A Rare Bird among Major Extracellular Matrix Proteins: EMILIN1 and the Tumor Suppressor Function

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Abstract

Extracellular Matrix (ECM) proteins constitute a complex network of macromolecules with distinctive physical, biochemical, and biomechanical properties. They are expressed dynamically and their cellular functions are highly dependent upon cues from the local environment. ECM proteins primarily by interaction with integrins on the cell surface initiate downstream signaling events that involve diverse cellular functions. Although tightly controlled under normal development, the ECM is commonly deregulated and becomes disorganized in diseases such as cancer. Abnormal ECM affects cancer progression by directly promoting cellular transformation, metastasis and facilitates tumor-associated angiogenesis and inflammation, and thus leads to generation of a tumorigenic microenvironment. In this review, we summarize and discuss the current knowledge of the diverse promoting or inhibiting role played by selected members (collagen, fibronectin, tenascin, thrombospondin, LTBP-2, fibulin, CCN1, decorin, EMILIN2) of the ECM play within the microenvironment that influences tumor progression with an emphasis on EMILIN1. This glycoprotein, a member of the gC1q domain superfamily, is involved in the maintenance of the blood pressure, the proper function of lymphatic capillaries and collecting vessels and, via the interaction with the α4β1 and/or the α9β1 integrins, regulates cell proliferation. This last function highlights the peculiar role of EMILIN1 as an anti-proliferative member of the ECM and likely a novel tumor suppressor.

Keywords: Extracellular matrix; EMILIN1; gC1q domain; integrin α4β1; Integrin α9β1; Proliferation; Lymphangiogenesis

Introduction

All cells are embedded in a supporting network of Extracellular Matrix (ECM) components that include collagens, elastin, proteoglycans, and glycoproteins. The interest for ECM in cancer processes is based on the general belief that the ECM does not constitute a mere structural scaffold for cells but it plays a significant role in regulating numerous cellular functions including cell shape, adhesion, migration, proliferation, polarity, differentiation, and apoptosis [1-3]. In physiological conditions, ECM is tightly regulated by a fine balance between synthesis and degradation, but under pathological conditions, such as cancer, both increased synthesis of certain ECM components and/or increased breakdown with consequent generation of fragments can contribute to tumor growth and progression [4,5]. A peculiar property of the ECM molecules is to function as reservoirs of growth factors (GFs), cytokines, Matrix Metalloproteinases (MMPs), and processing enzymes. When the ECM rearranges as occurs in cancer not only the relative availability of these elements may be affected but also the regulation of cell behavior and functions.

Among the receptors used by cells to interact with ECM, integrins translate chemical and physical cues from the ECM components into biochemical signals in order to regulate proliferation, apoptosis, and migration [6,7]. Integrins transduce signal both independently or in alliance with growth factor receptors, binding directly and/or indirectly with numerous intracellular signaling and scaffolding molecules that have been linked to oncogenesis.

Many evidences support the concept that the ECM has in general an advantageous role in the tumor progression and that the ECM components and their respective receptors favor the development and spread of tumor cells. Only very few ECM proteins are known to exert primarily a tumor suppressor function.

The overall goal of this review is to highlight the ability of some ECM molecules to concur in tumor growth and progression and the role of others to counteract proliferation and invasion of cancer cells with a particular attention to the ECM glycoprotein EMILIN1.

ECM Molecules Favoring Cancer and its Progression

The ECM is a highly dynamic structure undergoing a constant remodeling process where its components are deposited, degraded, or otherwise post-translationally modified. These modifications are crucial during restructuring of tissue architecture and matrix remodeling is an important mechanism whereby cell differentiation can be regulated. Conversely, abnormal ECM dynamics leads to a general deregulation of cell processes such as proliferation, differentiation and migration and contributes to the molecular etiology of cancer development. The action of most few representative ECM molecules will be described as examples of the multiple strategies and mechanisms of interaction adopted to promote directly and/or indirectly tumor initiation and progression.

A shining example to detail how ECM can diversely contribute to cancer is represented by collagens, the major constituents of the ECM, representing as much as 30% of total mammalian protein mass [8]. The abnormal expression of collagens is a frequent event in many types of cancer [9-11] and fibrosis that is an accumulation of ECM molecules,
including type I collagen, is often associated with cancer [12]. Moreover, increased expression of type I collagen and many of its modifying enzymes is frequently observed in the gene expression signature associated with increased risk of metastasis [13]. The architectural alterations of tumor-associated collagens that result crosslinked and consequently linearized following significant posttranslational modifications determine ECM stiffness and thereby diverse effects on cellular differentiation, gene expression, proliferation, survival and migration [14,15]. Collagen crosslinking during breast tumorigenesis stiffens the ECM to promote focal adhesion, enhancing PI3K activity, and fostering invasion of an oncogene-initiated epithelium [14]. The clustering of integrins induced by tissue stiffness perturbs epithelial morphogenesis and determines a tumor-like behavior by disrupting adherens junctions, destabilizing tissue polarity, and enhancing growth and migration [15,16].

