61, 86–88]. Talin is a well-established substrate of m-Calpain during the turnover of FAs [61, 89]. m-Calpain cleaves a site between the head and the rod domains and thereby triggers structural breakdown of the FA framework [61]. FAK is also cleaved by m-Calpain between the two C-terminal PR domains [87]. Breakdown of FAs by m-Calpain also requires MTs [90]. Even though the precise role of MTs in the breakdown of FAs by m-Calpain is unclear, ZF21 presumably plays a role as explained in next section [62, 91]. FAK can bind both ERK/MAPK and m-Calpain, and it might be a platform where m-Calpain can be activated by the ERK/MAPK [92]. Cleavage of components of FAs by m-Calpain presumably facilitates internalization of integrins by disrupting interconnected large structure of FAs.

4.7. ZF21. ZF21 contains a FYVE domain, which binds to phosphatidylinositol-3-phosphate that is enriched in the lipid layers of plasma membranes. Although there are 38 FYVE domain-containing proteins in mammals, they do not necessarily have common domain structures or functions [93]. ZF21 initially attracted our attention as a possible interaction partner of the cytoplasmic tail of the membrane type metalloproteinase, MT1-MMP, but it was later determined to be a regulator of FA turnover [62]. ZF21 is expressed almost ubiquitously in various types of adhesive cells. The FYVE domain of ZF21 is located in the middle in the protein, and the C-terminal region of the protein contains a novel protein fold that is similar to the PH domain but is lacking the positively charged amino acids necessary to bind phospholipid [94]. Interestingly, ZF21 binds multiple FA

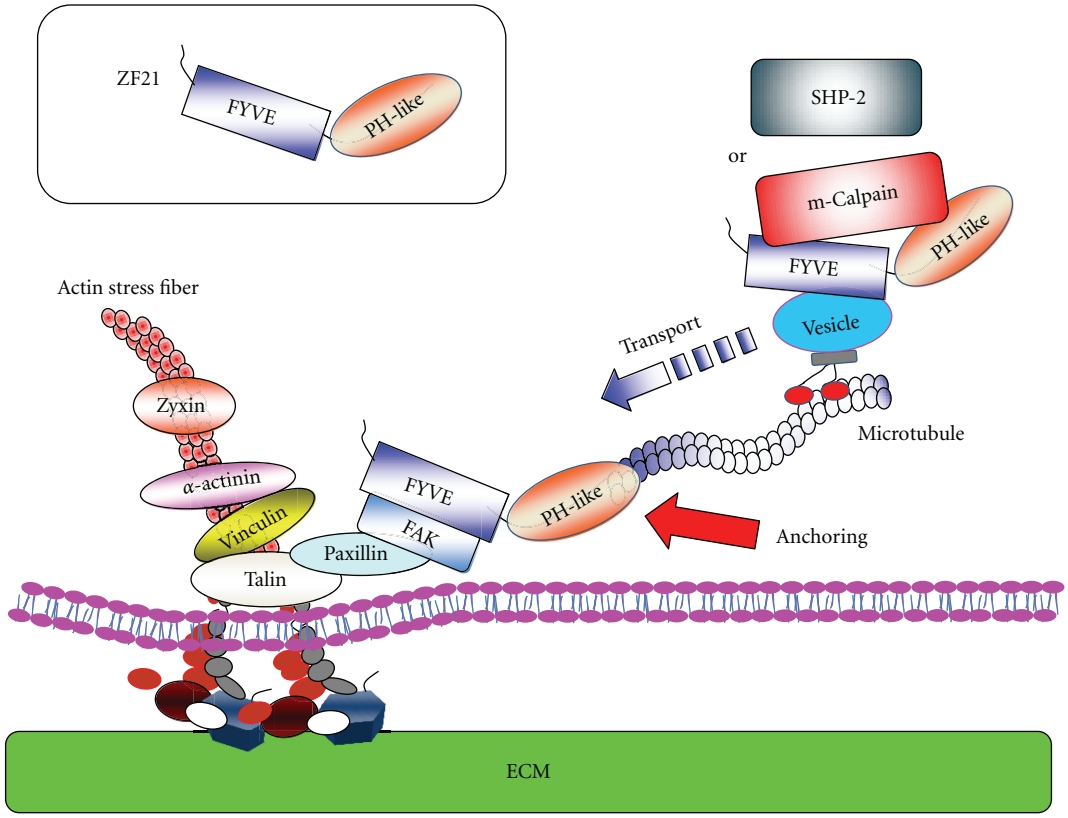

Figure 5: A model for the recruitment of disassembly factors to FAs. In this model, ZF21 conveys FA disassembly factors via intracellular vesicle transport on MTs. ZF21 associates with endosomal vesicles by binding to phosphatidylinositol-(3)-phosphate via its FYVE domain. m-Calpain and SHP-2 can be loaded onto ZF21 carried by the vesicles. ZF21 also can be found in FAs by interacting with FAK, and the ability of ZF21 to bind β-tubulin may act as a docking function for the extended MTs into FAs in order to unload the conveyed factors at the destination.

disassembly proteins, including FAK, β-tubulin, m-Calpain, and SHP-2 [62, 91, 94]. The FYVE domain of ZF21 binds FAK, and the PH-like domain binds β-tubulin. Almost the entire ZF21 polypeptide chain is required for binding m-Calpain and SHP-2. Substitution of the FYVE domain of ZF21 with a corresponding domain derived from EEA1, another member of the FYVE domain-containing proteins, abolishes its ability to bind FAK and abrogates its ability to mediate MT-induced FA disassembly [94].

Knockdown of ZF21 expression in cells prevents MT-induced FA disassembly, as well as disassembly-related events, such as dephosphorylation of FAK at $pTyr^{397}$ and internalization of integrins [62]. Binding of ZF21 to FAK is important for the regulation of FA disassembly because substitution of the FYVE domain with that of EEA1 abolishes both FAK binding and FA disassembly [94]. The PH-like domain is also indispensable for the activity of ZF21 [94]. Taken together, these findings suggest that ZF21 associates with endosomal vesicles moving on MTs via an interaction between the FYVE domain and phosphatidylinositol-3-phosphate within the vesicle membrane. The PH-like domain, which mediates an interaction with β-tubulin, may help stabilize the interaction of ZF21 with MTs and then ride on vesicles. The ability of ZF21 to bind SHP-2 and m-Calpain may facilitate the transport of the latter to FAs via vesicles loaded onto MTs (Figure 5). Upon targeting of MTs to FAs, ZF21 may be transferred to FAs since it can bind FAK, and the ZF21 transferred to the FAs may subsequently anchor the MTs to the FAs. Gab2 in FAs may facilitate the dephosphorylation of FAK by SHP-2 carried in on the MTs. These events are presumably followed by breakdown of FA components by the proteolytic activity of m-Calpain.

Importance of ZF21 for cell migration gave us a clue to understand its role in FA turnover [62]. Knockdown of ZF21 expression by shRNA in cancer cells induced cell spreading on the ECM and suppressed cell migration. Integrin-mediated cell adhesion and migration are important during cancer cell invasion and metastasis although FA-like structures are not obviously recognizable in most cells surrounded by ECM. Indeed, knockdown of ZF21 expression in human mammary carcinoma MDA-MB231 cells suppresses metastatic colony formation in the lung following injection of the cells into the tail vein of mice [94]. However, it is possible that ZF21 regulates metastasis of cancer cells by mechanisms distinct from the regulation of the turnover of FAs.

5. Conclusion

Our understanding of the mechanism of FA turnover remains fragmentary. However, the mechanisms governing the

migration of cells due to regulated adhesion are crucial to the understanding of cancer cell invasion and metastasis. Turnover of FAs is initiated by the extension of MTs to FAs and is completed by the internalization of integrins from the cell surface. Several factors have been implicated in the process of FA disassembly. In particular, the recently identified ZF21 has shed light on this process, owing to its ability to bind multiple proteins involved in FA disassembly. It is of note that FAs are not observed in cells cultured in a collagen lattice, indicating that the presence of integrin-based cell adhesion structures is dependent upon whether the cells are adhering to a rigid surface (2D) or are embedded within a 3-dimensional ECM (3D) [95, 96]. Advanced imaging technologies are powerful tools to elucidate the dynamic roles of FA disassembly factors during cell migration and invasion.

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Review Article

The Role of Immunoglobulin Superfamily Cell Adhesion Molecules in Cancer Metastasis

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Metastasis is a major clinical problem and results in a poor prognosis for most cancers. The metastatic pathway describes the process by which cancer cells give rise to a metastatic lesion in a new tissue or organ. It consists of interconnecting steps all of which must be successfully completed to result in a metastasis. Cell-cell adhesion is a key aspect of many of these steps. Adhesion molecules belonging to the immunoglobulin superfamily (Ig-SF) commonly play a central role in cell-cell adhesion, and a number of these molecules have been associated with cancer progression and a metastatic phenotype. Surprisingly, the contribution of Ig-SF members to metastasis has not received the attention afforded other cell adhesion molecules (CAMs) such as the integrins. Here we examine the steps in the metastatic pathway focusing on how the Ig-SF members, melanoma cell adhesion molecule (MCAM), L1CAM, neural CAM (NCAM), leukocyte CAM (ALCAM), intercellular CAM-1 (ICAM-1) and platelet endothelial CAM-1 (PECAM-1) could play a role. Although much remains to be understood, this review aims to raise the profile of Ig-SF members in metastasis formation and prompt further research that could lead to useful clinical outcomes.

1. Introduction

Cell proliferation, migration, and differentiation are critically important during the development of all organisms, and it is the overall coordination of these activities that leads to the formation of complex structures such as tissues and organs. These cellular processes are modulated by the interaction of cells with each other and with their microenvironment. Cell adhesion molecules (CAMs) facilitate these interactions and are essential during development and for maintaining the integrity of tissue architecture in adults [1, 2]. CAMs include cadherins, integrins, selectins, and the immunoglobulin superfamily (IgSF). In normal tissue, CAM expression is tightly regulated. However, aberrant expression of CAMs disrupts normal cell-cell and cell-matrix interactions, freeing cells from normal check points and constraints, and facilitating tumour formation and metastasis [3]. Although much has been written about the role of integrins and cadherins in cancer metastasis, the IgSF has received less attention. Here we explore the roles of some IgSF members in each step of the metastatic cascade.

2. Immunoglobulin Superfamily

With over 765 members, the IgSF is one of the largest and most diverse families of proteins in the body. Members of the IgSF include major histocompatibility complex class I and II molecules, proteins of the T cell receptor complex, virus receptors, and cell surface glycoproteins [4]. The definitive characteristic of the IgSF members is the presence of one or more immunoglobulin- (Ig-) like domains, which have a characteristic sandwich structure composed of two opposing antiparallel β-pleated sheets, stabilized by a disulphide bridge [5]. Most of the IgSF members are type I transmembrane proteins, which typically consist of an extracellular domain (which contains one or more Ig-like domains), a single transmembrane domain, and a cytoplasmic tail [6]. IgSF members mediate calcium-independent adhesion through their N-terminal Ig-like domains, which commonly bind other Ig-like domains of the same structure on an opposing cell surface (homophilic adhesion) but may also interact with integrins and carbohydrates (heterophilic adhesion) [7]. The C-terminal intracellular domains of IgSF members often

interact with cytoskeletal or adaptor proteins. In this way, the extracellular interactions of IgSF CAMs can lead to signaling within the cell, enabling these proteins to function in a wide range of normal biological processes, as well as pathological events such as tumourigenesis.

3. The IgSF and Metastasis

A number of IgSF members have been identified as biomarkers for cancer progression. For example, MCAM (also called CD146, Mel-Cam, Muc18, and S-Endo1) has been implicated in the progression of melanoma, as well as in breast and prostate cancer [8–10]. Similarly, IgSF members such as L1CAM (CD171), NCAM (CD56), PECAM-1 (CD31), ALCAM (CD166), and ICAM-1 (CD54) have been associated with metastatic progression in a range of cancers including melanoma, glioma, breast, ovarian, endometrial, prostate, and colon cancer [11–15]. In this paper, we will focus on the roles of these six IgSF members in the metastatic cascade (Table 1).

Metastasis is the endpoint of an evolutionary process in which cells acquire the ability to overcome intrinsic (genom ic) and extrinsic (microenvironmental) constraints imposed upon them and hence, are able to escape their preprogrammed behavior [16, 17]. During metastatic spread, tumour cells disseminate to sites distant from the primary tumour, using cell migration mechanisms that are similar, if not identical, to normal physiological processes [18]. The metastatic process consists of five sequential steps: (1) tumor cell proliferation and angiogenesis; (2) local cell invasion; (3) intravasation and dissemination; (4) extravasation; (5) metastatic colonization and proliferation [19]. Tumour cells may also have to withstand immunological attack during any of these stages. IgSF members have been implicated in most, if not all, of these processes.

Table 1: The role of IgSF members in the metastatic cascade.

Stages in metastasis	Involvement of IgSF members	
	Known role	Potential role
(1) Cell proliferation in primary tumour		
(i) Apoptotic evasion	NCAM [24] ALCAM [29]	MCAM [27, 28]
(ii) Angiogenesis	PECAM-1 [30, 31] ICAM-1 [33, 34]	VCAM [32]
(2) Local cell invasion		
(i) Cell-cell interactions	MCAM [8, 35, 36] ALCAM [37] L1CAM [14, 38]	
(ii) Directional cell migration and cell polarity	MCAM [39]	ICAM-1 [40] VCAM-1 [40] PECAM-1 [41] NCAM [27] MCAM [25, 42, 43] L1-CAM [44]
(iii) Matrix degradation	MCAM [35, 36, 45] NCAM [47]	ALCAM [46]
(3) Intravasation and dissemination		MCAM [48] ALCAM [49]
(4) Extravasation		MCAM [8, 35, 36] ALCAM [14, 37] NCAM [50] L1CAM [38, 51, 52] PECAM-1 [53, 54]
(5) Colonization and proliferation	As for (1) and (2)	As for (1) and (2)
(6) Immunological escape		MCAM [8, 36] ALCAM [14, 37] NCAM [50]

4. Cell Proliferation in the Primary Tumour

4.1. Apoptotic Evasion. The first step in metastasis is the transformation of cells from a normal to a cancerous phenotype. This is when cells acquire characteristics that help them to withstand factors that may limit their metastatic spread. These factors include genotypic stress, tissue hypoxia, nutrient depletion, the accumulation of toxic metabolites, haemodynamic shearing, and loss of adhesion [20, 21]. Most cells encountering these factors will undergo apoptosis (preprogrammed cell death) [21, 22]. However, genome expression analysis of metastatic tumours using cDNA microarrays has revealed a strong correlation between tumour progression and the loss of expression of proapoptotic genes, with a concomitant gain in expression of antiapoptotic genes [22]. Thus, the acquisition of apoptotic resistance in cells under stress is the first requirement in tumour progression toward metastasis.

Classically, genotypic stress due to genomic instability through DNA mutation, chromosomal rearrangement, and epigenetic alteration will trigger apoptosis through the tumour suppressor p53 (TS P53) pathway. In many tumour cells, the expression of TS P53 is lost, enabling them to avoid apoptotic death. However, this accounts for only 40% of cells that undergo malignant transformation [23]. Recent reports have indicated that aberrant expression of CAMs such as the IgSF members provides antiapoptotic signals that may account for the other 60% of malignant transformation. For example, Campodónico et al. [24] reported that the functional blockade of NCAM led to susceptibility to apoptosis in murine lung tumour cells and suggested that NCAM expression may be linked to apoptotic resistance in these cells. This resistance seems to be due to activation of the transcription factor, nuclear factor kappa B (NF-κB), whose downstream targets are antiapoptotic genes such as B-cell lymphoma/leukemia-x long (Bcl-x1), X-linked inhibitor of apoptosis protein (XIAP), and cellular inhibitor of apoptosis protein (C-IAP) [12, 25, 26]. MCAM expression by melanoma cells has also been shown to activate NF-κB via the upstream p38 mitogen-activated protein kinase (MAPK) [27]. Inhibition of MCAM using a blocking

monoclonal antibody led to downregulation of p38 MAPK phosphorylation, the suppression of NF-κB activation, and a decrease in tumour growth, possibly due to cell death through apoptosis [28]. ALCAM, another member of the IgSF, may also induce apoptotic resistance in tumour cells. For example, siRNA-mediated silencing of ALCAM on the MCF7 breast cancer cell line led to a decrease in the expression of the antiapoptotic protein B-cell-lymphoma-(BCL-) 2 and increased levels of markers of apoptosis [29].

4.2. Angiogenesis. After acquiring apoptotic resistance, tumour progression is dependent on the initiation of angiogenesis (the formation of new blood vessels from preexisting vasculature). This process is tightly regulated and involves endothelial cell proliferation, differentiation, and migration, in addition to the degradation of the extracellular matrix [16, 55]. These newly formed blood vessels supply nutrients and oxygen essential for tumour growth. The initiation of angiogenesis is triggered by an imbalance between multiple pro- and antiangiogenic molecules and is known as the "angiogenic switch" [56]. Some of the best-characterized proangiogenic molecules are vascular-endothelial-growth-factor-(VEGF-) A and hypoxia-inducible factor-1 alpha (HIF-1α). One of the major characteristics of solid tumours is tissue hypoxia, as the existing blood supply is not sufficient to supply the growing cell mass. Reduced cellular oxygen levels lead to decreased degradation and an accumulation of HIF-1α protein in the nucleus of tumour and stromal cells, which initiates transcription of *VEGF* and increases production of VEGF protein [57, 58]. The VEGF secreted by the tumour cells and stroma then stimulates the expression and modulates the function of IgSF members such as ICAM-1, VCAM-1, and PECAM-1 [40, 41, 59] in endothelial cells. For example, ICAM-1-mediated adhesion of leukocytes to endothelia is a key event in early angiogenesis and is also important in the development of endothelial cell polarity, thus mediating endothelial cell migration [33, 34]. VCAM-1 is believed to perform a similar role to that of ICAM-1 [32] while PECAM-1 regulates both endothelial adhesion and migration by modulating endothelial cell-cell and cell-matrix interactions [30, 31].

5. Local Invasion

5.1. Cell-Cell Interactions. Once tumour growth has reached a critical mass, the metastatic spread of tumour cells is dependent on their dissociation from the primary tumour and migration towards the systemic circulation. Primary tumours with invasive properties usually display reduced intercellular adhesion, which allows cells to break away from the parental cell mass. In tumours arising from epithelial cells, that is, carcinoma, E-cadherin is the major protein involved in cell-cell adhesion. Thus, the loss of E-cadherin expression enables cancer cells to dissociate from the primary tumour and migrate through the extracellular matrix [60, 61]. However, the detachment of cells from a primary tumour is not as simple as the loss of E-cadherin expression. Although some cells migrate as individuals, it has become increasingly clear that cells metastasizing from some solid tumours (e.g., breast and prostate cancer, melanoma, and rhabdomyosarcoma) often migrate together in tightly or loosely associated groups [62]. This suggests that cancer cells retain some cell-cell adhesion, even as they break away from the primary tumour. Other proteins that mediate cell-cell binding include IgSF members such as NCAM, MCAM, ALCAM, and L1CAM. These noncadherin systems are upregulated in cells following the loss of E-cadherin expression and are associated with an active, mobile state that retains enough cell-cell junctions to allow a group of cells to move as a unit [63]. For example, Johnson et al. [8] found an increase in homophilic cell-cell adhesion in melanoma cells transfected with MCAM compared to their MCAM-negative counterparts. Similar reports have indicated that upregulation of ALCAM and L1CAM mediates homophilic cell-cell cohesion in invading melanoma [37] and colorectal carcinoma [14, 38], respectively.

The benefits of collective cell migration include the production of relatively high local concentrations of growth factors, the protection of cells in the centre of a group from immunological attack, and the survival advantage of a mixed population that contains cells able to survive a range of different environmental challenges [18]. In addition, the mechanotransducing force of a migrating cell group exceeds that of a single migrating cell and results in enhanced cell motility.

5.2. Directional Cell Migration. Whether or not cells dissociated from the primary tumour migrate individually or collectively, to metastasize they must acquire the ability to migrate towards vascular or lymph vessels [64]. This migration is due, at least in part, to the interaction between chemokine receptors on cancer cells and chemokine gradients in the surrounding tissue. Although malignant cells from different types of cancers express different chemokine receptor profiles, the chemokine receptor most commonly expressed is CXC chemokine receptor 4 (CXCR4), which binds to the CXC chemokine ligand 12 (CXCL12), also known as stromal cell-derived factor-1 alpha (SDF-1α) [65]. CXCR4 expression is low or absent in many normal tissues but is expressed by at least 23 different types of tumour cells including cancers of epithelial, mesenchymal, and haemopoietic origin [66, 67]. Its ligand, CXCL12, is found in some primary tumour sites and sites of cancer metastasis and is also constitutively expressed by normal organs such as the bone marrow [65]. *In vitro* experiments have shown that the directional migration of a range of cancer cells (e.g., ovarian, pancreatic, rhabdomyosarcoma and melanoma) is stimulated by the interaction between CXCR4 and CXCL12 [65, 66, 68]. Furthermore, downregulation of CXCR4 through RNA interference or functional blockade using monoclonal antibodies showed a decrease in the invasiveness of breast cancer [69] and melanoma [70].

CXCR4 expression can be upregulated in cancer cells via a number of pathways, for example, hypoxia, VEGF, oestrogen, and stimulation of the transcription factor NF-κB pathway [65, 71, 72]. As previously mentioned, VEGF is

also known to stimulate the expression of the IgSF members ICAM-1, VCAM-1, and PECAM-1 [40, 41, 59], so it is possible there may be crosstalk between these molecules and CXCR4. Moreover, Zabouo et al. [2] reported that siRNA-induced downregulation of MCAM was associated with decreased expression of CXCR4 and decreased invasiveness of breast cancer cells. The expression of MCAM and NCAM has also been shown to activate NF-κB in endothelial and myeloid leukemia cell lines, respectively [25, 27].