Collagen crosslinking is catalyzed by enzymes such as Lysyl Oxidase (LOX). The increased expression of LOX correlates clinically with tumor progression and elevated metastatic risk [17,18]. Collagen linearization is observed close to the tumor vasculature and in areas of cancer cell invasion [14,19]. The abnormal linearized fibers are exploited by transformed cells as highways for migration and invasion into the interstitial matrix and towards vasculature [19].

Considering that collagens also induce chemoreistance in cancer cells by stimulating overexpression of anti-apoptotic genes [20,21], we could affirm that collagen is an “all seasons”-ECM molecule in cancer and an excellent example to recognize that the ECM is much more than a scaffold structure and a migration barrier.

Fibronectin (FN) represents another clear example of a matrix component contributing with its quantitative and qualitative modifications to the modulation of the malignant process. FN is a glycoprotein and it is considered the most suitable ECM component to study the role of “integrin-ECM” interaction on cell survival and proliferation. Integrin α5β1-mediated adhesion to FN is particularly efficient in stimulating cell-cycle progression [22,23]. In fact, FN can decrease the GF requirement for DNA synthesis up to 1000-fold [24]. Most cell types depend on integrin-mediated cell adhesion to ECM for survival and proliferation. This is especially true for endothelial and epithelial cells that rapidly undergo apoptosis when adhesion is disturbed. One of the important cell-matrix adhesion molecule that play a crucial role in integrin-mediated survival signaling is FAK that allows mechanical coupling between ECM and cytoskeleton: on rigid but not soft ECM substrates, FAK is activated causing Rac-mediated cyclin D1 gene induction and cyclin D1-dependent Rb phosphorylation [25]. This is also true in the case of FN: the process of fibrillogenesis leads to increased FN rigidity that in turns increases binding forces between FN and its major receptor α5β1 integrin [26]. Since FN binds collagen and regulates collagen fibril organization [27], the size, density and rigidity of FN fibrils in vivo influence the function of collagen fibrils and vice versa. This dynamics plays an important role in tumor progression: FN deposition has been implicated as an early step in metastatic process [28,29]. Binding of α5β1 to FN increases expression and secretion of MMP-1, MMP-3, and MMP-9 involved in tumor invasion [30,31]. Comparing benign breast tumors with malignant adenomas, the fragmentation of pericellular FN, besides its loss in tissue, was an early sign of malignancy [32].

Proteolysis and tumor progression

The quantitative and qualitative changes in the ECM are a key modification of the stromal tumor environment. It is well recognized that proteinases contribute actively to the elaboration of ECM. For example, the architecture of collagen is affected also by proteolysis carried out by MMPs and cathepsins. The importance of proteolysis in favoring migration and invasion is clear since ECM components of the basement membrane is a barrier for epithelial cells: ECM degradation generates pathways for cell to migrate through [33]. One of the most represented ECM molecules of the basement membrane is type IV collagen. In addition, to the important consequence of proteolysis on type IV collagen, it has been recently shown in a pancreatic model that this ECM protein is expressed close to the cancer cells in vivo, forming basement membrane like structures on the tumor cell surface that colocalize with the integrin receptors and providing essential cell survival signals to the pancreatic cancer cells through an autocrine loop [34]. Most importantly, proteolysis of type I and IV collagen can also reveal RGD sequences that are binding sites for αv integrins [35,36] and uptake of collagen fragments improves cancer invasion and epithelial-mesenchymal transition [9,37,38]. Degradation of collagen is fundamental in favoring angiogenesis: cleavage at the specific triple-helical site of type I collagen is required to fully manifest a growth factor effect for blood vessels [39,40]. Conversely, the proteolytic process can generate non-collagenous fragments able to counteract angiogenesis. Endostatin, a C-terminal fragment of type XVIII collagen, inhibits endothelial cell migration and thus the formation of new vessels [41]. The same effect is provided by tumstatin, a fragment of the type IV collagen, that affects endothelial cell functions by modulating αvβ5 and αvβ3 integrin signaling [42–47]. Arresten and canstatin are other type IV collagen fragments with anti-angiogenic activity [48,49].

Proteolysis of FN leads to the generation of bioactive fragments that promote cell growth [50] and inhibit tumor cell invasion, such as the FN13 amino acid peptide, that modulates αvβ3 integrin organization and inactivates ILK pathway [51]. This proteolytic process highlights the biological and pathological significance for a mechanism that discloses “matricryptic” sites in an increasing number of ECM components. A further example is laminin-5, a major component of the mammary basal lamina, and its cleavage by MMP-2 exposes a cryptic site that promotes cell migration and invasion [52,53]. Moreover, one of the peptides released by laminins degradation can bind and stimulate cells through the EGFR [54].

As already stated for the concept that ECM has in general an advantageous role in the tumor progression, also MMPs had been considered for many years as pro-tumorigenic enzymes. Despite the pro-tumorigenic function of certain MMPs, recent studies have shown that other members of these families, such as MMP-8 and MMP-11, have a protective role against tumor growth and metastasis in animal models (reviewed in [55,56]). Furthermore, antitumor effects or dual functions with protective roles can be extended to other proteinases including ADAMTS members [57,58].

Alternative splicing

The alternative splicing is a cell-, tissue-, and developmentally specific regulated process [59]. FN is a well defined and suitable example of how alternative splicing could play a significant role in cancer. In transformed cells and in malignancies, the splicing pattern of FN-pre-mRNA becomes altered [60], leading to an increased expression of oncotelic FN isoforms containing the IIICS, EDA and EDB sequences [61,62]. The presence of additional acceptor and donor splicing signals within the IIICS region allows generation of multiple IIICS polypeptide variants. CS1 isoform is one of these molecular variants which is a ligand for the αvβ3 integrin [63]. The CS1 site mostly mediates adhesion of lymphoid cells and some tumor cells in an
Alternative splicing is well documented also for tenasin C (TNC). Multiple isoforms of TNC can be generated: higher molecular weight TNC isoforms can be detected in a number of tissues, including bladder [66], brain [67], gastrointestinal tract [68] and lung [69] amongst others [70-72]. Moreover, in breast the expression profile of isoforms differs between cancers and normal tissue, with the fully truncated TNC isoform being predominant in normal and benign tissues and higher molecular weight isoforms induced predominantly in cancer [73,74]. How these spliced isoforms could exert their pro- or anti-tumor effect is not known.

While largely being complete, this overview highlights the direct mechanisms used by intact or degraded ECM molecules to promote tumor growth and progression mainly through the interaction with their cellular ligands, the integrins. By contrast, there are very few, if any, examples of direct integrin-ECM protein binding with suppressive activity.

**ECM Molecules with Anti Tumor Activity**

Only few ECM proteins exerts a tumor suppressor function through direct or indirect mechanisms.

**Indirect mechanisms**

Most ECM molecules counteract tumor growth indirectly by impacting angiogenesis. For instance, thrombospondin-1 (TSP-1) was the first and most studied naturally occurring protein inhibitor of angiogenesis. TSP-1 displays a direct apoptotic action on the remodeling of vascular endothelium [75]. The suppression of angiogenesis by TSP-1 involves also other mechanisms including the direct interaction with vascular endothelial cell growth factor (VEGF), the inhibition of MMP-9 activation [76], and the inhibition of endothelial cell migration [77,78]. Similar strategies are also used by trombospondin-2 (TSP-2) [77,78].

Convincing evidences have been recently provided for the tumor suppressive function of latent transforming growth factor β binding protein-2 (LTBP-2), a member of the LTBP-fibrillin gene family that encodes for glycoproteins sharing a similar overall domain structure for protein-protein interactions. This ECM molecule inhibits cancer cell migration and invasion [79] and decreases fibroblast adhesion to FN, revealing an important role of LTBP-2 as an anti-adhesion matrix component [80]. Pronounced suppression of several in vitro malignancy-related features by inducible expression of LTBP-2, including colony formation, growth in soft agar and migration, angiogenesis, plus in vivo tumorigenesis has been demonstrated in a model of nasopharyngeal carcinoma [81]. In the same study, the pleiotropic functions of LTBP-2 in this type of cancer reveal the pleiotropic functions of LTBP-2 in this type of cancer reveal the pleiotropic functions of LTBP-2 in this type of cancer reveal the pleiotropic functions of LTBP-2 in this type of cancer reveal the importance of LTBP-2 in promoting cell dormancy against metastatic growth induced by GFs [81]. Interrupting the tumor cell adhesion to the ECM via integrins results in dormancy [82], while production of FN props cells from dormancy to a highly proliferative status [83]. Since LTBP-2 binds to ECM components such as fibrillin-1 [84] and fibulin-5 [85], heparin, HSPG, syndecan-4, and perlecain [85], it is reasonable to speculate that LTBP-2 may modulate ECM conditions, thereby interfering with proper tumor cell-ECM adhesion, resulting in tumor cell dormancy and leading to a compromised cellular response towards the GFs in a permissive microenvironment.