In addition to a potential role in the regulation of chemokine receptors such as CXCR4, IgSF members themselves may act as an extracellular attractant. Li and Galileo [44] found that soluble L1CAM (sL1), produced by proteolytic cleavage of membrane-bound L1CAM, acted as a chemoattractant for breast cancer cells in transmigration assays and this effect was neutralized using sL1 blocking antibodies.

Lastly, for a nonpolarized and randomly oriented cell to migrate in response to a chemotactic stimulus such as CXCL12, it must display both front-rear polarization and direction sensing [73]. This is a complex process involving a large number of different molecules [73, 74] several of which have been linked with members of the IgSF. For example, melanoma cells exposed to Wnt5a (a cell polarity-associated signaling molecule) in the presence of a chemokine gradient formed an intracellular structure containing actin, myosin, and MCAM. This structure triggered membrane contractility and influenced the direction of cell movement [39]. MCAM has also been implicated in a reciprocal regulatory loop with AKT/PKB (protein kinase b), a molecule that has been associated with increased survival and directional migration in breast cancer cells [42]. In melanoma cells, phosphatidylinositol 3 kinase (PI3K) was found to upregulate MCAM expression via AKT expression and overexpression of MCAM also activated endogenous AKT [43]. It therefore appears that MCAM contributes to directional cell migration via several pathways.

5.3. Matrix Degradation. Although the extracellular matrix (ECM) serves as a niche for tumour cells to survive and proliferate, it is also a barrier to cell migration. Thus, degradation of the ECM is one of the first steps in tumour invasion and metastasis [75]. There are many types of proteases involved in ECM degradation, but the matrix metalloproteinases (MMPs) play a key role in metastasis and are upregulated in almost every type of human cancer [76]. Although more than 20 MMPs have been identified to date, the expression and activity of MMP-2 and MMP-9 are most frequently elevated in cancer and have been correlated with increased metastasis and poor prognosis [75]. MMP expression is regulated by both gene transcription and protein modification and activation.

At the transcriptional level, factors that can increase expression of MMP genes include growth factors, cytokines, hormones and the expression of other tumour promoting molecules such as IgSF members [77]. For example, in melanoma, elevated MMP-2 expression has been associated with increased levels of MCAM and NCAM. Forced expression of MCAM in MCAM-negative melanoma cells led to a significant increase in MMP-2 expression [35], and inhibition of MCAM using blocking antibodies decreased the expression of MMP-2 [36]. The mechanism behind the MCAM-MMP-2 axis was recently described by Zigler et al. [45], who found that MCAM regulated the expression of inhibitor of DNA binding-1 (Id-1), a transcription regulator, and that Id-1 expression controls MMP-2 transcription. In addition, Shi et al. [78] reported that the proinvasive function of NCAM is mediated through stimulation of both cyclic adenosine monophosphate (c-AMP) protein kinase (PKA) and PI3K/AKT pathways, which converge at the transcription factor CREB and increase MMP2 expression. Interestingly, CREB activity also upregulates the expression of MCAM [47], which suggests that MCAM may act as a downstream mediator of NCAM.

MMPs are also extensively regulated posttranslationally, as they are synthesized as preproenzymes and activated by proteolytic cleavage. Activation of most MMPs occurs in the extracellular space by serine proteases (e.g., plasmin and urokinase plasminogen activator) or by cell-surface membrane type (MT) MMPs such as MT1-MMP, a potent activator of pro-MMP2 [77]. It is also known that clustering of cell surface receptors such as β1 and $\alpha v\beta$3 integrins activates MMP2 [46]. Interestingly, recent data suggests that cell-cell contacts may influence the activation status of MMPs, with less confluent cells showing decreased MMP activity. Lunter et al. [46] found that cell-cell contacts, ALCAM and cell-matrix interactions were all critical for MMP2 activation, as cells transfected with truncated ALCAM showed less cell-cell adhesion and decreased MMP-2 activity due to reduced transcript levels and decreased processing of MT1-MMP.

6. Dissemination

The next step in metastasis is the dissemination of tumour cells via the systemic circulation. Intravasation of tumour cells is not well understood, but it is generally believed that tumour cells can pass easily into the irregular, highly permeable blood vessels formed during tumour angiogenesis [79]. Once inside the vasculature, less than 0.1% of these circulating tumour cells (CTCs) are estimated to remain viable after 24 hours and less than 0.01% survive to generate metastases [80]. This may be due to anoikis, the result of fluid shear forces, or immunological attack [79]. Anoikis is an apoptotic process triggered by the loss of cell-matrix interactions and the ability to overcome this is crucial for CTC survival [81]. The loss of cell-matrix attachment disrupts integrin receptors and results in the deactivation of focal adhesion kinase (FAK) and Src family kinases. This leads to the attenuation of prosurvival pathways, the upregulation of proapoptotic proteins, and the initiation of apoptosis [82].

Resistance to anoikis can be conferred by diverse mechanisms, including constitutive activation of FAK, epidermal-growth-factor-receptor- (EGFR-) mediated Src activation, and any disturbance to the apoptotic pathway. Although there is limited evidence that IgSF members confer resistance

to anoikis, it is possible they do—firstly, by their ability to provide antiapoptotic signals (as described above in *Apoptotic Evasion)* and secondly, through activation of FAK. Anfosso et al. [48] found that MCAM recruits the protein tyrosine kinase (PTK) FYN to its cytoplasmic tail, leading to the activation of downstream targets such as FAK. Thus, if tumour cells in the vasculature are present as a group (e.g., via collective migration), it is possible that cell-cell interactions mediated by MCAM may upregulate FAK and protect the cells from anoikis. L1CAM expression in ovarian carcinoma cells has also been linked with sustained phosphorylation of FAK and resistance to apoptosis [49].

7. Extravasation

The presence of CTCs within the vasculature is common in patients with advanced primary tumours, but these cells do not cause metastatic disease and subsequently exit the circulation [83]. One theory proposed to explain how tumour cells became lodged in the vasculature is that of mechanical entrapment where large tumour cells become stuck in the small vessels of capillary beds and then extravasate into surrounding tissue. This theory is supported by data showing that tumour cells that form homotypic aggregates are likely to be easily trapped in small capillaries and tend to exhibit higher metastatic potential than cells that do not form multicellular aggregates [84, 85]. MCAM, ALCAM, NCAM, and L1CAM have all been implicated in the formation of large cell aggregates and have been shown to increase the metastatic capability of tumour cells [8, 14, 36–38, 50].

However, it seems clear that mechanical entrapment is not the only factor influencing the site of extravasation. If this was the case, tumour cells or cell aggregates would become trapped in the first capillary bed they encounter after being released into the venous circulation; in most cases this would be the lung [83]. Although the lung is a common site of metastases, CTCs also colonize other organs, suggesting that a significant number of tumour cells escape arrest in the pulmonary microcirculation. A recent report showing that cancer cells are capable of adjusting their shape to pass through narrow vessels supports this conclusion [86]. Furthermore, a number of studies have shown that tumour cells can adhere to the walls of precapillary arterioles, whose diameters far exceed cell size [87, 88]. Taken together, these data suggest that specific adhesion occurs between tumour cells and vascular endothelial cells and that the arrest of tumour cells in the capillary beds of particular organs is likely due to a combination of both mechanical trapping and cancer-cell adhesion to specific molecules on the vasculature [83, 86].

Glinskii et al. [86] propose a multistep model of tumour-endothelial cell adhesion, where carbohydrate-lectin interactions, which tend to be weak and transient, initiate an adhesion cascade that subsequently involves more stable interactions. Specifically, they suggest that the Thomsen-Friedenreich (TF) glycoantigen (a β-galactoside) on tumour cells leads to clustering of galectin-3 on the surface of endothelial cells and transient adhesion. The association of endothelial galectin-3 with $\alpha 3\beta 1$ integrin [89] on the tumor cells then stabilizes this adhesion and may mediate multiple downstream signals that determine the fate of the cell deposit and organ-specific metastasis. This work involved primarily breast and prostate cancer cells in bone vasculature and lung vasculature. As it is known that the endothelia in different organs express different cell-surface receptors [90], it is probable that different glycosylation structures and/or different integrins may mediate tumour-endothelial cell interactions in different capillary beds.

Cell-surface glycosylation is upregulated in many different cancers [91], and a number of glycoproteins have been identified as ligands for galectin-3, including integrin $\beta 1$, lysosome-associated membrane proteins 1 and 2 [92], and the IgSF members carcinoembryonic antigen (CEA) and L1CAM [51, 52]. There is also evidence that MCAM expression facilitates melanoma-endothelial cell adhesion [35, 36] although it is not known if this is mediated via carbohydrate or protein binding. In addition, PECAM-1 is located at the cell junctions on endothelial cells and may also contribute to tumour cell arrest and extravasation. PECAM-1 has been described as engaging in both homophilic and heterophilic adhesive interactions, and it is possible that the interaction of PECAM-1 with heparan sulfate proteoglycans on tumour cells could contribute to extravasation. Carcinoma, melanoma, lymphoma, and leukemia cells have been described as overexpressing heparan sulfates of the glypican family compared to that seen in their normal counterparts [53]. Although the possibility that PECAM-1 bound heparan sulfate was controversial for many years, it is now clear that the hypoxic conditions found in tumours would favour this interaction [54]. Thus, IgSF members may contribute to the arrest of tumour cells via both cell aggregation (leading to mechanical trapping) and specific tumour-endothelial cell adhesion.

Most models of metastasis propose that extravasation occurs soon after cell arrest, by degradation of the endothelial basement membrane and the surrounding ECM [93]. However, Al-Mehdi et al. [87] propose that tumour cells may also proliferate intravascularly to form metastatic foci without the need for extravasation. In time, these metastatic colonies are likely to outgrow the vessels, destroy the vascular walls, and invade the surrounding tissue [83].

8. Colonization and Proliferation

It is well known that different cancers show an organ-specific pattern of metastases. This is probably due to, firstly, the lodgment of cells in the vasculature, as a result of both entrapment and specific adhesion and, secondly, the ability of the cancer cells to grow in their new environment. Many of the features that allow tumour cells to proliferate in the primary lesion (e.g., apoptotic evasion and the ability to move through the ECM) will also be essential for growth as a secondary lesion. However, metastasizing cells must also adapt to a new microenvironment that is likely to be very different from that of the primary tumour, and their ability to do this will influence whether or not secondary

tumours successfully develop at the site of extravasation. The metastasizing cells will need to respond to growth factors and cytokines in the host tissue, proliferate, recruit the necessary supportive stromal cells, and develop an appropriate blood supply [83]. Indeed, all of the characteristics required to facilitate growth of the primary tumour will also be required for the development of a successful metastatic lesion. It is expected that the contribution of IgSF members to these processes in the metastatic lesion will be as described for proliferation of the primary tumour.

The occurrence of metastases of metastases should not be discounted and for some tumours (e.g., melanoma) they may be expected. For the clinician it is of little consequence whether a metastatic lesion arose from the primary tumour or from another metastatic lesion, as the difficulties of treatment are similar. However, from a drug development perspective if metastases of metastases are a possibility, adhesion molecules like MCAM on melanoma, for example, remain a viable drug target even after the first metastases have been diagnosed.

9. Immunological Escape

Over a century ago, Paul Ehrlich hypothesized that cancer would be more common in long-lived organisms if the immune system did not protect against cancer (described in [94]). However, it was not until the 1990s, with improved mouse models of immunodeficiency, that the role of cancer immunosurveillance by the immune system was determined. It became clear that those mice lacking the cells of the adaptive immune system (T and B cells) and natural killer (NK) cells were more susceptible to tumour formation and dissemination [94, 95]. As our knowledge has increased, it has become apparent that, although cancer immunology is very complex, NK cells and T and B lymphocytes are able to recognize tumour cells as abnormal and target them for destruction (the elimination phase) [94]. However, it appears that rare cancer cell variants survive elimination by the immune system and that tumour cell clusters increase the probability of cells being protected from immunological attack. These tumour cell clusters are held in check by the immune system but not all the cells are destroyed (equilibrium or dormant phase). This dormant phase can last for years, until the tumour cells acquire the ability to escape immune recognition or there is a change in the immune system of the host [94].

While there is no evidence that IgSF members directly lead to immunoescape of tumour cells, molecules such as MCAM, ALCAM, and NCAM mediate cell-cell cohesion, enabling the formation of cell aggregates [36, 37, 50]. It is believed that the formation of tumour aggregates ensures the survival of the inner cells, particularly during migration and dissemination, as the outer cells protect them from the immune-mediated cell death [16, 18].

10. Conclusion

The metastatic cascade is very complex and most research in this area has focused on the role of integrins and cadherins in cell migration and invasion, using carcinoma as a model system. In writing this paper, our goal was to examine the potential role of a selection of IgSF members in the metastatic pathway in different types of cancer, including carcinoma, melanoma, and sarcoma. Although most of these molecules have been described as tumour biomarkers, the extent and nature of their contribution to the metastatic pathway has not been clear. We have examined aspects of each step in the pathway and have suggested ways in which one or more of the six IgSF members could contribute. Much of this is conjecture based on what is known about the behaviour of these proteins in nontumour systems. However, as tumours commonly use existing molecular interactions in inappropriate or aberrant ways, we feel our conclusions indicate some interesting possibilities for further research. Performing these studies, however, will not be easy because of the difficulties of accurately dissecting a system as complex as the metastatic cascade *in vivo* and the limitations of the *in vitro* assays used to support *in vivo* conclusions. It is for these reasons that much remains to be understood, particularly about the role of IgSF members in the metastatic cascade. Yet the need to understand metastasis is high because most patients that succumb to cancer succumb to metastasis or the complications of its treatment.

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Review Article

Alterations in Cell-Extracellular Matrix Interactions during Progression of Cancers

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Cancer progression is a multistep process during which normal cells exhibit molecular changes that culminate into the highly malignant and metastatic phenotype, observed in cancerous tissues. The initiation of cell transformation is generally associated with genetic alterations in normal cells that lead to the loss of intercellular- and/or extracellular-matrix- (ECM-) mediated cell adhesion. Transformed cells undergo rapid multiplication and generate more modifications in adhesion and motility-related molecules which allow them to escape from the original site and acquire invasive characteristics. Integrins, which are multifunctional adhesion receptors, and are present, on normal as well as transformed cells, assist the cells undergoing tumor progression in creating the appropriate environment for their survival, growth, and invasion. In this paper, we have briefly discussed the role of ECM proteins and integrins during cancer progression and described some unique conditions where adhesion-related changes could induce genetic mutations in anchorage-independent tumor model systems.

1. Introduction

Cancer afflicts an organ or a tissue by inducing abnormal and uncontrolled division of cells that either constitute it or migrate to it. At the cellular level, this is caused by genetic alterations in networks that regulate cell division and cell death. The increased rate of proliferation of transformed cells causes further mutations in genes that regulate other cellular processes. For example, transformed cells eventually gain the capacity to invade into other tissues by modulating their own kinetic properties without losing the capacity to divide rapidly and avoid cell death, despite internal and external perturbations.

Cancer cells adopt diverse mechanisms to cope with the various physiological insults, such as low oxygen and metabolic stress, that they encounter [1]. These mechanisms have been discussed in a recent review [2], and based upon that discussion, six important hall marks of cancer cells can be identified. These are (a) sustained proliferative signalling, (b) evasion of growth suppressors, (c) resistance to cell death, (d) replicative immortality, (e) copious angiogenesis, and (f) active invasion and metastasis. In addition to these, cancer cells can exhibit two other properties, that is, tumor promoting inflammation and gene instability that assist the cells in the transition from normal to oncogenic phenotype [2]. Eventually transformed cells undergo somatic evolution and generate diverse populations that tend to harbor genetic and epigenetic instabilities and alterations [3]. These changes also assist the cells in adapting to the variations in the surrounding microenvironment and even to alter it. As a consequence of these alterations, the tumor milieu or microenvironment becomes an "enabling element" for defining some characteristics of cancer cells. For example, the tumor microenvironment can induce cancer cells in acquiring anoikis resistance and in selecting new sites to colonize and grow. Sometimes these cells remain unresponsive until signals generated from the ECM reach the cell's nucleus and they determine whether the cell would proceed to the next stage in cancer progression or not. This response of cancer cells to ECM-generated signals similar to the "dynamic reciprocity" proposed by Bisell for normal cells. An example of such an adaptation of cancer cells to their microenvironment and the resultant clonal selection of invasive cells has been recently reported [4–6].

Metastatic invasion is generally the final phase of cancer progression, and it involves formation of new blood vessels either by neovasculogenesis, in which endothelial cell precursors (angioblasts) migrate to the tumor site and differentiate and assemble into primitive blood vessels, or by angiogenesis in which we observe sprouting of new blood vessels from preexisting ones, or their longitudinal bifurcation, in the tumor [7]. The invasive tumor cells migrate through these newly formed blood vessels to other sites such as lung and liver brain and this leads to the death of tumor-bearing patients or animals as the case may be.

Based on available evidence, the entire process of cancer formation can be divided into four different stages: initiation, progression, epithelial mesenchymal transition (EMT), and metastasis (see Figure 1). At the initiation stage, a normal cell acquires oncogenic properties mainly through genetic alterations, which lead to changes in cell structure, adhesion properties, and response to signals from ECM proteins. In the second stage, transformed cells respond to cues from the altered environmental conditions and acquire properties of adhesion-independent growth and colonization. The third stage is also referred to as a transitional or the EMT stage, and, in this stage, the fully transformed cells begin to exhibit mesenchymal gene expression patterns which induce them to invade into the neighbouring tissue and enter into blood circulation [8]. The fourth and prominent stage is metastasis in which the invasive mesenchymes like cells move from the primary site and colonize in a new location. This stage spreads the disease into different parts of the body and involves several alterations in the adhesion properties of cells.

From all earlier observations, it is clear that the cell adhesion in transformed cells plays an important role in all four stages of cancer formation. This paper highlights recent studies done on the integrin-mediated interaction of transformed cells with the ECM and discusses its role in cancer progression.

2. ECM Components and Properties

Over the past two decades, research in the field of cancer biology has focussed extensively on the role of ECM constituents during cancer progression. These molecules comprise the cell's microenvironment, and they can affect the mechanical and biophysical properties of cells as well as that of the ECM such as its mechanics, geometry, and topology [9].

In some tissues, mainly of epithelial origin, ECM constituents are present in the basement membrane that defines the boundaries of that tissue. In this location, the organization of these components is different than in the matrix. In the basement membrane, we notice molecules such as collagens, proteoglycans, laminins, and fibronectins associate strongly with certain carbohydrate polymers and generate a membrane-like structure which facilitates the formation of a framework of cells and ECM constituents [10]. Specific domains in ECM proteins that are created by partial gene duplication and exon shuffling during the process of evolution [11] play a critical role in keeping the cells attached to the ECM and the basement membrane and initiating signalling cascades in the cells.

Inside the cells, the ECM-induced signaling pathways are transmitted mainly through integrin molecules that are transmembrane multifunctional ECM receptors. Integrin-mediated signaling in association with many cofactors, for example, cytokines, growth factors, and intracellular adapter molecules, can significantly affect diverse cell processes such as cell cycle progression, migration, and differentiation. The interplay between the biophysical properties of the cell and ECM establishes a dynamic, mechanical reciprocity between the cell and the ECM in which the cell's ability to exert contractile stresses against the extracellular environment balances the elastic resistance of the ECM to that deformation [4, 9]. The ECM in association with the available growth factors activates a sequence of reactions with a complex network of proteases, sulfatases, and possibly other enzymes to liberate and activate various signalling pathways in a highly specific and localized fashion. The maintenance of ECM homeostasis therefore involves a tight balance between biosynthesis of ECM proteins, their 3D organization, cross-linking, and degradation.

3. Modulation of ECM-Generated Signaling in Cancer

During cancer progression, we observe significant changes in the structural and mechanical properties of ECM constituents. It has been reported that changes in matrix stiffness, which offers resistance to cell traction forces [12] and also influences "shape dependence" in cells, can contribute actively to the tumor formation [13]. Deregulation of cell shape and alterations in the interactions with the ECM are considered as important hallmarks of cancer cells. These changes in ECM homeostasis can be brought about by the properties of tumor cells themselves or by the secretions of other surrounding cells such as fibroblasts, macrophages, and leukocytes [14]. Integrin ECM interactions are significantly modulated by crosstalk with several other signal-generating molecules, some of them are receptor molecules on the cell surface whereas others are present in the cytoplasm as adaptor proteins and actin-binding proteins [15]. These signaling crosstalks in which integrin molecules lie at the center are very useful for the transition of transformed cells to metastatic cells [16]. Integrin-generated ECM remodelling is further controlled by the localization and activity of proteases [17]. One example of such an integrin-directed cancer progression is seen in breast cancers, where adhesion-independent mammary epithelial cells secrete laminin-5 and luminal cells secrete laminin-1. This leads to aberrant polarity in cells, causing upregulation of metalloproteinases (MMPs, such as MMP9) and induction of tumor invasion and metastasis [18–20].

4. Integrins: Its Ligands and Signalling

Integrins are heterodimeric cell-surface receptors that mediate adhesion to ECM and immunoglobulin superfamily molecules. At least 24 distinct integrin heterodimers are formed by the combination of 18 α-subunits and

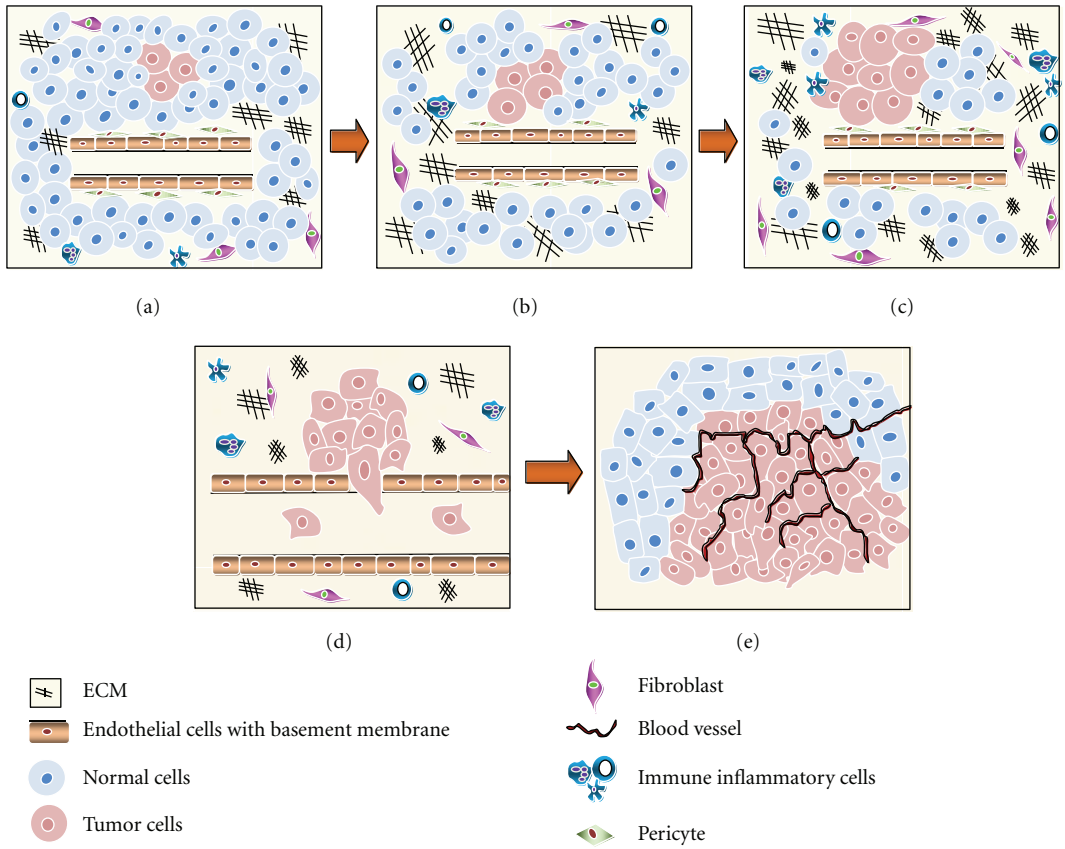

Figure 1: Various steps in tumor initiation and progression where panel (a) represents initiation of tumor by transforming normal cells, panel (b) shows the modulation of ECM proteins allowing transformed cells to multiply, panel (c) shows progression of cancer by replacing normal cells, panel (d) represents the invasion, where cancer cells migrate into the blood stream by modulating ECM and cell adhesion molecules, and panels (e) shows metastasis where the cancer cells are localized at different sites enabling angiogenesis.

8 β-subunits. Specific integrin heterodimers preferentially bind to distinct ECM proteins like laminin, collagen IV, fibronectin, and so forth. The level of integrin expression on the cell surface dictates the efficiency of cell adhesion and migration on different matrices. While some integrins selectively recognise primarily a single ECM protein ligand (e.g., $\alpha 5\beta 1$ recognises primarily fibronectin), others can bind several ligands (e.g., integrin $\alpha v\beta 3$ binds vitronectin, fibronectin, fibrinogen, denatured or proteolysed collagen, and other matrix proteins). Several integrins recognise the tripeptide Arg-Gly-Asp (e.g., $\alpha v\beta 3$, $\alpha 5\beta 1$, αIIb$\beta 3$), whereas others recognise alternative short peptide sequences (e.g., integrin $\alpha 4\beta 1$ recognises EILDV and REDV in alternatively spliced CS-1 fibronectin). Inhibitors of integrin function include function-blocking monoclonal antibodies, peptide antagonists, and small molecule peptide mimetics matrix [21–23].

The positioning of integrin receptors acts as a direct bridge between the extracellular matrix and the internal cell cytoskeleton by transducing key intracellular signals by associating with the clusters of kinases and adaptor proteins in focal adhesion complexes. Integrins thus act as mediators in transmitting different signals from "inside out" (intracellular to extracellular) and "outside in" (extracellular to intracellular) between ECM to cells and vice versa. Through these pathways, ECM proteins are able to control proapoptotic and antiapoptotic cascades by regulating the activity of caspase 8 and caspase 3 [24–26]. ECM-integrin interactions thus determine the balance of apoptotic and cell survival signals and maintain the homeostasis of organs and tissues. Although integrins lack kinase activity, by inter- and intramolecular clustering, they recruit and activate kinases, such as focal adhesion kinases (FAKs) and src family kinases (sFKs) to a focal adhesion complex. In addition to scaffolding molecules, such as p130 CRK-associated substrate (p130CAs; also known as BCAR1), integrins also couple the ECM to actin cytoskeleton by recruiting cytoskeletal proteins, including talin, paxillin, α-actinin, tensin, and vinculin. Additionally, they form a ternary complex consisting of an integrin-linked kinase, PINCH, and parvin to regulate many scaffolding and signalling functions required for integrin-mediated effects on cell migration and survival [27].

5. Integrin Expression and Signalling in Cancer Progression

Although anchorage-independent growth is a hallmark of malignant transformation, integrins expression levels and activity are an important role in different steps of tumour

progression including initiation [28]. Higher expression of $\alpha 3$, $\alpha 5$, $\alpha 6$, αv, $\beta 1$, $\beta 4$, $\alpha 6\beta 4$, $\alpha 9\beta 1$, $\alpha v\beta 5$, and $\alpha v\beta 3$ integrins is directly correlated with the progression of the disease [10]. Several epithelial cell tumors showed the altered $\alpha 6\beta 4$, $\alpha 6\beta 1$, $\alpha v\beta 5$, $\alpha 2\beta 1$, and $\alpha 3\beta 1$ integrin expression [29]. Integrin recruitment to membrane microdomains has been shown to be regulated by tetraspanins and crucially regulate integrin function in tumour cells [30]. Recent studies have demonstrated that cell signalling generated by growth factors and oncogenes in transformed cells requires collaboration with specific integrins, especially during tumour initiation. In tumor cells, several survival signals are upregulated upon integrin ligation, which includes increased expression of BCl-2 or FlIP (also known as CFlAR), activation of the PI3K-AKT pathway or nuclear factor-κB (nF-κB) signaling, and/or p53 inactivation [24].

Invasive cancer cells evacuate from the primary site and migrate to the secondary site by the process of tissue invasion and cell migration. Integrin-mediated pathways involving focal adhesion kinase (FAK) and src family kinase (SFK) signaling play a major role in this. In order to survive in the new location and to withstand the stressful conditions of hypoxia, nutrient deprivation, and inflammatory mediators the migratory cells increase the blood supply to themselves by neoangiogenesis. This is achieved by increased expression of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins and by deposition of provisional matrix proteins such as vitronectin, fibrinogen, von Willebrand factor, osteopontin, and fibronectin in the tumour microenvironment. Interaction between these molecules plays a critical role in the process of generating new blood vessels in the newly formed tumor site [31, 32].

Integrins found on tumour-associated normal cells, such as the vascular endothelium, perivascular cells, fibroblasts, bone-marrow-derived cells, and platelets, also have a profound effect in tumor progression via integrin-mediated pathways. A summary of these has been given in Table 1 [33–47].

6. Genetic and Chromosomal Aberrations at the Onset of Cancer

Neoplasia occurs when cells are exposed to cancer-promoting substances that cause single or multiple premalignant genetic/epigenetic changes which may coalesce to form a large lesion. These genetic changes may have neutral, deleterious, or advantageous effects on the proliferation of a clone or clones of cells. Neutral or deleterious genetic changes may result in stagnation or cell death, whereas the cell receiving advantageous events may result in higher proliferation, recruitment of blood vessels to the developing tumors, and gain the ability to metastasize [48]. The model of Braakhuis et al., 2004 [49], advanced this idea by suggesting that initial genetic alterations occur in stem cells, forming a patch and expanding field of cells with the original and subsequent genomic and or chromosomal alterations. Then, clonal selection of one or more cells within this field of preneoplastic cells leads to the development of a carcinoma. There is a considerable cytogenetic variability among cells reflecting heterogeneity due to clonal evolution within the original tumor [50]. Initial heterogeneity or cell-to-cell differences in cancer are due to cytoskeletal alterations which result in defecting chromosomal segregation and lead to karyotypic variations during mitosis, causing chromosomal aberrations, for example, NUMA1 gene at 11q13, which results in multipolar spindles, leading to daughter cells that differ from each other and their mother cells [51]. Structural chromosome alterations also occur due to deletions, translocations, isochromosomes, dicentric chromosomes, and endoduplicated chromosomes. The gain or amplification of chromosomal segments is driven by more than one gene [52]. Structural rearrangements involving the cleavage and fusion of centromeres from participating chromosomes, also referred to as Robertsonian translocations, are the most frequently observed alterations. Chromosomal aberrations identified with the help of cytogenetic methods including FISH, cCGH, or aCGH showed the gain of the entire long arm of chromosome 3 which amplifies the EGFR gene in SCCHN [53], 8q24 gain to amplify MYC and PTK2 in primary tumors, 11q13 amplifications to amplify cyclin D1 gene, loss of 3q14 causes deletion of fragile site FRA3B/FHIT, necessary to protect cells from accumulation of DNA damage [48]. Aberrations mainly in chromosome 13 and also involving chromosomes 6, 11, 12, and 17 are associated with B-CLL [54].

7. Anchorage-Independent Tumor Model System

The wide range of *in vivo* tumor models like syngeneic, human tumor xenograft, orthotopic, metastatic, and genetically engineered mouse models is available from the basis of the compounds selected and treatments that go into clinical testing of patients [55]. The ability to exhibit anchorage-independent cell growth (colony-forming capacity in semisolid media) has been considered to be fundamental in cancer biology because it has been connected with tumor cell aggressiveness *in vivo* such as tumorigenic and metastatic potentials and also utilized as a marker for *in vitro* transformation. Although multiple genetic factors for anchorage-independence have been identified, the molecular basis for this capacity is still largely unknown [56, 57]. During the process of *in vitro* tumorigenesis, various oncogenes with distinct pathways have been shown to transform anchorage-dependent cells to anchorage-independent cells [5, 57]. For example, transfer of c-Myc (a transcription factor), v-Src (a tyrosine kinase), or H-Ras (a small GTPase) into spontaneously immortalized mouse embryonic fibroblasts (MEFs) provides the cells an ability to grow in an anchorage-independent manner [56, 57]. Anchorage-independent multicellular spheroids made by Ewing tumor cell lines were more closely related to primary tumors with respect to cell morphology, cell-cell junctions, proliferative index, and kinase activation [58].

However, changes in ECM and cell adhesion molecular interaction and genetic variations were observed till the date only with the primary or secondary tumors. We have

Table 1: Various integrins in association with different ligands to induce different signaling pathways in generation of tumor and metastasis.

Integrin type	Interacting ECM protein	Activated signaling cascade	Tumor/metastasis	Reference
$\alpha 3\beta 1$	Laminin	MMP9 and oncogenic Ras, VEGF, FAK-paxillin signaling cascade	Invasion in keratinocytes, Induces angiogenesis, Human hepatoma cells	[33, 34]
$\alpha 6\beta 1$	Laminin	Urokinase plasminogen activator and MMP-2, PI3Kinase, Src	Tumor invasion in pancreatic cells	[35, 36]
$\alpha 7\beta 1$	Laminin	Rho-A signaling cascade	Invasion in breast cancer	[37]
$\alpha 2\beta 1$, $\alpha 1\beta 1$, $\alpha 10\beta 1$, $\alpha \mathrm{II}\beta 1$	Collagen	FAK and src signaling	Invasion of melanoma cells, cancer progression, and invasion of lung adenocarcinoma	[38, 39]
$\alpha v\beta 1$, $\alpha v\beta 6$, $\alpha v\beta 3$	Vitronectin, syndican, thromospondin-1	MMP9, urokinase signaling, MEK/Erk/NF-κB, PKCa, FAK	Metastatic breast Cancer, pancreatic, cervical, colon, lung/liver metastasis	[40–42]
$\alpha 9\beta 1$	CCN3, osteopontin	Src, P130 Cas, Rac, NOS signaling	Metastatic potential	[43]
αIIb3, αvb3	Von Willebrand factor	Interacts with thrombospondin-1 and induces VEGF/FGF signaling	Breast cancer	[44, 45]
$\alpha 5$	Fibronectin	FAK, ERK, PI-3 K, ILK, and nuclear factor-kappa B -	Metastatic lung and cervical cancer	[46, 47]
$\alpha \mathrm{L}\beta 2$	Intercellular cell adhesion molecules		Breast cancer	[1]

developed a cellular model system by using normal, adherent rat fibroblast cell lines. These cells lose their cytoskeletal organization and specificity to fibronectin as $\alpha 5\beta 1$ integrins are constantly recycled between cytoplasm and plasma. In the drastic unfavorable stressful conditions, the mechanical, phenotypic, and genetic characteristics are altered/modified to sustain their identity [5, 26]. We observed that this cellular model system represents a tumor with all characteristics of cancer described by Hanahan and Weinberg 2011 as hallmarks of cancer.

The cells during the nonadhesion process can evade from cell death by caspase 3 interaction with unligated $\alpha 5\beta 1$ integrins inducing resistance to integrin-mediated death (IMD) and also gain the ability to metastatise. Mutational changes mainly with 2;6 Robertsonian's translocation and activated Ras, FAK, and PKC provide self-sufficient growth signals potential for uncontrollable growth of the cells (Figure 2). Upregulated Spp1, MMP3, Egfr, Rb1, Ddit3, Egln3, Vegfa, Stc1, Hif1a, MMP3, and altered pathways like glycolysis/gluconeogenesis and hypoxia (Pfkm, HK2, Pdk1, Adh1, aldh3a1, and Slc2a) lead the cells to invade, metastasize, and sustain angiogenesis. We observed another phenomenon of dedifferentiation by gaining the stem-cell-like and multidrug resistance properties by expressing Cd133 and ABCG-2 when the cells are exposed to unfavorable condition [5].

The anchorage-independent cellular model system represents a multicentric tumor model system apprehended with genes related to tumor progression, angiogenesis, and metastasis (Figure 2). It is very advantageous, convenient, and possible model system to study the effect of various cancer-mediated drugs at the initial stage itself for the proper diagnosis.

8. Integrin Signalling as a Target in Cancer Treatment

Several studies showed the correlation of integrin inhibition at any point of its action will lead to the inhibition of tumor progression [24]. Therefore, integrins are focused pharmacologically in the treatment and prevention of cancer. Antagonists of these integrins suppress cell migration and invasion of primary and transformed cells by inducing apoptosis in primary cells could block tumor angiogenesis and metastasis. Recycling integrins present on the surface of endothelial cells are targeted in the blood stream by exposing to the circulating drugs and agents [59]. Various antibodies, cyclic peptides, disintegrins, and peptidomimetics are meant to bind the targeted integrins to prevent integrin ligation. cRGD, cyclic arginine-glycine-aspartic acid; RGDK, arginine-glycine-aspartic acid-lysine; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand are being used as antagonists integrins to hit the integrin ligand function. The function of upregulated $\alpha v\beta 3$ integrin can be blocked by function-blocking monoclonal antibodies, such as LM 609 [60]. The human αv integrin specific monoclonal antibody CnTo 95, which targets both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins to induce endothelial apoptosis, also had antitumour and antiangiogenic effects in xenograft tumour models [15].

Cilengitide, inhibitor of both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins and volociximab, a function-blocking monoclonal antibody against integrin $\alpha 5\beta 1$, inhibits angiogenesis and impedes tumour growth [61, 62]. $\alpha v\beta 3$ is targeted by various therapeutic antibodies like LM609, vitaxin, humanized mouse monoclonal derived from LM609, CNTO 95, humanized IgG1, c7E3, chimeric mouse human, 17E6, mouse monoclonal antibodies to inhibit tumour growth, and angiogenesis

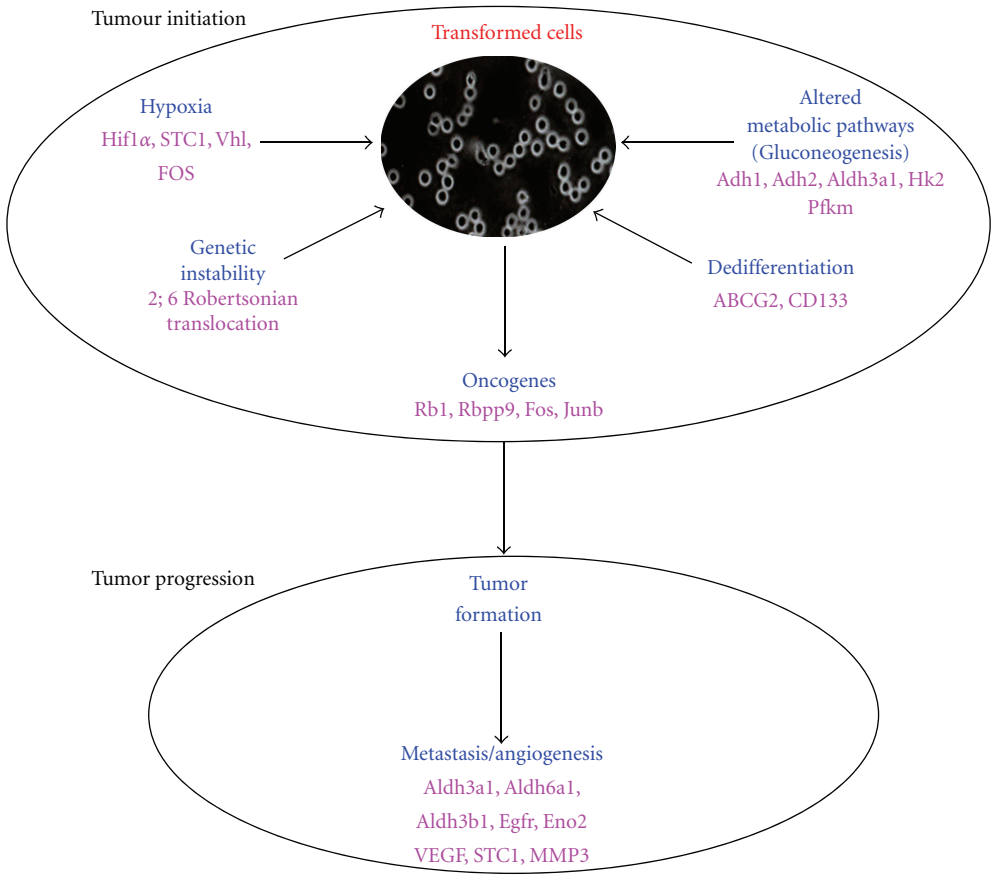

Figure 2: This figure shows the hallmark characteristics of transformed cells at initial stages.

in tumour xenografts and preclinical studies [63]. The prognostic $\alpha 5\beta 1$ integrins in ovarian cancer can be targeted effectively both *in vitro* and *in vivo* by using specific antibodies. Cell-mediated $\alpha 5\beta 1$ adhesion can be blocked with a small molecule antagonist (SJ479), a fibronectin-derived peptides in prostate, and colon cancer models [64, 65]. Recent activity is extended to detect tumors and angiogenesis and deliver the drugs to the site of cancer by coupling integrin antagonists to a paramagnetic contrast agent or radionuclide in rabbit and mouse tumour models [66, 67].

9. Conclusion

Normal cells lead to the transformation when exposed to adverse conditions such as anchorage independence and effects are found to be similar to the effect of carcinogens and mutagens. These cells could alter the ECM and other cell adhesion molecules by showing altered integrins expression on their surface and are associated with different kinds of growth factors and oncogene. ECM-integrin interactions along with other growth factors provide the diversified anchorage-independent signals to the transformed cells to progress as cancers, metastasis, and angiogenesis. Several tumours are sustained with diversified integrins found to be specific to the tumour-host microenvironment. In future, these integrins can be targeted at the initial stages of cancer by using integrin antagonists to minimize the growth of tumour and metastasis.

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Review Article

The Role of Lipid Rafts in Cancer Cell Adhesion and Migration

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Lipid rafts are cholesterol-enriched microdomains of the cell membrane and possess a highly dynamic nature. They have been involved in various cellular functions including the regulation of cell adhesion and membrane signaling through proteins within lipid rafts. The dynamic features of the cancer cell surface may modulate the malignant phenotype of cancer, including adhesion disorders and aggressive phenotypes of migration and invasion. Recently, it was demonstrated that lipid rafts play critical roles in cancer cell adhesion and migration. This article summarizes the important roles of lipid rafts in cancer cell adhesion and migration, with a focus on the current state of knowledge. This article will improve the understanding of cancer progression and lead to the development of novel targets for cancer therapy.

1. Introduction

The alternation of cell adhesion and highly migratory behavior are the most prominent features of cancer cells, and play critical roles in their aggressive invasion and metastatic spread [1]. These processes appear to be facilitated by remodeling of the extracellular matrix (ECM) of the tumor microenvironment and adhesion molecules at the cancer cell surface and affected by both the interaction between ECM and adhesion molecules and by growth factor signaling [2, 3]. The proteolytic ectodomain cleavage and release (shedding) of adhesion molecules are also critical regulatory steps in cancer cell adhesion and migration [4, 5].

To date, cholesterol-enriched membrane microdomains called "lipid rafts" have been implicated in a variety of pathogeneses [6]; neurological diseases including Alzheimer's [7], Parkinson's [8], and prion diseases [9]; cardiovascular diseases; immune disorders such as systemic lupus erythematosus [10] and HIV infection [11]. Lipid rafts have been also implicated in signaling pathways in cancer progression [12], but how these microdomains affect the adhesion and migration of invasive cancer cells remains obscure. In this paper, recent findings on the roles of lipid rafts in cancer cell adhesion and migration will be reviewed.

2. Lipid Raft Structure

The prevailing model of cellular membrane structure was proposed by Singer and Nicolson, and this model is known as the fluid mosaic model, where globular proteins float in a lipid bilayer with a basic structure [13]. Later, the model was improved by Simons and van Meer, who suggested the existence of microdomains or "rafts" in the plasma membrane of epithelial cells [14]. In the current understanding of the lipid raft model, cholesterol- and sphingolipid-enriched microdomains of the plasma membrane exhibit a biophysical state comparable to the liquid-ordered phase floating in the liquid-disordered phase of the membrane [15]. One subtype of lipid rafts exists in flask-shaped plasma membrane invaginations called caveolae [16].

Lipid rafts consist of assemblies of cholesterol, sphingolipids including sphingomyelin and gangliosides, and certain types of proteins [15]. Sphingolipids contain saturated fatty acyl chains in their structure, thereby allowing cholesterol to be tightly intercalated in the sphingolipid assemblies to form liquid-ordered microdomains. The most important properties of lipid rafts are that they are small, dynamic, and heterogeneous and can include or exclude proteins to variable extents [17, 18]. Proteins with raft affinity include

glycosylphosphatidylinositol-anchored proteins, palmitoylated proteins, doubly acylated proteins, such as Src family kinases (SFKs), and transmembrane proteins such as CD44. Lipid rafts have been implicated in various physiological cellular processes, such as protein membrane trafficking and signal transduction [18, 19].

3. Tools for Lipid Raft Analyses

3.1. Lipid Raft Markers. Lipid rafts can be fractionated as detergent-resistant membrane (DRM) fractions using nonionic detergents such as Triton X-100 [18, 20]. Cholesterol- and sphingolipid-enriched rafts are insoluble in Triton X-100 at 4°C and float to a low-density area during gradient centrifugation. Notably, the constitution of DRM is affected by the type and concentration of detergents, and lipid rafts contained in DRM are nonnative aggregates. Marker molecules for lipid rafts are frequently used in biochemical and cytochemical analyses. The ganglioside GM1 is the most commonly used marker among putative lipid components of rafts; it is detected using the GM1-binding molecule, cholera toxin subunit B (CTxB) [21]. Protein markers such as caveolins and flotillins are also used for identifying lipid rafts [22].

3.2. Cholesterol Clathrate. Membrane cholesterol serves as a spacer for the hydrocarbon chains of sphingolipids and maintains the assembled microdomains of lipid rafts. Thus, cholesterol depletion leads to the disorganization of lipid raft structure. Methyl-β-cyclodextrin (MβCD), a torus-shaped cyclic oligosaccharide composed of 7 D-glucopyranosyl units linked by α-1,4 glycosidic bonds, is used to extract membrane cholesterol selectively and to disrupt lipid rafts [23]. MβCD is a practical tool for membrane studies as it neither binds to nor inserts into the plasma membrane. MβCD-mediated manipulation of membrane cholesterol is now a standard methodology in the research of lipid rafts [18, 24]. However, MβCD may deplete cholesterol from both the raft and nonraft domains of the membrane as well as alter the distribution of cholesterol between the plasma membrane and organelle membranes under high concentrations (i.e., >10 mM). Thus, it is recommended that a cholesterol-repletion experiment using cholesterol-MβCD complex and raft disruption with other cholesterol-sequestering agents as described below would be performed for confirmation.

3.3. Cholesterol-Binding Antibiotics. Filipin, a fluorescent polyene macrolide antibiotic from *Streptomyces filipinensis*, binds cholesterol and disperses it in the membrane. Filipin is thus used as a cholesterol probe and a cholesterol sequestration agent in the research of lipid rafts [25, 26]. Other than filipin, nystatin and amphotericin are also used in lipid-raft analyses.

3.4. Inhibitors for Cholesterol Biosynthesis. Statins are widely used inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, the key rate-limiting enzyme in the biosynthesis of cholesterol. Statins lower cellular cholesterol content and thus are useful in the analysis of lipid-raft function. Prevention studies using statins have confirmed its significance in the prevention of cardiovascular diseases [27]. It has also been demonstrated that statins may be an effective preventive medicine for neurodegenerative diseases, including Alzheimer's disease [28]. Although the various population-based reports of the effects of statins on cancer are controversial, recent epidemiologic studies suggest that statins inhibit the progression of certain cancers [29]. Recent evidence suggests that statins blocked the adhesion and migration processes of cancer cells [30, 31]. Cholesterol reduction is a potential therapy for suppressing cancer cell adhesion and migration.

4. Lipid Rafts and Proteolytic Processing of Adhesion Receptors

CD44 is a major cell adhesion molecule expressed in cancer cells and implicated in cancer cell adhesion, migration, and metastasis [32–34]. A number of reports have demonstrated that CD44 is present in lipid rafts [35–40], but the role of lipid rafts in cancer cell adhesion and migration has not been elucidated.

Recently, it was demonstrated that lipid rafts play a crucial role in the localization and functionality of CD44, which regulates cancer cell adhesion and migration [31]. Treatment of human glioma cells with the lipid-raft-disrupting agent MβCD resulted in an increase in CD44 shedding (Figure 1(a)) [31]. Similar patterns are observed when cells were treated with another lipid-raft-disrupting agent, filipin, and also in the case of pancreatic cancer cells. Analyses of Triton X-100 solubility of CD44 and its processing enzyme, a disintegrin and metalloproteinase 10 (ADAM10), revealed that CD44 was present in both Triton-X-100-insoluble and Triton-X-100-soluble fractions of untreated cells, whereas ADAM10 was largely in Triton-X-100-soluble fraction [30, 31]. Treatment with MβCD or filipin, however, led to loss of CD44 from the Triton-X-100-insoluble fraction. These results suggest that the perturbation of the ordered distribution of CD44 and ADAM10 on the membrane increased the probability of enzyme-to-substrate contact that leads to enhanced CD44 shedding. Membrane microdomains such as lipid rafts serve as platforms for the nanoscale assembly of membrane proteins. Simvastatin, one of the statins most frequently used in the clinical treatment of hypercholesterolemia, also enhanced CD44 shedding (Figure 1(b)). Moreover, simvastatin blocked the stimulation of glioma cell migration by hyaluronan oligosaccharides or epidermal growth factor (EGF) (Figure 1(c)) [41–43]. Taken together, these results suggest that lowering cholesterol levels may disturb the regulated CD44 membrane localization that is necessary for enhanced cancer cell adhesion and migration (Figure 2).

Recent studies on the shedding of various membrane proteins revealed that cholesterol depletion triggers the shedding of these molecules, including amyloid precursor protein (APP) [44], IL-6 receptor [45], CD30 [46], L1-CAM [47], and collagen types XVII [48] and XXIII [49]. It is especially noteworthy that APP and CD30 were found to be strongly associated with lipid rafts, whereas their processing enzymes, ADAM10 and ADAM17, respectively, are excluded from lipid

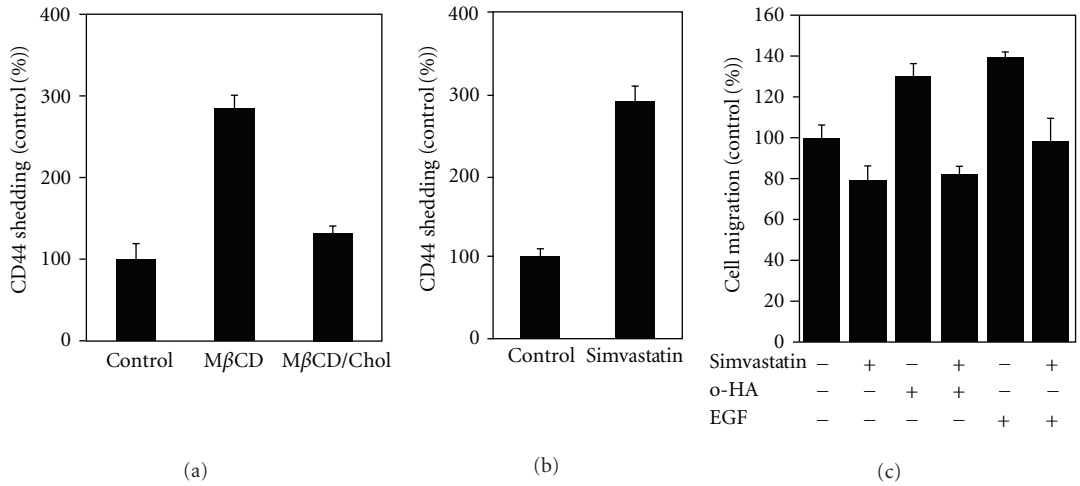

Figure 1: Cholesterol lowering stimulates CD44 shedding and suppresses cancer cell migration. (a) Modulation of cellular cholesterol affects CD44 shedding from human glioma cells. Cells were cholesterol-depleted (MβCD), cholesterol-replenished (MβCD/Chol), or left untreated (control), and CD44 shedding was assessed by measurement of soluble CD44 in the culture medium. (b) Effect of simvastatin on CD44 shedding. Cells were incubated in the presence or absence of simvastatin, and CD44 shedding was assessed by measurement of soluble CD44 in the culture medium. (c) Effect of simvastatin on CD44-dependent cell migration. Cells were incubated in the presence or absence of simvastatin, and treated with hyaluronan oligosaccharides (o-HA) or EGF.

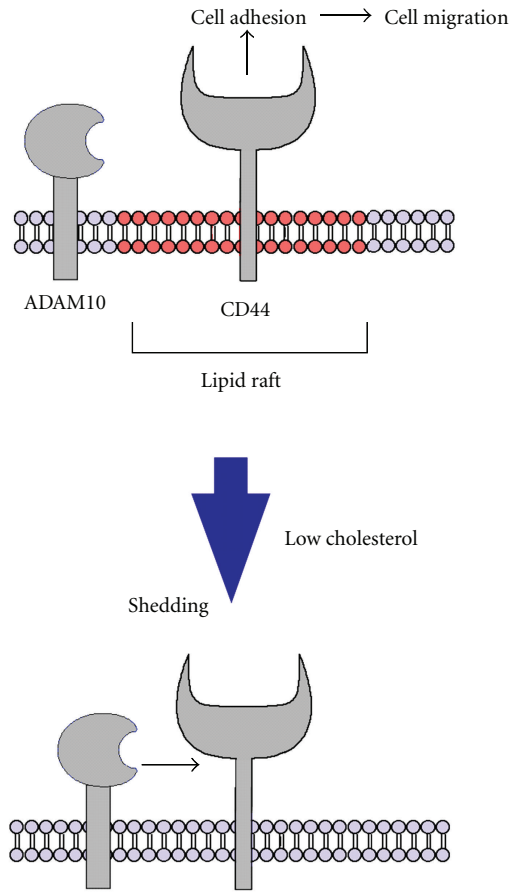

Figure 2: A putative model of the lipid-raft-related cancer cell adhesion and migration.

rafts [44, 46]. These findings suggest that lipid rafts may play a critical role in regulating the accessibility of processing enzymes to their substrate proteins during both constitutive and regulated shedding [50].

Na^+-H^+ exchanger interacts with CD44 in lipid rafts and may regulate cancer cell migration [39]. Complement component receptor gC1qR is a lipid raft protein that is concentrated in the lamellipodia along with CD44, regulating A549 lung adenocarcinoma cell migration and metastasis [51].

5. Cell Adhesion Signaling in Lipid Rafts

Integrins are transmembrane adhesion receptors composed of α and β subunits that facilitate the anchorage of cells to components of the ECM or bind to ligands on other cells to support cell-cell adhesion. Recent evidence suggests that the microorganization of lipids in the plasma membrane can affect integrin-mediated cellular functions [52]. Integrin-mediated cell adhesion to the ECM is regarded as one of the primary stages of SFKs' function. SFKs are activated in lipid rafts, and lipid-raft-specific inhibition of SFKs abrogates adhesion of breast cancer cells [53]. The transmembrane phosphoprotein, Cbp, a C-terminal Src kinase-binding protein, serves as a sensor of SFK activity in integrin-mediated cell adhesion signaling [54].

CD44 is an important marker for various cancer stem cells (CSCs), such as pancreatic [55], breast [56], ovarian [57], colon [58], and bladder CSCs [59]. However, why CD44 is a CSC marker remains largely unknown. Recently, it was reported that lipid-raft-associated CD44 is required for the survival of CSCs in the suspension condition through CD44-SFK-integrin signaling, leading to tumor metastasis [60].

Lipid rafts are necessary platforms for membrane receptor redistribution and the acquisition of a polarized phenotype during MCF-7 mammary adenocarcinoma cell migration [61]. Disruption of lipid rafts with MβCD abolishes lamellipodia formation and inhibits the chemotactic migration of MCF-7 cells [61].

6. Invasion Machinery and Lipid Rafts

A variety of invasive cancer cells form invadopodia, subcellular structures with ventral membrane protrusions that induce ECM degradation, a pivotal process in cancer invasion [62]. The ECM degradation activity of invadopodia is mainly mediated by membrane type 1-matrix metalloproteinase (MT1-MMP) concentrated at the surface of invadopodia [4]. Localization to lipid rafts is essential for the internalization of MT1-MMP. Lipid rafts are required for invadopodia formation in breast cancer cells and ECM degradation [63]. Caveolin-1 is predominantly expressed in invasive breast cancer cell lines and is well correlated with invadopodia activity, implying that caveolin-1 plays important roles in the trafficking of the components of invadopodia including MT1-MMP [63].

Concluding Remarks

I have summarized here the nature of lipid rafts and their role in cancer cell adhesion and migration focusing on the current state of knowledge, although many questions about the nature of lipid rafts remain unsolved. Future studies may corroborate a variety of aspects of the role of lipid rafts in the regulation of adhesive and migratory properties of invasive cancer cells.

The elucidation of the mechanism underlying the lipid-raft-mediated regulation of cancer cell adhesion and migration will provide new insights into the mechanism of cancer invasion and metastasis and also provide a wealth of new targets for cancer prevention and therapy for clinical medicine.

Abbreviations

ADAM:	A disintegrin and metalloproteinase
APP:	Amyloid precursor protein
CSC:	Cancer stem cells
CTxB:	Cholera toxin subunit B
DRM:	Detergent-resistant membrane
ECM:	Extracellular matrix
MβCD:	Methyl-β-cyclodextrin
SFK:	Src family kinase
EGF:	Epidermal growth factor
MT1-MMP:	Membrane type 1-matrix metalloproteinase.

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Review Article

Modulation of Tumor Cell Survival, Proliferation, and Differentiation by the Peptide Derived from Tenascin-C: Implication of β1-Integrin Activation

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Cell adhesion to extracellular matrix (ECM) participates in various biological processes, such as cell survival, proliferation, differentiation, and migration. Since these processes are essential for keeping homeostasis, aberration of these processes leads to a variety of diseases including cancer. Previously, we found that a peptide derived from tenascin- (TN-) C, termed TNIIIA2, stimulates cell adhesion to ECM through activation of β1-integrin. It has been shown that TNIIIA2 can modulate cell proliferation and differentiation. Interestingly, TNIIIA2 could not only enhance cell proliferation but also induce apoptotic cell death, depending on cellular context. In this review, we show the function of the peptide TNIIIA2 in cell survival, proliferation, and differentiation and refer to the possibility of new strategy for tumor suppression by regulating cell adhesion status using the ECM-derived functional peptides.

1. Introduction

Tenascin- (TN-) C, one of extracellular matrix (ECM) proteins, is expressed predominantly during embryogenesis, wound healing, and neoplastic processes. Since TN-C mRNA is alternatively spliced within the fibronectin type III-like (FN-III) repeats (Figure 1), various isoforms of TN-C could be generated. It has been identified that TN-C shows multifunctional properties including effects on cell adhesion, migration, proliferation, survival, and differentiation. Since this ECM protein works as a modulator of cell-matrix interaction but does not seem to contribute directly to the structural elements formation, TN-C is classified as a member of the matricellular protein family. Matricellular proteins regulate cellular function and matrix production through multiple interactions with their cellular receptors, and through modulating expression and activity of cytokines, growth factors, and proteinase [1, 2]. For cell adhesion, the functions of TN-C are particularly complex; the TN-C substrate supports attachment of some cell types but is nonadhesive or even repulsive for other cell types. Various domains of TN-C molecule, including alternative splicing domains, have been implicated in its multifunctional properties. However, the details of their contribution to the adhesion modulatory effects of TN-C are still unclear.

The ECM proteins often harbor functionally active sites within their own molecules. Since these cryptic active sites (matricryptic sites) are disclosed by proteolytic degradation with inflammatory proteinases, the relations between the exposure of matricryptic sites and the development of various diseases have been investigated. We previously found a 22-mer peptide termed FNIII14 from fibronectin (FN), which plays an important role in promoting cell adhesion. FNIII14 strongly suppresses FN-mediated cell adhesion by inhibiting the activation of $\alpha 4\beta 1$ (VLA-4) and $\alpha 5\beta 1$ (VLA-5) integrin [3, 4]. It has been determined that the antiadhesive

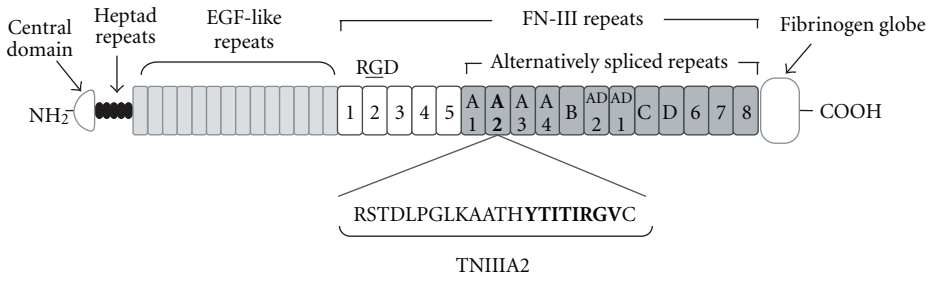

Figure 1: Schematic illustration of tenascin-C domain structure. Sequences analogous to antiadhesive peptide, FNIII14 (YTIYVIAL), are presented in alternative splicing region of TN-C.

activity of FNIII14 depends on its C-terminal amino acid sequence, YTIYVIAL [3]. We thought that this matricryptic antiadhesive site should be exposed by either FN degradation with matrix metalloproteinase- (MMP-) 2, or FN interaction [5]. Subsequently, we found several sequences similar to the YTIYVIAL sequence of FN in TN-C. Two analogous sequences, YTITIRGV and YTIYLNGD, are present in the FN-III repeat A2 of the alternative splicing region and the C-terminus fibrinogen-globe, respectively (Figure 1). Surprisingly, we observed that a 22-mer TN-C peptide containing YTITIRGV, termed TNIIIA2, stimulates cell adhesion to FN by inducing conformational and functional activation of β1-integrin. We also observed that the active site of TNIIIA2, which is also cryptic and exposed by MMP-2 processing, may induce a lateral interaction of β1-integrin with the cell surface heparan sulfate proteoglycans (HSPGs), including syndecan-4 ectodomein. Additionally, it has been reported that cytokine-stimulated adhesion via VLA-4 and VLA-5 to FN is rapid (reaching a max within 30 minutes) but transient (returning to basal levels after several hours) [6]. In sharp contrast, TNIIIA2 has the ability to strongly activate β1-integrins and to sustain this activated status, probably due to stabilization of the active β1-conformation through lateral association with syndecan-4 [7]. Moreover, we observed that TNIIIA2 has a potential to induce apoptotic cell death in nonadherent tumor cells, whereas this peptide also induces aggressive cell growth in nontransformed adherent cells. The evidence from the series of studies with TNIIIA2 shows the possibility that the effect of TN-C in tumor progression has close relation with the behavior of TNIIIA2. In this review, we describe in detail about current knowledge of the effect of TNIIIA2 on various tumor cell phenotypes.

2. Host-Beneficial Effects of TNIIIA2 in Hematopoietic Progenitor Cell Types

2.1. Induction of Apoptotic Cell Death in Leukemic Cell by TNIIIA2. In ordinary proliferation and survival of hematopoietic stem and progenitor cells, it has been reported that FN plays an important role via the FN-receptors, such as VLA-4 and VLA-5 [8]. Like their normal counterparts, transformed hematopoietic progenitor cells need signals from the FN for their survival and proliferation during their malignant progression [9–11]. This survival effect of FN/ECM interaction is due to prevention of apoptosis [12, 13]. Additionally, increasing evidence has demonstrated that adhesion of hematopoietic tumor cells to FN via VLA-4 and VLA-5 confers a multidrug resistance phenotype, commonly referred as cell adhesion-mediated drug resistance (CAM-DR) [14]. These facts indicate that integrin signal is important for regulating tumor progression.

Constitutive expression of TN-C has been observed on lymphoid tissues, such as adult bone marrow and lymph nodes [15, 16]. It has also reported that the expression of TN-C is transiently upregulated in pathological states, including inflammation and tumorigenesis [17, 18]. Therefore, lymphoid tissues of patients with hematopoietic malignancy should show highly increased expression of TN-C. Since TNIIIA2 can induce cell adhesion to FN also in hematopoietic tumor cells (Figure 2(a)), it is easily presumed that this peptide may induce enhancement of cell survival and proliferation. However, when hematopoietic tumor cells are forced to adhere to FN substrate by TNIIIA2, these cells undergo apoptotic cell death (Figures 2 and 3). We found that VLA-4 expression is essential for TNIIIA2-induced apoptosis in hematopoietic tumor cell lines. For example, U937 cells, expressing both VLA-4 and VLA-5, underwent apoptosis only when adhered to FN fragments containing the VLA-4-binding sites, and this apoptosis was specifically abrogated by the VLA-4 antagonist, but not by VLA-5 agonist [19]. These results suggest that TNIIIA2-induced forced adhesion to FN via $\alpha 4\beta 1$ integrin leads to apoptotic cell death in hematopoietic tumor cells.

Our observation seems to be inconsistent with the theory "CAM-DR". However, there have been also several reports demonstrating the negative effects of cell adhesion on cell survival. Integrin-mediated adhesive interaction with FN was shown to lead apoptosis in myeloid [20, 21] and erythroid progenitor cell lines [22]. To explain this discrepancy, we hypothesized that a moderate adhesion to FN may be favorable for continuous survival in hematopoietic tumor cells. We previously demonstrated that leukemic cell adhesion to bone marrow FN via VLA-4 generated CAM-DR, which could be a major cause of recurrence in acute leukemia patients [23, 24]. Additionally, we recently demonstrated using *in vitro* and *in vivo* experiments that combination therapy with an anticancer drug and antiadhesive peptide, FNIII14, which is capable of inactivating β1-integrins, effectively overwhelms the CAM-DR of AML [25]. In a series of previous reports investigating CAM-DR demonstrated that

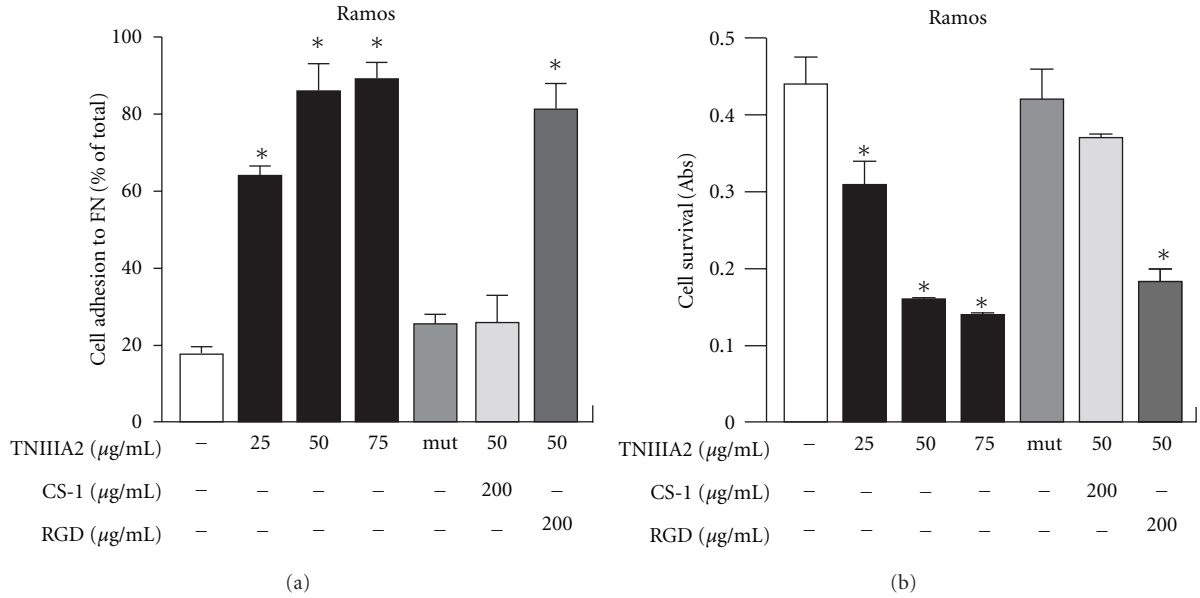

Figure 2: Effect on cell growth of forced adhesion of Ramos cells to FN. (a) shows the results of cell adhesion assay. The percentage of adhere cells are shown relative to the total number of cells seeded into the well. In (b), the effect of induced adhesion to FN on Ramos cell survival was shown. CS-1: connecting segment 1 peptide. (figures were modified from Figure 1 of [19]). $^*P < 0.05$ versus untreated control.

hematopoietic tumor cells show chemoresistancy through spontaneous adhesion to FN without addition of integrin activators [14, 24, 26, 27]. It has been shown that spontaneous adhesion of hematopoietic tumor cells is induced mainly by β1-integrin activation through the interaction between cytokine and G protein-coupled receptor (GPCR) [28]. Additionally, it has also been reported that cytokine-stimulated adhesion through VLA-4 and VLA-5 to FN is rapid (reaching a max within 30 minutes) but transient (returning to basal levels after several hours) [6]. Therefore, it appears likely that CAM-DR may be induced through weak or moderate adhesion to FN. In sharp contrast, TNIIIA2 has the ability to strongly activate β1-integrins and to sustain this activated status. We suppose that this difference in the state of β1-integrin activation should produce the difference in adhesion-induced cellular responses.

How does TNIIIA2 transmit their signal into hematopoietic tumor cells? We previously found that TNIIIA2 requires syndecan-4 as a membrane receptor for activation of β1-integrin [7]. Actually, syndecan-4 expression, besides VLA-4, was essential for TNIIIA2-induced apoptosis [19]. Syndecan-4 probably contributes to the sustained activation of VLA-4 through a lateral association with it [7]. Interestingly, TNIIIA2 exhibited no remarkable pro-apoptotic effects on normal peripheral blood cells, such as neutrophils, monocytes, and lymphocytes. It is well known that expression of syndecans is highly regulated with respect to developmental expression and cell-type specificity. Actually, it has been reported that very little syndecan-4 is present on polymorphonuclear leukocytes and peripheral blood mononuclear cells (PBMCs) [29, 30]. Moreover, we tested several hematopoietic tumor cell lines with various expression levels of VLA-4 and syndecan-4 and suggest that syndecan-4 is a key molecule in adhesion-regulated apoptosis induced by TNIIIA2 administration (Table 1).

Although the molecular mechanisms underlying TNIIIA2-induced apoptosis were not defined in detail, these data clearly showed that integrin-mediated adhesion plays a negative role in the survival of hematopoietic progenitor/tumor cells. TNIIIA2 activity embedded in TN-C molecule could contribute, once exposed, to preventing prolonged survival of hematopoietic malignant progenitors. Further study is needed to examine whether the TNIIIA2-related matricryptic site is exposed at its functional level in lymphoid tissues with hematopoietic malignancy.

2.2. Acceleration of Erythroid Differentiation by TNIIIA2. Besides hyperproliferation, incomplete differentiation of blood cells is the major phenomena observed in myeloid leukemia. Similar to the proliferation, differentiation of hematopoietic stem and progenitor cells occurs in the bone marrow and fetal liver [8, 31–35]. Although cytokines and growth factors are strong regulator of hematopoiesis, it is generally accepted that the adhesive interactions between hematopoietic stem/progenitor cells and the microenvironment also influence hematopoiesis. Stromal cells of the bone marrow and fetal liver form a hematopoietic microenvironment, called a "niche". This microenvironment niche plays a pivotal role in the regulation of proliferation and differentiation of hematopoietic stem and progenitor cells. In addition to stromal cells, ECM proteins in lymphoid tissues, such as FN, TN, collagen, laminin, and proteoglycans (PGs), have been implicated as essential components of the microenvironment that regulates hematopoiesis. Among these macromolecules, FN is known as the most important

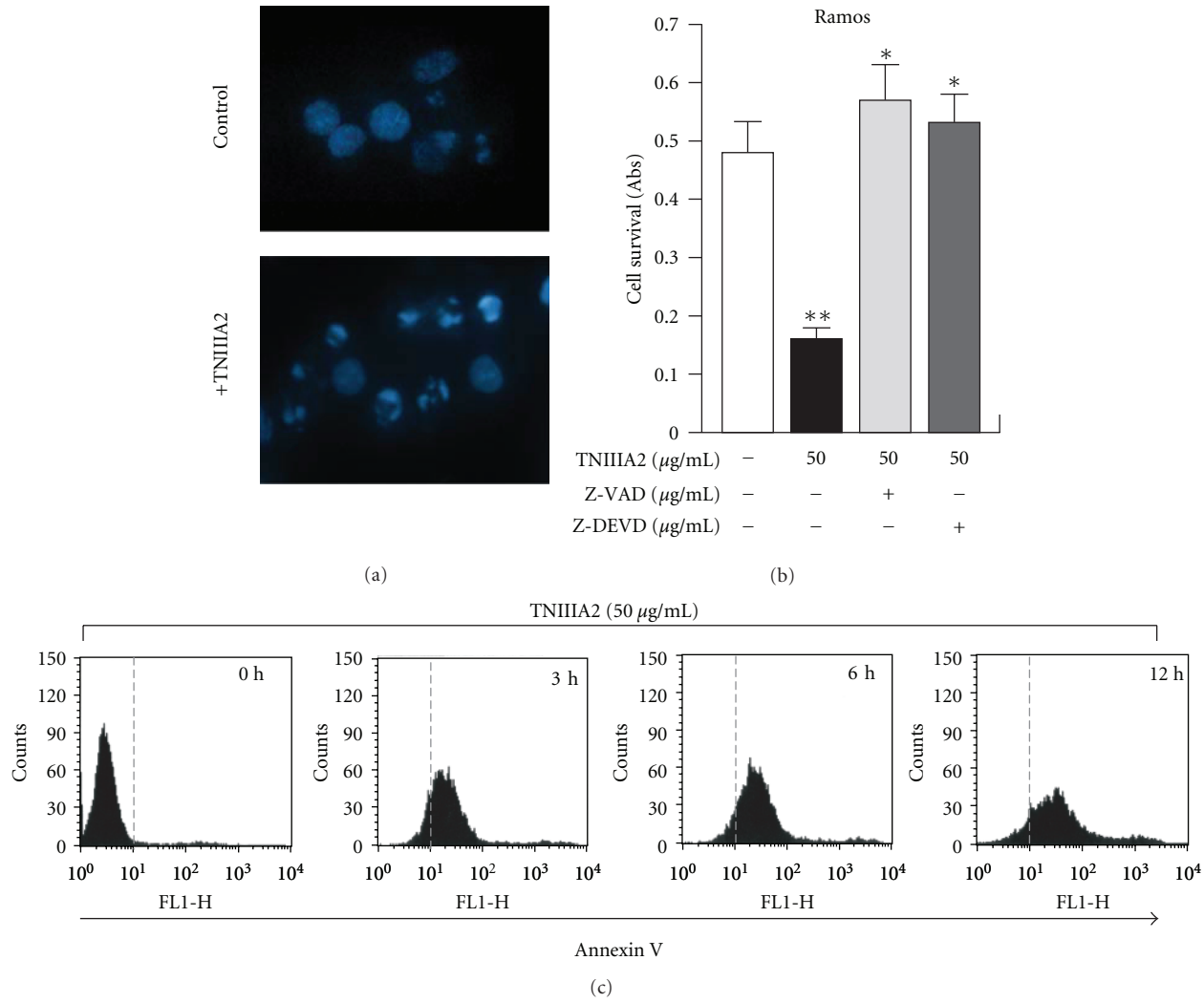

Figure 3: Apoptosis was induced on Ramos cells with TNIIIA2 administration. (a) Hoechst staining of Ramos cells treated with TNIIIA2. (b) Effect of caspase inhibitors on Ramos cell survival, treated with TNIIIA2. (c) Cell surface exposure of phosphatidylserine on Ramos cell treated with TNIIIA2 (figures were modified from Figure 2 of [19]). $^{*}P < 0.05$ versus TNIIIA2 single treated sample, $^{**}P < 0.05$ versus untreated control.

protein of the microenvironment niche in the bone marrow and fetal liver [36–40].

In the case of erythropoiesis, the importance of the cell adhesion of erythroid progenitors to FN via the FN-receptors VLA-4 and VLA-5 has been demonstrated [22, 40–44]. A number of previous studies demonstrated direct adhesion of erythroid progenitor cells to FN. FN functions as an adherent substrate scaffolding erythroid progenitor cells to support their survival and proliferation [22, 40]. Furthermore, it has been postulated that adhesive interaction with FN via FN receptors contributes to the regulation of erythroid differentiation [22, 40–44]. In particular, the importance of VLA-4-mediated adhesion to FN and/or VCAM-1 on stroma cells has been implicated by *in vitro* and *in vivo* studies using antagonist for VLA-4 and VLA-5 [22, 40–44]. However, the substantial role of these FN receptors and their functional assignment in erythroid differentiation were not fully understood.

We recently reported that hemin-induced erythroid differentiation was greatly enhanced when K562 cells were forced to adhere to FN by activating VLA-5 with TNIIIA2 (Figures 4 and 5). Since FN receptor antagonists abrogated the acceleration of erythroid differentiation, the stimulatory effect of TNIIIA2 on erythroid differentiation might be dependent on adhesion of K562 cells to FN (Figures 4, 5(a) and 5(b)). The adhesion-dependent acceleration of hemin-induced erythroid differentiation may be responsible for the VLA-5-mediated adhesion to FN, because K562 cells reportedly express only VLA-5 as the FN receptor [45, 46]. Nevertheless, the stimulatory effect of TNIIIA2 on hemin-induced erythroid differentiation was abrogated not only by a VLA-5 antagonist (RGD peptide) but surprisingly also by a VLA-4 antagonist (CS-1 peptide) (Figure 5(c)). This conflicting result was explained by the observations that forced adhesion to FN resulted in the induction of VLA-4 expression in K562 cells [45].

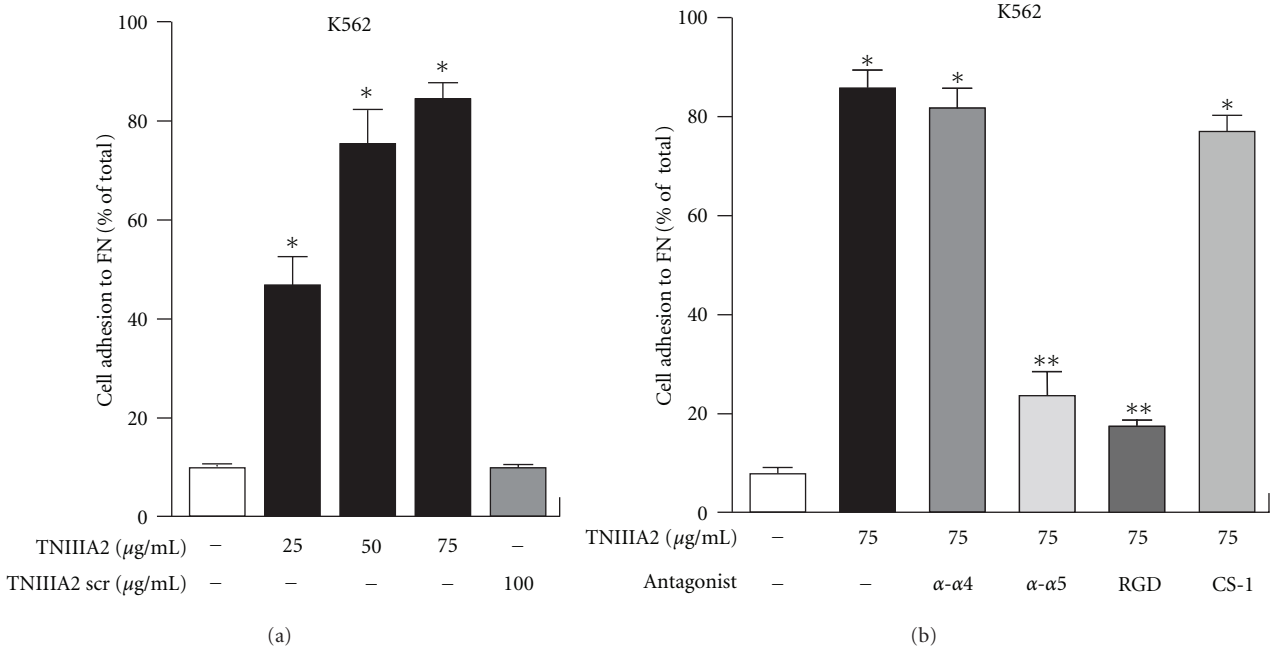

Figure 4: Adhesion of K562 cells to FN through $\alpha 5\beta 1$ integrin activation. (a) Dose dependency of TNIIIA2-induced adhesion of K562 cells. (b) Effects of antagonist for VLA5 (α-$\alpha 5$ Ab and RGD) and VLA4 (α-$\alpha 4$ Ab and CS-1) on TNIIIA2-induced adhesion to FN in K562 cells (figures were modified from Figure 1 of [45]). $^{*}P < 0.05$ versus untreated control, $^{**}P < 0.05$ versus TNIIIA2 single treated sample.

Table 1: Expression level of cell adhesion-relating molecules (VLA-4, 5, and syndecan-4) and induced cell adhesion or apoptosis by TNIIIA2 or Mg^{2+} administration in fresh AML cells from patients, peripheral blood cells from healthy adults, and hematopoietic tumor cell lines (the table was modified from [19]).

Cells	Expression (%)			Adhesion		Apoptosis	
	VLA-4	VLA-5	Syndecan-4	+TNIIIA2	$+Mg^{2+}$	+TNIIIA2	$+Mg^{2+}$
"Fresh AML cells"							
Patient A	98.2	88.8	48.8	++	++	+	++
Patient B	97.5	98.5	9.5	–	+	–	++
"Peripheral blood cells"							
Neutrophil	6.7	N.D.	3.2	–	–	–	–
Monocyte	48.6	N.D.	2.0	–	++	–	++
Lymphocyte	40.5	N.D.	4.4	–	++	–	+
"Cell lines"							
B cell							
Ramos	96.5	3.2	92.1	+++	++	+++	++
Raji	94.3	65.3	2.3	–	++	–	++
T cell							
Jurkat	92.8	96.6	42.4	+++	++	+++	++
Erythroid							
K562	9.3	97.2	66.7	+++	++	–	–
Myeloid							
U937	98.1	98.2	87.5	+++	++	++	+++
HL60	99.8	99.7	75.3	+++	++	++	+
THP1	68.5	20.7	30.1	++	++	++	+
THP1 (+PMA)	10.5	18.7	99.2	++	++	–	–

Expression of VLA-4, 5 and syndecan-4 was evaluated by flowcytometric analysis.

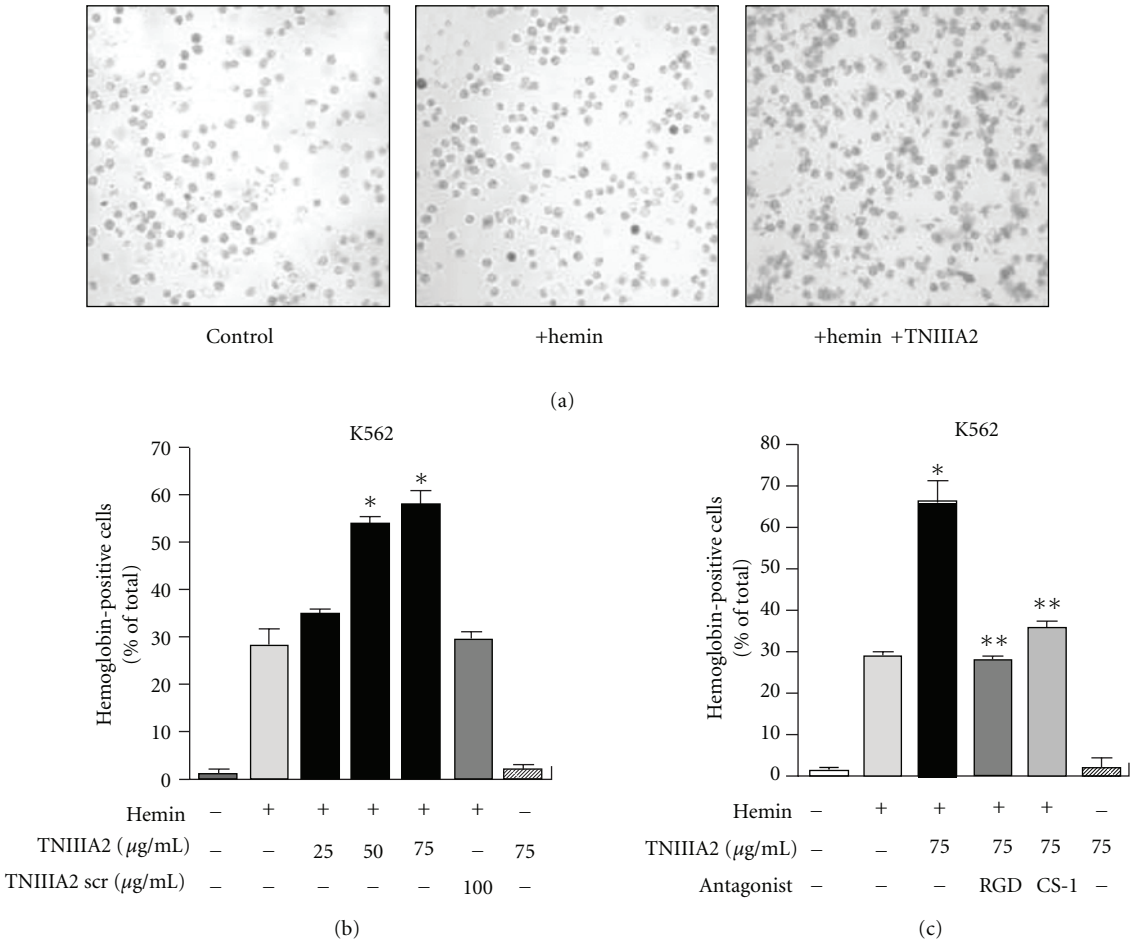

Figure 5: Adhesion dependent acceleration of hemin-induced erythroid differentiation of K562 cells. (a, b) Effect of TNIIIA2 on erythroid differentiation of K562 cells, induced by hemin. Typical image of erythroid differentiation was shown in (a). (c) Effects of antagonist for VLA5 (RGD) and VLA4 (CS-1) on TNIIIA2-induced acceleration of erythroid differentiation (figures were modified from Figures 2(a), 3(a), and 3(c) of [45]). $^{*}P < 0.05$ versus Hemin single treated sample, $^{**}P < 0.05$ versus Hemin and TNIIIA2 treated sample.

Several studies demonstrated that activation of p38 and/or JNK but not ERK is required for erythroid differentiation induced by butyrate [47], erythropoietin [48], hydroxyurea [49], or hemin [50], although another study reported the involvement of ERK in erythroid differentiation induced by hemin [51]. We also observed that the phosphorylation of p38, which was shown to play a crucial role in hemin-induced erythroid differentiation and its acceleration by TNIIIA2, was suppressed by antagonists for VLA-4 and -5 [45]. From these observations, we supposed that prolonged adhesion to FN, mediated through VLA-5, induced VLA-4 expression in K562 cells and the resulting adhesive interaction of FN with newly expressed VLA-4 participated in differentiation via phosphorylation/activation of p38 MAP kinase, which was shown to serve as a signaling molecule crucial for hemin-induced erythroid differentiation. It has also been demonstrated that TN-C on bone marrow stromal cells may play an important role in erythropoiesis [52]. As mentioned above, our observations suggest that the peptide derived from TN-C, TNIIIA2, can accelerate hemin-induced erythroid differentiation. TN-C is known to be abundantly expressed in the stromal cells of immune organs including the bone marrow [15, 16] and is susceptible to proteolytic modification [53]. Therefore, it might be possible that inducing the exposure of TNIIIA2 region works as a beneficial therapeutic treatment reducing one of symptoms in tumor, "poor differentiation".

3. Hyperstimulation of Nontransformed Cell Proliferation by TNIIIA2

It is well known that normal adherent cell types, such as fibroblastic and epithelial cells, undergo apoptosis like cell death when the β1-integrins of these cells lose the interaction with ECM. This process is termed "anoikis" [54, 55] and plays a fundamental role preventing dissemination of the cells to inappropriate site. It is also well accepted that tumor cells develop anoikis-resistance, resulting in acquisition of metastatic ability. Thus, understanding the mechanisms how tumor cells evade anoikis is important.

Recently, we found that detachment-induced cell death, which was repressible by Z-VAD, general caspase inhibitor,

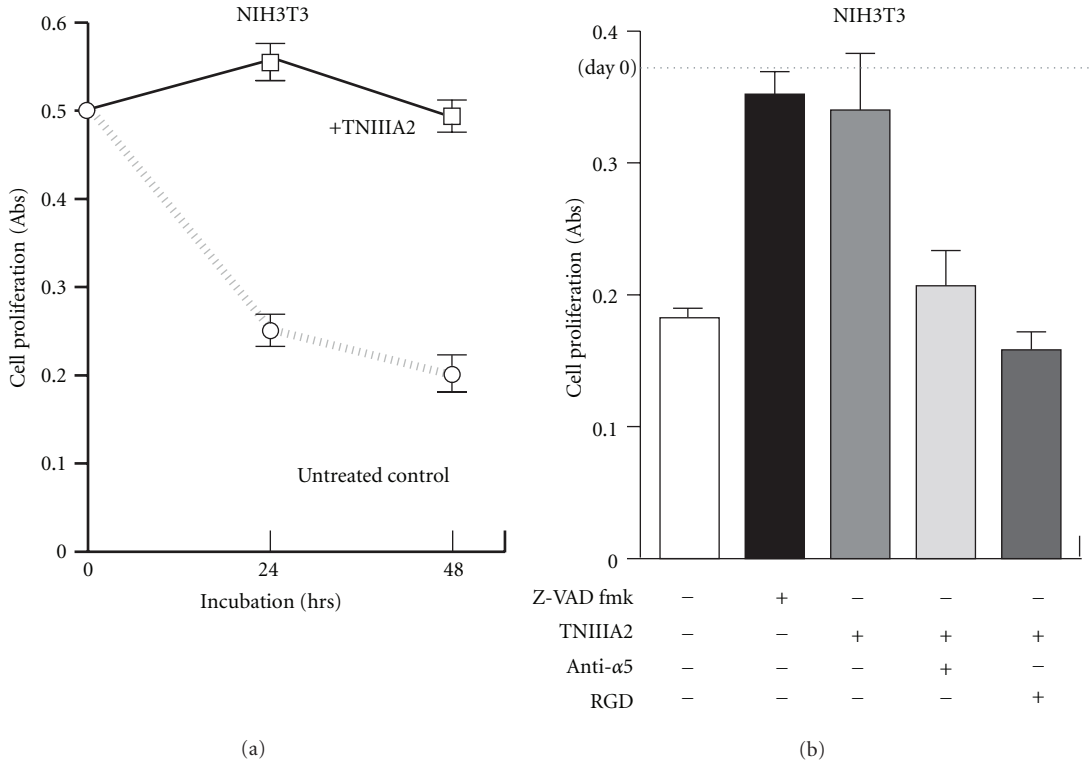

Figure 6: TNIIIA2 protects cells from anoikis by activating the β1-integrin. (a) Time-course study of the effect of cell-detachment in survival/proliferation of NIH3T3 cells. (b) Effect of inhibition of apoptosis (Z-VAD) or $\alpha5\beta1$ integrin signal (anti-α5 Ab and RGD peptide) in detachment-induced cell death (our unpublished observation).

was completely blocked by TNIIIA2 administration (Figures 6(a) and 6(b)). This antianoikis effect of TNIIIA2 was abolished by inhibition of $\alpha5\beta1$ integrin (VLA-5) (Figure 6(b)). Activation of Akt and upregulation of Bcl-2 were observed in consistent with inhibition of the detachment-induced cell death by TNIIIA2 (data not shown). These results suggest that TNIIIA2 has a potential to render cells resistant to anoikis.

Platelet-derived growth factor (PDGF) works as a potent mitogen for both untransformed and transformed mesenchymal cells. The binding of PDGF to its receptor PDGFR induces the activation of its intrinsic kinase, which infers activate the Ras/MAP kinase pathway [56]. However, it has also been established that cell proliferation does not occur unless the cells are adhered to the extracellular matrix (ECM) via integrins [57]. Thus, adhesion receptor integrins, as well as growth factor receptors, play an indispensable role in cell proliferation. The collaboration of signaling by integrin ligation with signaling by growth factor receptors is known to enable to amplify the magnitude and duration of activation status in the MAP kinase/ERK pathway.

In our investigation, TNIIIA2 showed the ability to accelerate PDGF-induced proliferation of NIH3T3 cell on FN-coated culture dish (Figure 7). Similar to the effect of TNIIIA2, 9EG7, an anti-β1 integrin monoclonal antibody, which has the ability to activate β1-integrin, also enhanced the PDGF-dependent cell proliferation. Inhibition of α5-integrin mediated cell adhesion, but not of α4- and β3-integrin, could attenuate the effect of TNIIIA2 (unpublished observations), suggesting that stimulation of NIH3T3 cell proliferation by TNIIIA2 is due to activation of β1-integrins. In this condition, it was also observed that stimulation of NIH3T3 cell proliferation by TNIIIA2 promotes the autophosphorylation of PDGFR, in which both PDGFR and β1-integrin were colocalized in caveolae (data not shown). These observations suggest the existence of crosstalk between ECM signaling and PDGF signaling in cell proliferation. Therefore, the antagonistic drug targeting TNIIIA2-related active site in TN-C molecule, such as anti-TNIIIA2 antibody, might become a new therapeutic drug candidate for diseases relating hyperstimulated cell growth, such as in tumor progression.

4. Summary and Future Perspectives

Besides the developing cancer, several researchers recently reported the parallel relationship between TN-C expressions and severity of various diseases, such as chronic liver disease, cardiac infarction, and arthritic joint disease [58–60]. In fact, it has been shown that the ECM proteins, such as TN-C, harbor functional sites within their molecular structure, and

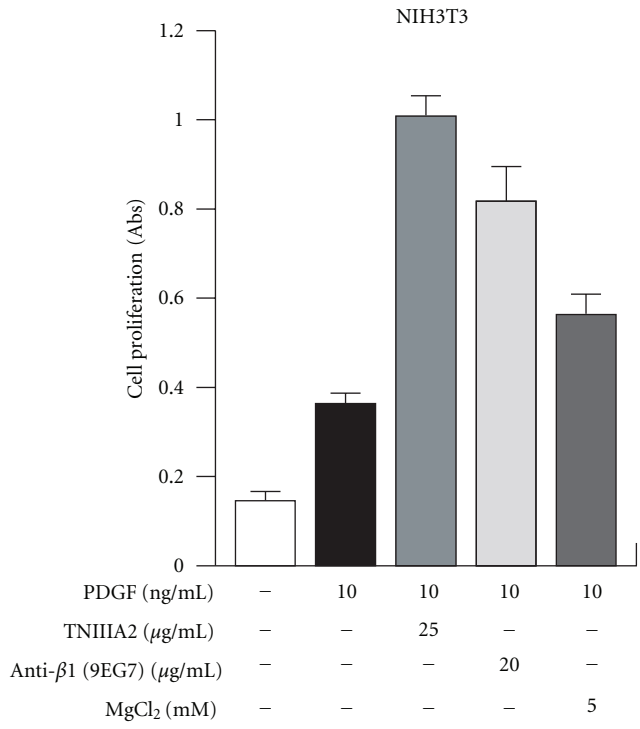

Figure 7: TNIIIA2 accelerates PDGF-induced cell proliferation. PDGF-induced cell growth was enhanced by addition of TNIIIA2, as well as administration with integrin activators (anti-β1 and $MgCl_2$) (our unpublished observation).

these cryptic active sites are disclosed by proteolytic degradation with inflammatory proteinases, including MMPs [5, 61, 62]. Thus, the peptide TNIIIA2 might become a powerful tool for understanding these diseases through the concept "signaling disorder by unusual cell adhesion".

In the case of cancer progression, we mentioned in this review that TNIIIA2 shows a host-beneficial effect in leukemic situation by inducing apoptosis and/or differentiation. Consistent with our results, it has been reported that the loss of integrin-mediated adhesion resulted in decreased sensitivity to chemotherapy in melanoma [63, 64]. On the other hand, Stupack et al. have reported that unligated integrins trigger apoptotic cell death without any death-inducing signals [65]. Moreover, Ileć et al. have also been reported that integirin-mediated adhesion can promote cell survival although these cells are exposed to stress-associated apoptotic signals [54]. From these facts, we presumed that the ability of TNIIIA2 to induce strong and sustained activation of β1-integrins is the key factor in modulating cell survival. We already found a cryptic peptide, FNIII14 from FN, and reported that simultaneous administration of this peptide itself with anticancer drug effectively overcomes CAM-DR of AML [25]. In a series of observations using TNIIIA2, this peptide might be capable for regulating cell survival, growth, and differentiation via controlling cell adhesion to ECM. Since tumor cell is characterized by its immortality, hyper-proliferation, and poor differentiation, there is a possibility that the peptide TNIIIA2 might become a useful therapeutic target for cancer treatment. However, at present, several questions still remain unclear. For example, the regulatory mechanism of TNIIIA2 exposure is not fully explored. Effect of TNIIIA2 or its antagonist *in vivo* should also be tested using tumor transplantation model. Further examinations are expected.

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Review Article

HIC-5: A Mobile Molecular Scaffold Regulating the Anchorage Dependence of Cell Growth

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HIC-5 is a multidomain LIM protein homologous to paxillin that serves as a molecular scaffold at focal adhesions and in the nucleus. It forms mobile molecular units with LIM-only proteins, PINCH, and CRP2 and translocates in and out of the nucleus via a nuclear export signal (NES). Of note, NES of HIC-5 is distinctive in its sensitivity to the cellular redox state. Recently, the mobile units of HIC-5 have been suggested to be involved in the regulation of the anchorage dependence of cell growth. On loss of adhesion, an increase in reactive oxygen species in the cells modifies NES and stops shuttling, which leads to cell-cycle control. More specifically, the system circumvents nuclear localization of cyclin D1 and transactivates $p21^{Cip1}$ in detached cells, thereby avoiding anchorage-independent cell growth. Thus, the HIC-5-LIM only protein complex has emerged as a fail-safe system for regulating the anchorage dependence of cell growth.

1. Introduction

Hydrogen peroxide-inducible clone 5 or *Hic-5* is a gene we isolated by subtractive hybridization in 1994 as a cDNA clone induced by transforming growth factor β (TGF-β) or hydrogen peroxide [1]. At that time, we studied TGF-β signalling and pursued the possibility that reactive oxygen species (ROS) function was an intracellular TGF-β signal. After isolating the gene, we conducted a number of studies of *Hic-5* at a molecular as well as cellular level. Its amino acid sequence revealed that HIC-5 is a homologue of paxillin, which is a multidomain LIM (Lin-11, Isl-1, and Mec-3) protein that is localized at focal adhesions and was originally identified as a substrate of the *v-src* oncogene [2] (Figure 1). Together with its family members (Leupaxin specifically expressed in lymphocytes, PaxB, an orthologue of paxillin in slime mold, and HIC-5), paxillin has now been established as a molecular adaptor that transduces signals in response to changes in the adhesion environment of cells. A famous example of a molecular adaptor is the Grb2-SOS system that transduces signals from growth factor receptors to RAS. Paxillin transduces signals from extracellular matrix receptors, integrins, to intracellular downstream molecules such as MAP kinase.

Of these family members, HIC-5 is most homologous to paxillin, and thus, analyses of HIC-5 have been conducted in reference to and in comparison with paxillin. For example, the intracellular localization of HIC-5 is, like paxillin, mainly confined to so-called focal adhesion sites where cells adhere to the extracellular matrix via integrins. In terms of expression in tissues and cell types, paxillin is relatively ubiquitously expressed, whereas expression of HIC-5 is prominent in the smooth muscle layer of tissues such as the large intestine and uterus [3]. Furthermore, expression of HIC-5 is relatively high in the lung and spleen [1]. In cell culture systems, HIC-5 expression is detectable in most cell lines with varying degrees of expression. High expression of HIC-5 is detected in mesenchymal cell lines including fibroblastic and osteoblastic cell lines; however, it is generally low in epithelial cell lines. In a knockout mouse model, HIC-5 was suggested to be inessential for the development and maintenance of homeostasis of the animal, and no remarkable functional abnormality was found under standard rearing

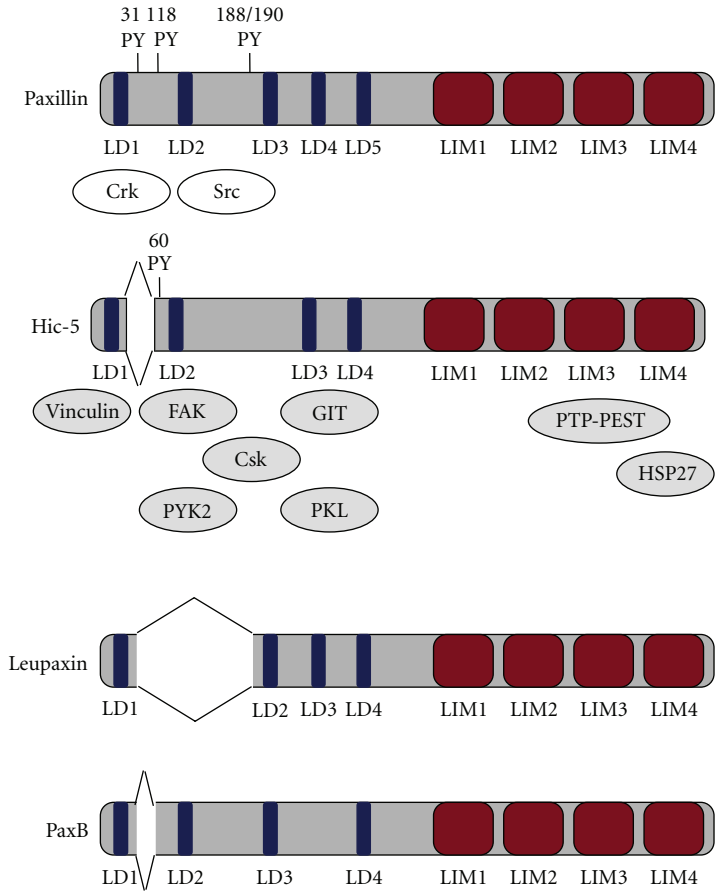

Figure 1: The paxillin/focal adhesion-associated adaptor protein family; domain structure and binding factors. The paxillin family includes HIC-5, Leupaxin, which is preferentially expressed in hematopoietic cells, and PaxB, an orthologue of paxillin in the slime mould *Dictyostellium discoidium*. The family members share many of their structural characteristics and binding factors. They have four to five leucine-rich motifs (LD repeats) in the N-terminal half and four cysteine-rich regions composed of two zinc fingers (LIM domains) in the C-terminal half. These domains mediate the protein-protein interactions that allow paxillin to bind a variety of structural and signalling molecules, such as the structural protein vinculin, the SH2-SH3 adaptor protein Crk, Src, focal adhesion kinase (FAK), PTK2B protein tyrosine kinase 2 beta (PYK2), a negative regulator of Src, the Csk nonreceptor tyrosine kinase, the G protein-coupled receptor kinase interactor Arf GAP1 (GIT-1), paxillin-kinase linker (PKL), protein tyrosine phosphatase-PEST (PTP-PEST), and heat shock protein 27 (HSP27). The LIM domains also mediate the localization of Hic-5 at the nucleus and at focal adhesions. HIC-5 has the same binding partners, except for Crk and Src, as paxillin.

conditions [4]. In contrast, the paxillin knockout mouse is reportedly embryonic lethal [5]. Similar to fibronectin, it exhibits abnormal development of extraembryonic tissues and heart and body segmentation, resulting in death at 9.5 foetal days. The embryonic lethality of the paxillin knockout mouse means that HIC-5 cannot substitute the functions of paxillin, at least those associated with development. These results together with the abovementioned differences in expression patterns indicate that it is most likely that paxillin and HIC-5 have different functions in mammals.

2. Structure of HIC-5 and Interacting Factors

The genomic structure of *Hic-5* features a long intron between the N-terminal and C-terminal domains, a sign that *Hic-5* evolved from the fusion of two different genes [6]. Accordingly, the protein structure can also be broadly divided from the centre into N-terminal and C-terminal regions.

The N-terminal region comprises four domains, the LD domains, which are rich in Leu and Asp; LD1 is deleted in one isoform. The C-terminal region comprises four LIM domains having two zinc fingers (Figure 1). These features are almost identical to those of paxillin, with minor differences in the number of LD domains in the N-terminal region (five for paxillin and four for HIC-5). Given that both the LD and LIM domains are protein-protein interacting domains, it is naturally assumed that paxillin family members are adaptor molecules that provide multiple proteins with interfaces to facilitate their interaction and cooperation.

Based on these structural features, further analyses have successfully identified a number of interacting factors. In particular, in the case of adaptor molecules, identification

of their interacting factors is crucial for inferring their functions. Several interacting factors of paxillin have been identified previously. Therefore, we began to study their interaction with HIC-5. Isolation of new interacting factors was also attempted, and we found an array of proteins that interact with each of the LD and LIM domains of HIC-5 [2] (Figure 1). Consistent with the localization of HIC-5 at focal adhesions, most of the factors identified are those contained in the cytoplasmic domain complex of integrins (discussed below) and are involved in integrin signal transduction and/or in the control of actin cytoskeleton dynamics. More specifically, the factors include signalling molecules such as protein tyrosine kinase 2 beta (PYK2), *c-src* tyrosine kinase (Csk), and focal adhesion kinase (FAK) [7, 8]. Using a yeast two-hybrid method with the N-terminal and C-terminal domains of HIC-5 as bait, we originally characterized G protein-coupled receptor kinase interacting Arf GAP1 (GIT-1) as an LD3 interacting factor [9] and protein tyrosine phosphatase, nonreceptor type 12 (PTP-PEST) as an LIM3 interacting factor [10]. Vinculin and talin are structural proteins involved in the architecture of focal adhesions and the actin cytoskeleton, and these also interact with HIC-5 [8].

Most of the factors that bind to HIC-5 also interact with paxillin although paxillin additionally binds Src and Crk (*v-crk* sarcoma virus CT10 oncogene homologue), which do not bind to HIC-5 [2, 8]. Accordingly, HIC-5 and paxillin hypothetically compete for these interacting factors. This difference is presumably because of the presence of tyrosine residues (Y31, Y118, Y188, and Y190) in paxillin, which do not exist in HIC-5 although no definitive answer has been obtained yet. Furthermore, it is known that paxillin binds the cytoplasmic domains of $\alpha 4$, $\alpha 9$, and $\beta 3$ integrins [11], and HIC-5 interacts with the domain of $\alpha 4$. No particular pattern of interaction has been identified for the LD and LIM domains of HIC-5 and paxillin and their corresponding interacting factors. Thus, the study as above identified only factors whose localization and functions are related to focal adhesions. Furthermore, no factors that interact specifically with HIC-5 were identified.

HIC-5 has also been found to be localized in actin stress fibers, while paxillin is not [3]. Here, HIC-5 appeared to interact with cysteine-rich protein (CRP) 2, which belongs to the LIM-only group of proteins [3]. This finding prompted us to search for other LIM-only proteins that interact with HIC-5, and we identified a particularly interesting new cysteine and histidine-rich protein (PINCH) that binds HIC-5 [12]. Further investigation demonstrated that LIM4 in the C-terminal is important for HIC-5 interaction with these LIM-only proteins. In other words, the fourth LIM domain located at the endmost C-terminal region has a unique property, whereby it interacts with the same LIM domain, thus enabling HIC-5 to form LIM-LIM homo- and hetero-oligomers [12]. Consequently, HIC-5 exists as homo-oligomers in cells, which appear to be important for HIC-5 localization in the nuclear matrix (unpublished data). Hetero-oligomer formation between HIC-5 and PINCH/CRP2 is essential for the two LIM-only proteins to localize at particular sites in cells [12]. This means that HIC-5 directs their subcellular localization (as discussed below). It should be noted that LIM4 of paxillin cannot form oligomers and does not interact with PINCH or CRP2. This difference is surprising given the high structural homology between HIC-5 and paxillin. Another molecular difference is that paxillin is highly tyrosine-phosphorylated during adhesion and growth signalling, whereas HIC-5 is not generally tyrosine-phosphorylated to a significant level. This might be explained by the fact that HIC-5 has no tyrosine residues *per se* to be phosphorylated [13]. Thus, unlike paxillin, HIC-5 appears to not have a function in adhesion and growth signalling based on tyrosine phosphorylation although in human platelets, HIC-5 has been shown to be tyrosine-phosphorylated with aggregation.

Studies at a molecular level have characterized paxillin and HIC-5 as being tyrosine-phosphorylated and forming LIM-LIM oligomers, respectively, which possibly underlies the functional difference between them. Paxillin may transduce adhesion/growth signalling, and HIC-5 organizes a fail-safe system for the adhesion dependence of cell growth, as discussed below.

3. Functions of HIC-5 at Focal Adhesions and in the Nucleus

Adhesion between cells and the extracellular matrix is achieved by integrins, a group of transmembrane proteins that act as receptors for the extracellular matrix. The resultant adhesion of cells to the extracellular matrix, in turn, promotes aggregation between integrins, resulting in the formation of a protein complex known as a focal adhesion at the site of adhesion. Importantly, a focal adhesion is not just a physical structure but also a molecular apparatus that senses the adhesion status between cells and the extracellular matrix and sends signals to the inside of cells, thereby coordinating their motility, survival, and proliferation in response to adhesion status [14, 15]. During signalling by integrins whose cytoplasmic domain is very short and contains no enzymatic catalytic domain, multiple proteins assemble at the cytoplasmic domain through protein-protein interactions, forming an undercoat structure. These proteins include several enzymes such as FAK and SRC, through which the signal is emitted to the inside of the cell [14, 15].

HIC-5 and paxillin act as adaptor molecules in this integrin undercoat complex and play roles in controlling enzyme activity by mediating the above-mentioned interactions between binding factors, thereby participating in the regulation of integrin signalling. A detailed study has shown that HIC-5 plays a role in negatively regulating FAK by competing with paxillin [16]. However, under normal culture conditions, cellular behaviours including growth and motility are hardly affected by HIC-5 expression in most cases. Therefore, HIC-5 appears to not be required for any particular function at focal adhesions under normal cellular conditions so long as the adhesion status is maintained. Normal phenotype observed in the knockout mice strongly supports this idea. Rather, HIC-5 may have a critical function in suppressing excess changes in adhesion and the cytoskeleton structure by antagonizing paxillin when the maintenance of healthy adhesion is in jeopardy under

stressful conditions affecting adhesion [3, 16]. In sharp contrast, abnormal cell motility and spreading is observed under normal conditions in paxillin$^{-/-}$ cells in which the focal adhesions are disorganized and integrin signalling by FAK and the downstream MAP kinase is disturbed [5].

In parallel with localization at focal adhesions, HIC-5 simultaneously shuttles between focal adhesions and the nucleus, as described below. This fact lead us to investigate the possible function of HIC-5 in the nucleus, and a series of study demonstrated that HIC-5 was capable of regulating expression of the *c-fos* and p21^{Cip1} genes [17, 18]. This involvement in transcription was not observed with paxillin. Further study demonstrated that HIC-5 promotes the formation of a transcriptional complex, specifically with the transcriptional coactivator p300 and transcription factors Sp1 and Smad3, on DNA [18]. In other words, HIC-5 appears to function as an adaptor for transcription-associated factors in the nucleus by acting as a scaffold for transcriptional complex formation, as is the case for integrin signalling at focal adhesions. A similar function of HIC-5 in the nucleus has been suggested by other researchers who recloned HIC-5 as an androgen receptor coactivator 55 kDa protein (ARA55), in 1999 [19, 20]. It has also been reported that HIC-5/ARA55 is a coactivator of PPARγ and others [21, 22]. Taken together, these results indicate that HIC-5/ARA55 may serve as a scaffold for a fairly wide range of transcriptional activities in the nucleus.

In summary, a growing body of evidence suggests that the function of HIC-5 in the nucleus is to facilitate transcription complex formation by interactions with several transcription-associated factors. However, taking into consideration the fact that under normal conditions, HIC-5 is mainly localized at focal adhesions and not in the nucleus at a detectable level, we should be cautious about this conclusion. Rather, we postulate that HIC-5 has some roles in the nucleus, primarily under conditions where focal adhesions have been disrupted and that localization of HIC-5 in the nucleus then becomes discernable.

4. Nuclear-Cytoplasmic Shuttling of HIC-5 and Its Biological Significance: A Fail-Safe System for Anchorage Dependence of Cell Proliferation

In 2003, we found that HIC-5 shuttles between focal adhesions and the nucleus [23]. Our initial observations indicated that HIC-5 changed its localization to the nucleus following treatment with hydrogen peroxide, suggesting that besides focal adhesions, HIC-5 can also localize in the nucleus based on the cellular conditions. Finally, we found that HIC-5 shuttles between focal adhesions and the nucleus constantly in normal adhesion cells and that it does so in company with PINCH and CRP2, its LIM-LIM hetero-oligomer partners.

This molecular-level analysis of the shuttling ability of HIC-5 identified NES around the LD3 domain [23]. A nuclear localization signal is carried by four LIM domains in the C-terminal region. Of note, there are specific cysteine residues upstream of the NES consensus sequence in HIC-5 that enable NES to sense the intracellular redox state. Because of this, NES is interrupted to stop the export of HIC-5 from the nucleus under conditions such as hydrogen peroxide treatment, which results in the accumulation of the protein in the nucleus [23]. In short, HIC-5 shuttles between focal adhesions and the nucleus in normal situations, but it accumulates and becomes functional in the nucleus when the intracellular redox state becomes more oxidised. Of interest, when cells are deprived of adhesion to a substrate, the ROS concentration in the cells increases [24].

These findings indicate that HIC-5 is a shuttle protein and an ROS effector, just as the name suggests. Most ROS effectors are signalling molecules centred on tyrosine phosphatase or transcription factors. HIC-5 is probably the first ROS effector to act as an adaptor. In addition, HIC-5 is unique in that it uses ROS as a switch for changing its localization by sensing ROS in NES. A group of LIM proteins including paxillin, zyxin, LLP, and a thyroid hormone receptor-binding factor, Trip 6, has also been shown to communicate between focal adhesions and the nucleus although the controlling mechanisms and significance of this shuttling are poorly understood [25].

Next, we explored the significance of the shuttling of HIC-5 between the two compartments. Hypothetically, by shuttling, HIC-5 connects the adhesion status with nuclear activity. We focused on the adhesion (anchorage) dependence of cell proliferation as an example of a phenomenon that requires such a coupling mechanism.

Numerous studies have defined the roles of adhesion signals mediated by integrin-extracellular matrix (ECM) interaction in cell-cycle progression, Basically, integrin-ECM-mediated signaling potentiates and prolongs the growth factor receptor-mediated mitogenic signalling and is required from mid- to late-G_1 phase in various events associated with cell-cycle progression, such as upregulation of G_1-phase cyclin-dependent kinase (CDK) activity, Cip/Kips downregulation, association of cyclin E with CDK2, pRB phosphorylation, and cyclin A expression. As a result, loss of adhesion generally causes complete G_1 phase cell-cycle arrest in nontransformed cells; moreover, in susceptible cells, it leads to anoikis, a specific type of apoptosis caused by the detachment of a cell from its supportive matrix, which was first described in epithelial and endothelial cells [26, 27]. In contrast, transformed cells usually circumvent the anchorage requirement in cell-cycle progression. Their anchorage-independent survival and growth is well known as a hallmark of cellular transformation and correlates with tumorigenicity *in vivo*. Mechanistically, the anchorage-independent growth is considered to be based on an abnormal activation of the G_1-phase cyclin CDKs uncoupled from anchorage. In general, an oncogenic pathway activates a robust and/or constitutive mitogenic signal, which is presumed to reduce the requirement for integrin-ECM-mediated signalling and its importance as a booster of growth factor receptor-mediated mitogenic signalling in the transformed cells. Among the downstream pathways of oncogenic signals, the activation of the phosphatidylinositol 3-kinase/Akt pathway is crucial for the induction of anchorage-independent growth and cell survival.

Among the events required for the cell-cycle progression, activation of CDK4/6 by cyclin D is one of the most important events, because it promotes the G_1/S transition; that is, it serves as a determinant of cell-cycle progression along with cyclin E/CDK2 activity. Importantly, activation of this complex is sensitive to adhesion status as well as growth stimuli, and it plays a role in coupling adhesion status with cell-cycle progression. Thus, under conditions of inappropriate adhesion, it operates by circumventing progression of the cell cycle. In other words, besides acting as an engine of cell-cycle progression, CDK4/6-cyclin D functions as a security system for the anchorage dependence of cell proliferation. Mechanistically, the expression level of cyclin D is regulated not only by growth signals but also by adhesion signals, and an insufficiency of either signal type decreases its expression [28, 29]. The level of expression of other cyclins rather automatically cycles up and down with progression of the cell cycle, as their name suggests. Thus, Cyclin D is a key molecule in regulation of the cell cycle, and abnormal cyclin D behaviour is directly linked to aberrant proliferation of cells, which is supported by the fact that cyclin D is a proto-oncogene.

Accordingly, not only the amount of cyclin D in cells but also its subcellular localization is rigorously regulated by signalling, and cyclin D localizes in the nucleus only during G_1/S phase, when nuclear localization is essential for its function; it is exported from the nucleus during the other phases [30]. When this does not occur, it becomes oncogenic, as has been shown by observations, where the nuclear localization of cyclin D promoted cell proliferation; its oncogenic potential is closely related to its nuclear localization ability [31]. Regulation of the nuclear localization of cyclin D is so important that we hypothesized that it may also be regulated by adhesion status. Therefore, we examined the relationship between the nuclear localization of cyclin D1 and the adhesion status of cells. In adherent cells at G_1 phase, cyclin D1 is localized in the nucleus. However, this localization changes immediately to the cytoplasm when cells are suspended [24]. This result supports the above hypothesis that the nuclear localization of cyclin D1 is also regulated by the adhesion status. This regulation would be of great help in avoiding unacceptable cell growth when cells are detached; most interestingly, HIC-5 is deeply involved in this mechanism. Basically, HIC-5 and cyclin D1 translocate between the cytoplasm and nucleus through the same CRM 1-dependent nuclear export system, and for this reason, they compete for this export system. The presence of PINCH with HIC-5 is important for the competition between HIC-5 and cyclin D1. A critical step is that HIC-5 stops shuttling in cells on loss of adhesion, the consequence of which is the export of cyclin D1 in place of HIC-5 outside the nucleus. The arrest of shuttling in nonadherent cells is caused by an elevated level of ROS, which inactivates NES [24].

To summarize, the biological significance of the shuttling of HIC-5 is competitive localization of cyclin D1 in the nucleus in adherent cells with the aid of PINCH. Importantly, the shuttling of HIC-5 is in the off state in nonadherent cells. This results in critical acceleration of the export of cyclin D1 to the cytoplasm and in the arrest of cell proliferation under abnormal conditions. This mechanism, which is regulated by the on/off status of HIC-5 shuttling, functions as a fail-safe system for ensuring the anchorage dependence of cell proliferation (Figure 2).

Most recently, we found an additional mechanism involving HIC-5 for inducing growth arrest in detached cells. The mechanism targets $p21^{Cip1}$, a cyclin-dependent kinase inhibitor that is famous as the downstream target of the tumour-suppressor gene p53. This gene is well known to be transactivated by p53 in response to a variety of stresses and to stop G_1/S progression by inhibition of cyclin D-CDK4/6 or cyclin E-CDK2. Of note, when cells are deprived of adhesion, $p21^{Cip1}$ is upregulated and plays a critical role in growth arrest [29]. Our recent investigation suggests that HIC-5 participates in the transactivation of $p21^{Cip1}$ in response to loss of anchorage. In this case, HIC-5 cooperates with CRP2, another LIM-only protein shuttling partner, to transactivate the KLF4 and Runx1 sites upstream of the gene (unpublished data).

In conclusion, HIC-5 has emerged as a critically important molecule for regulation of the anchorage dependence of cell proliferation. More specifically, HIC-5 organizes two types of mobile LIM-LIM oligomer platforms with PINCH and CRP2, which collaboratively prevent anchorage-independent cell growth. For this purpose, HIC-5/PINCH regulates the nuclear localization of cyclin D and HIC-5/CRP2 is operational in the transactivation of $p21^{Cip1}$ (Figure 3).

5. Adhesion Status of Cells and HIC-5

A series of studies by us and others have suggested interesting possibilities for the function of HIC-5 at focal adhesions and in the nucleus. We also have originally addressed its role as a mobile scaffold for the anchorage dependence of cell growth. However, in terms of an active role of HIC-5 under normal adhesion conditions, the evidence presented thus far is not fully convincing for either localization. We speculate that under normal adhesion conditions, HIC-5 senses the adhesion status based on the fact that its NES is protected from attack by ROS in the adhesion complex and is able to assist HIC-5 shuttling. Once the cells lapse into a state of abnormal adhesion, such as detachment from the matrix, the adhesion structure becomes disrupted along with ROS production; under such conditions, NES of HIC-5 is unmasked, becomes exposed to ROS, and is modified to stop the shuttling. When shuttling of HIC-5 is stopped, cell growth is arrested through the mechanisms involving cyclin D and $p21^{Cip1}$. In other words, HIC-5 organizes a fail-safe system that ensures the anchorage dependence of cell growth. The activation mechanism of this system is oxidative modification of NES of HIC-5 in response to increased production of ROS as well as the collapse of the focal adhesion complex. At this point, it should be noted that the interaction between FAK and HIC-5 is also sensitive to ROS. This is because FAK and HIC-5 interact through LD3 of HIC-5, which contains NES, and this interaction is regulated by the upstream cysteines in the same manner as for NES [32]. Therefore, the interaction between FAK and HIC-5 is

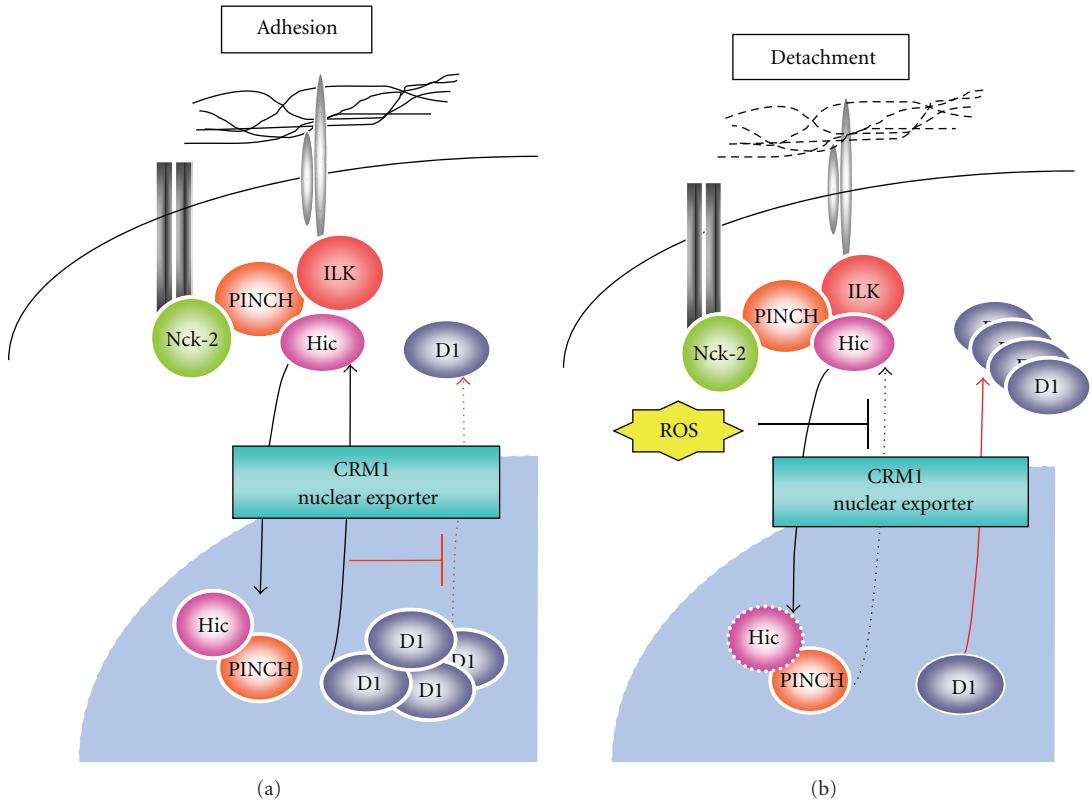

Figure 2: A mobile HIC-5/PINCH scaffold regulating cyclin D1 subcellular localization in response to adhesion status. Cyclin D1 is retained in the nucleus in adherent cells such as mouse primary fibroblasts, C3H10T1/2 fibroblasts, and mammary epithelial NMuMG cells by the CRM1-dependent nuclear export of HIC-5 with the aid of PINCH, which counteracts the nuclear export of cyclin D1. The higher affinity of HIC-5 for CRM1 favours the export of HIC-5. In detached cells, the nuclear export of HIC-5 is inhibited by elevated levels of ROS, and cyclin D1 is actively transported out of the nucleus, which results in a decrease in its nuclear localization (see further details in [24]).

also lost under abnormal adhesion conditions, suggesting that the functionality of NES and the complex formation of HIC-5, at least with FAK, in the focal adhesion complex are intimately interrelated.

In an earlier section, we discussed the role of HIC-5 at focal adhesions and on actin stress fibres. Considering that most of these results were observed in cells exposed to stresses such as detachment and stretching but not in cells under normal conditions, HIC-5 functions can, after all, be interpreted as being closely related to the abnormity of the adhesion environment and devoted to suppression of extreme changes in the cytoskeleton as well as to prevention of proliferation under abnormal adhesion conditions.

A group studying glomerulosclerosis has reported that HIC-5 is significantly induced and involved in the induction of cell death in a pathological model, where mesangial cells were cultured on collagen that was of a different type to the original extracellular matrix [33]. This observation may have captured the essence of HIC-5's function of sensing abnormal adhesion status.

6. Cancer Cells and HIC-5

The organization of HIC-5 as a fail-safe system for ensuring the anchorage dependence of cell growth is expected to be involved in the cancer development process. In this point, it should be noted that although HIC-5 is actively involved in the growth arrest of fibroblasts via induction of $p21^{Cip1}$ on loss of anchorage, it is not involved in the anoikis or cell death of epithelial cells (unpublished data). This may be concerned with the fact that $p21^{Cip1}$, which is the target of HIC-5 action and functions in growth arrest, was not induced in normal epithelial cells under detached conditions. Instead, epithelial cells undergo anoikis that is governed by a group of factors specialized for cell death execution. Given these findings together with its relatively high expression in mesenchymal cells, HIC-5 might be an important molecule in the development of sarcoma. Another interesting possibility is that HIC-5 may be involved in the epithelial-mesenchymal transition, as was pointed out by Tumbarello and Turner [34]. The ability of HIC-5 to act as a steroid hormone receptor coactivator has led to studies of its involvement in prostate cancer and endometriosis. Furthermore, a study of a human breast cancer cell line showed that both paxillin and HIC-5 have distinct impacts on cancer cell phenotypes related to metastatic potential. According to this study, in which siRNA was used for knockdown of the genes, both paxillin and HIC-5 function in promoting metastatic potential although through different mechanisms. However, in our recent experiment using

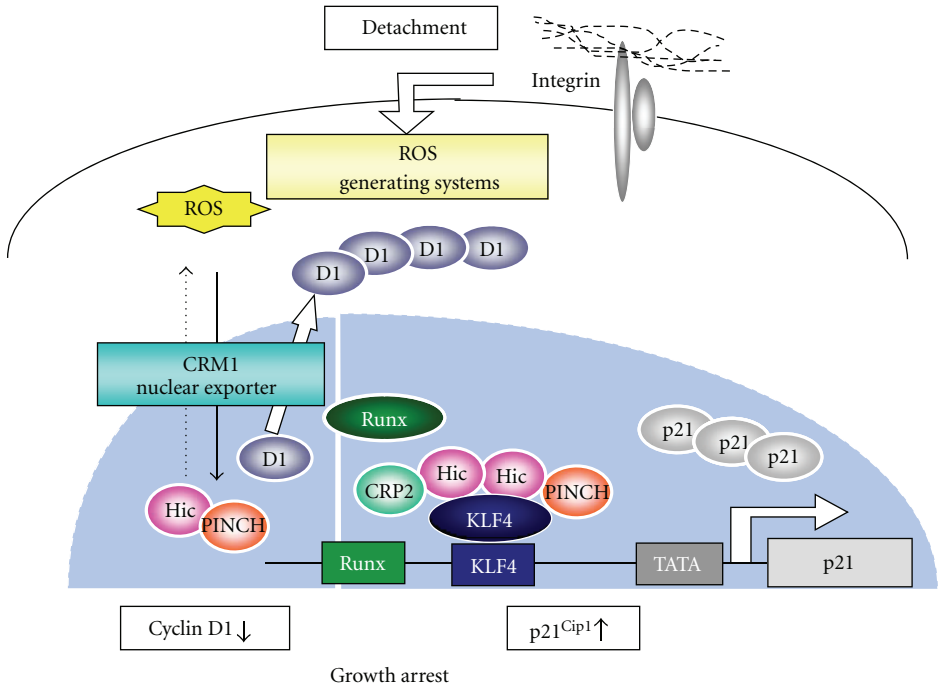

Figure 3: A fail-safe system where HIC-5 ensures the anchorage dependence of cell growth through LIM-LIM interactions. On loss of adhesion, HIC-5 activates two modes of a fail-safe system in collaboration with PINCH and CRP2 to prevent anchorage-independent cell growth. The HIC-5-PINCH unit halts its nuclear-cytoplasmic shuttling and makes way for cyclin D1 to be exported from the nucleus by CRM1, as illustrated in Figure 2, while simultaneously transactivating $p21^{Cip1}$ in the nucleus together with another HIC-5 unit, HIC-5-CRP2. As such, the dual systems, which target cyclin D1 and $p21^{Cip1}$, respectively, cooperatively stop detached cells from proliferating and thus avoid unacceptable anchorage-independent growth (see further details in [24]).

shRNA, which would be more adequate for long-term observation than siRNA, we found contradictory results. These studies suggest that HIC-5 is potentially involved in the development of certain carcinomas. However, HIC-5 and cellular transformation remains a controversial issue and awaits further investigation.

In our earlier study, HIC-5 expression dropped sharply and dramatically during immortalization of mouse fibroblasts [35]. In contrast, forced expression of HIC-5 induced a senescence-like phenotype with decreased proliferative potential after repeated passages in some immortalized human fibroblasts [36]. Recently, it was found that the anchorage-independent growth of cancer cells over a long period of time may be negatively regulated by HIC-5 (unpublished data). Thus, the interaction of cell adhesion and the mortality/senescence process would be an interesting subject for future study of HIC-5 biology in association with cancer cells.

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Review Article

Integrin Trafficking and Tumor Progression

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Integrins are major mediators of cancer cell adhesion to extracellular matrix. Through this interaction, integrins play critical roles in cell migration, invasion, metastasis, and resistance to apoptosis during tumor progression. Recent studies highlight the importance of integrin trafficking, endocytosis and recycling, for the functions of integrins in cancer cells. Understanding the molecular mechanisms of integrin trafficking is pivotal for understanding tumor progression and for the development of anticancer drugs.

1. Integrins and Cancer

Most of the cells in multicellular organisms are surrounded by a complex mixture of nonliving materials that make up the extracellular matrix (ECM). The ECM of vertebrates is composed of complex mixtures of proteins (collagens, laminins, fibronectin, etc.) and proteoglycans (chondroitin sulfate, keratan sulfate, etc.) [1, 2]. ECM plays a significant role in regulating numerous cellular functions, including cell shape, adhesion, migration, proliferation, polarity, differentiation, and apoptosis [1]. In pathological conditions such as cancer, increased synthesis of certain ECM components and/or increased breakdown with consequent generation of ECM cleavage products can contribute to cancer growth and progression [3].

Cells attach to the ECM by means of integrins. Integrins are transmembrane glycoproteins that are composed of a set of noncovalently associated α and β subunits. There are at least 18α and 8β subunits capable of forming more than 24$\alpha\beta$ heterodimers that account for the structural and functional diversity of the integrin family [4–6]. Integrins consist of a large extracellular domain, a single transmembrane domain, and a cytoplasmic tail [7]. The extracellular portion of integrins binds to ECM proteins, and the intracellular portion binds to cellular cytoskeletal elements such as actin filaments. This connection reinforces tissue integrity and cell adhesion and stabilizes cell protrusions during migration. The connection also constitutes a signaling platform through which integrins can relay information for major processes such as transcriptional control, cell death, proliferation, and cell migration [8, 9]. There is a growing body of evidence suggesting that alterations in the adhesion properties of neoplastic cells endow them with an invasive and migratory phenotype. Indeed, changes in the expression and/or function of integrins have been implicated in all steps of tumor progression, including detachment of tumor cells from the primary site, invasion of ECM, intravasation into the blood stream, dissemination through the circulation, extravasation into distant target organs, and formation of the secondary lesions [5, 10–13].

Although at least 24$\alpha\beta$ integrin heterodimers are known, $\alpha5\beta1$, $\alpha6\beta4$, $\alpha v\beta3$, and $\alpha v\beta6$ integrins have been extensively studied in cancer and their expression is correlated with cancer progression in various tumor types [14–18]. Upregulation of these integrins renders cancer cells more motile, invasive, and resistant to anticancer drugs [5, 18]. Unlike these integrins, expression levels of some integrins, such as $\alpha2\beta1$ and $\alpha1\beta1$, decrease in tumor cells, which potentially increase tumor cell dissemination [18–21]. In addition to changes in expression, changes in the function of these integrins also play a critical role in cancer progression.

2. Integrin Trafficking

The way in which integrins are trafficked by the endosomal pathway is now recognized to influence their function [6, 22]. Certain integrin heterodimers are continually internalized

from the plasma membrane into endosomal compartments and subsequently recycled back to the cell surface, which indicates that the endocytic and recycling pathways have the potential to exert minute-to-minute control over integrin function. Abundant evidence suggests that integrin trafficking regulates cell adhesion to ECM, establishes and maintains cell polarity, redefines signaling pathways, and controls migration [6, 23]. Therefore, transcriptional changes, mutational alterations, and deregulated cellular signaling changing endocytosis and recycling of integrins confer invasive and metastatic properties to tumor cells.

Integrin trafficking is regulated by members of the Ras-associated binding (Rab) family of small GTPases, which function as molecular switches regulating vesicular transport in eukaryotic cells [24, 25] (Figure 1). Through their indirect interactions with coat components, motors, and other proteins, the Rab GTPases serve as multifaceted organizers of almost all membrane trafficking processes including integrin trafficking [25, 26]. Approximately 70 types of Rab GTPases have now been identified in humans [27]. Among these, several Rab GTPases regulate endocytosis and recycling of integrins. For example, Rab21 mediates integrin endocytosis (Figure 1). In addition, Rab11 mediates slow integrin recycling through recycling endosomes, whereas Rab4 mediates fast integrin recycling directly from early endosomes (Figure 1) [25].

The pathophysiological roles of Rab GTPases in human malignancies have been less studied compared to members of the Ras and Rho GTPase families. However, more attention has been paid to the roles of Rab GTPases in cancer in recent years, and several members of the Rab family such as Rab11 and Rab25 have been shown to be aberrantly expressed in various cancer types [25, 28, 29]. Because of the important roles of Rab GTPases in integrin trafficking, deregulation of Rab GTPases is closely related to cancer development and progression [24, 25, 29].

2.1. Integrin Endocytosis. There are several major endocytosis mechanisms, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolin-independent endocytosis [30–33]. Clathrin-mediated endocytosis is mediated by small vesicles that have a morphologically characteristic crystalline coat made up of a complex of proteins associated with the cytosolic protein clathrin [33]. Clathrin-coated vesicles (CCVs) are found in virtually all cells and form domains of the plasma membrane termed clathrin-coated pits. Clathrin-coated pits can concentrate large extracellular molecules and receptors responsible for the receptor-mediated endocytosis of ligands, for example, low-density lipoprotein, transferrin, growth factors, and antibodies [30, 33]. In contrast, caveolae-mediated endocytosis is mediated by small flask-shape pits, caveolae, in the membrane. Caveolae are the most common reported non-clathrin-coated plasma membrane buds which exist on the surface of many, but not all cell types [33]. They consist of the cholesterol-binding protein caveolin (Vip21) with a bilayer enriched in cholesterol and glycolipids [30]. Clathrin- and caveolin-independent endocytosis includes macropinocytosis and circular dorsal ruffles [9, 30, 33]. Clathrin-dependent endocytosis and caveolin-dependent endocytosis require dynamin and exhibit small vesicles. However, macropinocytosis does not require dynamin and displays highly ruffled structures. Like macropinocytosis, circular dorsal ruffles show highly ruffled structures, but this endocytosis is dependent on dynamin.

Integrins are known to be endocytosed by clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolin-independent endocytosis (Table 1). It is probable that a given type of integrin heterodimer follows more than one route to internalization depending on regions within a cell, cell conditions, and cell type [6, 34]. For instance, a subpopulation of integrin $\alpha5\beta1$ is internalized into clathrin-coated structures near focal complexes at the cell front, whereas the bulk of integrin $\alpha5\beta1$ follows a nonclathrin pathway from other parts of the cell surface [34].

Deregulation of integrin endocytosis is closely related to cancer development and progression [9, 30]. For example, chromosomal deletion and loss of Rab21, a regulator of endocytic trafficking of integrins, has been found in cancer that leads to the accumulation of multinucleate cells in cancer. The correlation with multinucleate cells is thought to reflect the requirement of Rab21-mediated integrin endocytosis for correct cytokinesis [35]. Rab21 also enhances cancer cell adhesion and migration by regulating integrin endocytosis [36].

2.2. Integrin Recycling. Once internalized, integrins are predominantly recycled back to the plasma membrane, although a fraction of integrin $\alpha5\beta1$ has been shown to traffic to lysosomes for degradation during migration [6, 37]. Following endocytosis, integrins travel to early endosomes from which they can either be returned directly to the plasma membrane in a Rab4-dependent manner (the short loop) or further trafficked to the perinuclear recycling compartment (PNRC) before recycling through Rab11-dependent mechanisms (the long loop) (Table 1). Rab11 GTPase functions have been linked to tumorigenesis and tumor progression. Rab11 is upregulated during skin carcinogenesis [38] and is linked to Barrett's dysplasia [39]. However, the function of the Rab11 family member Rab25 (or Rab11C) is controversial. Rab25 shows highly restricted expression under normal physiological conditions but is upregulated in invasive cancer cell lines and metastatic tumor cells [40], and its elevated expression is further linked to the aggressiveness of breast and ovarian cancers [28]. Rab25 is a determinant of tumor progression, and the aggressiveness of epithelial cancers and is strongly associated with decreased survival [28]. In contrast, recent studies showed that Rab25 expression is decreased in human colon cancers and triple-negative (negative for estrogen receptor (ER), progesterone receptor (PR), and Her2/Neu) breast cancers, and Rab25 functions as a tumor suppressor in these cancers [41–43]. The key roles of Rab GTPases in tumorigenesis and tumor progression are closely related to integrin recycling. For example, Rab25 contributes to tumor progression by directing the localization of integrin-recycling vesicles and thereby enhancing the ability of tumor cells to invade the extracellular matrix [44].

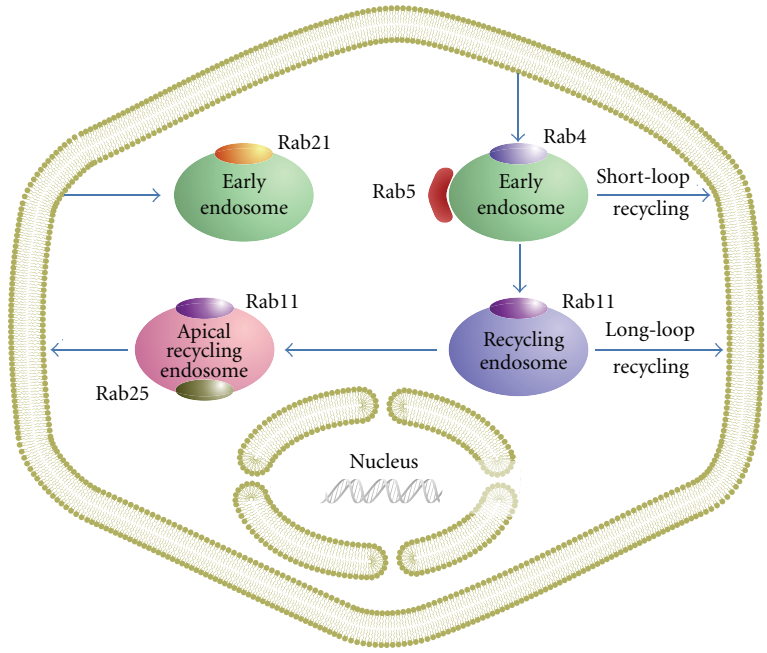

Figure 1: The roles of Rab GTPases involved in integrin trafficking. Integrins can be internalized by a clathrin-dependent, caveolin-dependent, or clathrin- and caveolin-independent pathway. For example, some integrins are internalized by a Rab21 and clathrin-independent pathway. Once internalized, integrins can be recycled back to the plasma membrane by a Rab4-dependent manner or can be transported to the perinuclear recycling compartment. Rab11 family members (Rab11 and Rab25) regulate recycling of integrins from perinuclear recycling compartment.

Table 1: Mechanisms of the internalization and recycling of integrins $\alpha5\beta1$, $\alpha6\beta4$, $\alpha v\beta3$, and $\alpha v\beta6$.

Integrin	Internalization		Recycling	
	Associated proteins	Comments	Associated proteins	comments
$\alpha5\beta1$	Numb	Clathrin-dependent	Rab11	Akt/GSK-3β-dependent
	AP2 associated with tetraspanin	Clathrin-dependent	Rab11 RCP	Akt-dependent
	NRP1	Clathrin-dependent	VAMP3	SNARE-mediated
	Rab21	Clathrin-dependent	Rab21	Required for cytokinesis
	?	Caveolin-dependent	Rab25	Promote invasion in 3D
$\alpha6\beta4$			Rab11	Akt/GSK-3β-dependent
$\alpha v\beta3$	Numb	Clathrin-dependent	Rab4, PKD1	PDGF- or VEGF-driven
	?	Caveolin-dependent	Rab4, RABIP4	PDGF-driven
			Rab11	Akt/GSK-3-dependent
$\alpha v\beta6$	HAX1	Clathrin-dependent		

3. Trafficking of Integrin $\alpha5\beta1$

Integrin $\alpha5\beta1$ is a receptor for fibronectin and contributes to cancer cell invasion, metastasis, resistance to anticancer drugs, and decreased survival in patients [17, 45].

Integrin $\alpha5\beta1$ is internalized by clathrin-dependent, caveolin-dependent, and clathrin- and caveolin-independent mechanisms. For clathrin-dependent endocytosis, $\alpha5\beta1$ integrin binds to NUMB, an endocytic protein that influences clathrin-coated pit assembly [46]. Integrin $\alpha5\beta1$ can also internalize with tetraspanin protein, which interacts with AP-2, an adaptor for clathrin-mediated endocytosis [47]. Clathrin-dependent internalization of $\alpha5\beta1$ integrin with NUMB or tetraspanin has a profound effect on cell migration. In addition, $\alpha5\beta1$ integrin can undergo Rab21- and clathrin-independent endocytosis that is required for successful cytokinesis [35]. In some cell types, integrin $\alpha5\beta1$ localizes to caveolae for caveolin-mediated endocytosis [6, 48]. Caveolin-dependent endocytosis of integrin $\alpha5\beta1$ is critical for fibronectin turnover [48].

Internalized integrin $\alpha5\beta1$ is transported through Rab4-positive early endosomes and arrives at the Rab11-positive perinuclear recycling compartment [49]. Akt-mediated glycogen synthase kinase (GSK)-3 phosphorylation is known to deliver $\alpha5\beta1$ from the Rab11 compartment to the plasma membrane [50]. One of the Rab11 effectors, Rab11

FIP1/RCP, associates with integrin $\alpha5\beta1$ and regulates recycling of this integrin [51]. Rab-coupling protein (RCP) provides a scaffold that promotes the physical association and coordinated trafficking of $\alpha5\beta1$ and epidermal growth factor receptor 1 (EGFR1). This association drives migration of tumor cells into three-dimensional matrices [51]. Recently, it was shown that mutant p53 can promote invasion, loss of directionality of migration, and metastatic behavior by regulating the interaction of $\alpha5\beta1$ integrin to Rab-coupling protein, which enhances $\alpha5\beta1$ trafficking and signaling [52]. Since Rab25 (Rab11C, Rab11 family member) binds to Rab11 FIP1/RCP, it is hypothesized that interaction between them may control integrin $\alpha5\beta1$ trafficking. Recently, it has been shown that Rab25 associates with $\alpha5\beta1$ integrin to enhance migration and invasion of cells in three-dimensional microenvironments and directs $\alpha5\beta1$ integrin recycling to dynamic ruffling protrusions at the leading edge of migrating cells, which promotes invasive migration [29, 44]. In addition to Rab11 and Rab25, Rab21 is required for carcinoma-associated fibroblasts to promote invasion by cancer cells and facilitates integrin $\alpha5\beta1$ accumulation for force-mediated matrix remodeling at the plasma membrane [53]. It has also been shown that Rab21-dependent recycling of integrin $\alpha5\beta1$ is critical for proper activation of RhoA during cytokinesis [35].

Although most endocytosed integrin $\alpha5\beta1$ is known to recycle back to plasma membrane, a subset of this integrin moves to lysosomes for degradation [37]. This process is very slow, but it is important for $\alpha5\beta1$-dependent cell motility [37].

4. Trafficking of Integrin $\alpha6\beta4$

Integrin $\alpha6\beta4$ is a receptor for laminin. Overexpression of $\alpha6\beta4$ integrin was seen in several types of cancers including breast cancer and correlated with tumor invasion, increased tumor size and grade, and a poor prognosis [54–57].

We showed that integrin $\alpha6\beta4$ integrin recycles back to the plasma membrane via the Rab11-positive perinuclear recycling compartment [58]. Hypoxia stimulated carcinoma invasion by promoting Rab11 trafficking of integrin $\alpha6\beta4$, which is dependent on hypoxia-inhibited glycogen synthase kinase (GSK)-3 signaling [58].

5. Trafficking of Integrin $\alpha v\beta3$

Integrin $\alpha v\beta3$ is a receptor for fibronectin and vitronectin. Integrin $\alpha v\beta3$ is expressed in a variety of tumors such as melanoma, prostate cancer, and breast cancer [59]. Integrin $\alpha v\beta3$ is overexpressed in activated endothelial cells during tumor-induced angiogenesis, whereas it is absent on quiescent endothelial cells and normal tissues. It is known that integrin $\alpha v\beta3$ promotes cancer cell survival, migration, invasion, and metastasis [4, 60, 61].

Integrin $\alpha v\beta3$ is endocytosed via clathrin-dependent, caveolin-dependent, or clathrin- and caveolin-independent mechanisms. NUMB is an alternative clathrin adaptor and can interact with $\beta3$ integrin, which controls $\alpha v\beta3$ integrin endocytosis and cell migration [46]. In some cell types, integrin $\alpha v\beta3$ is internalized by caveolin-dependent mechanisms [62]. In this case, MT1-MMP is clustered together with caveolin-1 and $\alpha v\beta3$ integrin at motility-associated structures, resulting in increased proteolytic activity, which is important for cell migration and invasion [62]. A recent study shows that upon growth factors stimulation, integrin $\beta3$ abruptly redistributes to circular dorsal ruffles where it is internalized through macropinocytosis, which plays an important role in growth factor-induced cell migration [63].

Internalized integrin $\alpha v\beta3$ recycles back to the plasma membrane via Rab4-dependent mechanisms or the Rab11-positive perinuclear recycling compartment [23]. Following treatment with PDGF, integrin $\alpha v\beta3$ was rapidly recycled directly back to the plasma membrane from early endosomes via a Rab4-dependent mechanism without the involvement of Rab11 [49]. The PKC-related kinase PKD1 influences cell migration by this fast recycling of integrin $\alpha v\beta3$. It is known that PKD1 directly interacts with $\beta3$ integrin and this interaction promotes fast recycling of $\alpha v\beta3$ integrin from recycling endosomes to the plasma membrane upon growth factor stimulation [64]. Activation of VEGFR1 also enhances a Rab4A-dependent pathway that transports $\alpha v\beta3$-integrin from early endosomes to the plasma membrane [65]. Recent studies link PKD and VEGF signaling in which VEGF-A induces recycling of integrin $\alpha v\beta3$ in a PKD1-dependent manner [66]. Because of the involvement of Rab4 in the recycling of $\alpha v\beta3$ integrin, inhibition of Rab4 effector protein (Rab IP4) blocks integrin recycling, leading to inhibition of cell adhesion and cell spreading [67]. Another study suggests that supervillin, an actin and myosin binding protein, regulates rapid $\beta3$ integrin recycling through collaboration with Rab4 and Rab5 [68]. The short-loop recycling of integrin $\alpha v\beta3$ via Rab4 does not directly contribute to migration by moving $\alpha v\beta3$ to the cell front, but by antagonizing $\alpha5\beta1$ recycling, which, in turn, influences the cell's decision to migrate with persistence or to move randomly [69]. Integrin $\alpha v\beta3$ is also recycled to the plasma membrane in a Rab11-dependent manner (long loop recycling) in which Akt promotes this recycling by phosphorylating and inactivating GSK-3 [50].

6. Trafficking of Integrin $\alpha v\beta6$

Integrin $\alpha v\beta6$ is a receptor for fibronectin, vitronectin, and tenascin. Integrin $\alpha v\beta6$ is usually expressed at low or undetectable levels in most healthy adult epithelia but is upregulated in many cancers such as colon cancer [70]. The expression of integrin $\alpha v\beta6$ inhibits apoptosis and promotes tumor cell invasion and metastasis, which is often associated with a more aggressive disease outcome and a poor prognosis [18, 70].

Recently, the mechanism of endocytosis of integrin $\alpha v\beta6$ was revealed. Integrin $\alpha v\beta6$ is internalized by a clathrin-dependent mechanism by interaction with HS1-associated protein X1 (HAX1) [71]. HAX1 is found in clathrin-coated vesicles, and the cytodomain of $\beta6$ integrin interacts with HAX1 and is endocytosed, which increases carcinoma migration and invasion [71].

7. Conclusion and Future Direction

Integrins are key regulators of cell adhesion, migration, and proliferation. Therefore, deregulation of their expression and altered functions play critical roles in cancer progression by enhancing cancer cell invasion, metastasis, and survival. There are now clear indications that integrin trafficking is important to modulate integrin distribution and function. However, more studies are needed to define the molecular mechanisms of integrin trafficking in tumor progression. Many questions remain to be answered. One important question is whether endosomal integrins can signal cell proliferation and migration. It is known that unligated integrins can positively or negatively regulate tumor cell survival and metastasis, and, therefore, signaling arising from endosomal compartments may be important for tumor cell survival. Another question is how trafficking of specific integrins affects other integrins. For instance, it has been shown that rapid recycling of $\alpha v\beta 3$ via the Rab4 pathway antagonizes the Rab11-mediated $\alpha 5\beta 1$ recycling, which influences the cell's decision to migrate with persistence or to move randomly [69]. Because of the critical roles of integrins in cancer progression, integrins are potential targets for the development of targeted anticancer therapeutics. Understanding the mechanism of integrin trafficking will provide valuable information for the development of new anticancer drugs and clues to increase the efficacy of current anticancer therapeutics.

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