Fibulin-5 (FBLN-5) is a suppressor of tumor angiogenesis and a potential tumor suppressor [86,87]. Its effects on angiogenesis were first described using mouse brain microvascular endothelial cells: FBLN-5 overexpression inhibited sprouting, proliferation, and invasion in matrigel [87]. In mediating its angiogenic function, FBLN-5 targets multiple endothelial activities most likely via direct and indirect mechanisms. For instance, by antagonizing VEGF stimulation of ERK1/ERK2 [87], which couples to MMP expression [88], and p38 MAPK [87], which couples to actin cytoskeleton reorganization [89], FBLN-5 reduces endothelial cell migration and invasion. FBLN-5 also significantly stimulates TSP-1 expression in endothelial cells [87], thereby enhancing angiogenesis resolution via TSP-1-mediated induction of apoptosis and the inhibition of MMP-9 activation. An indirect mechanism is provided by the down-regulation as well as enzymatic activity of MMP-2 in endothelial cells overexpressing FBLN-5 during tubulogenesis in collagen gels [90]. FBLN-5 controls angiogenesis through the regulation of integrin-induced production of reactive oxygen species (ROS) which have pro-angiogenic properties [91]. FBLN-5 prevents ROS production by blocking the interaction between FN and β1 integrins. It has been reported that FBLN-5 inhibits FN-mediated cell spreading, migration and proliferation by competing with FN for β1 integrin binding. Interestingly, binding of FBLN-5 to β1 integrins does not induce integrin activation [92].

The antitumor activity of FBLN-5 has been clearly demonstrated in several models showing that FBLN-5 mRNA expression is dramatically down-regulated in prostate, kidney, breast, ovary, colon cancers and in metastatic lung colonization [93-95]. In this context, the loss of inactivation of FBLN-5 could have a role in cancer progression. An additional mechanism has been shown in lung cancer where FBLN-5 functions as a suppressor of cell invasion by inhibiting MMP-7 expression [96].

Among fibulin members, also FBLN-3 can be considered an angiostatic agent capable of reducing tumor angiogenesis and, consequently, tumor growth in vivo [90]. Recently, anti-angiogenic and tumor-suppressive roles have been disclosed for FBLN-2 in nasopharyngeal carcinomas but the precise molecular mechanisms remain still to be elucidated [97].

However, functional relevance of fibrulins in cancer is still unclear and in some cases contradictory. So far, different studies conclude that both tumor suppressive functions and oncogenic activities can be elicited by fibrulins (reviewed in [98,99]).

**Direct mechanisms**

A direct action in inhibiting tumor growth is played by very few ECM molecules: CCN1, decorin and EMILIN2 impair cancer cell viability by increasing cell death and/or apoptosis.

CCN1 induces fibroblast apoptosis through its adhesion receptors, integrin α6β1 and syndecan-4, triggering the transcription-independent p53 activation of Bax to induce cytochrome c release and activation of caspase-9 and -3 [100].

Decorin evokes protracted internalization of the EGFR via a caveolar-mediated endocytosis, which leads to EGFR degradation and attenuation of its signaling pathway. Decorin specifically targets the tumor cells enriched in EGFR and causes a significant down-regulation of EGFR and attenuation of its activity. Furthermore, decorin induced apoptosis via activation of caspase-3 [101].

EMILIN2 triggers the apoptosis of different cell lines. Cell death depends on the activation of the extrinsic apoptotic pathway following...
EMILIN2 binding to the TRAIL receptors DR4 and, to a lesser extent, DR5. Binding is followed by receptor clustering, colocalization with lipid rafts, death-inducing signaling complex assembly, and caspase activation [102]. This is the first example of the direct activation of death receptors by an ECM molecule that mimics the activity of the known death receptor ligands, disclosing an additional mechanism by which ECM cues can negatively affect cell survival.

**EMILIN1**

At the ultrastructural level, the molecule was first detected in elastic fibers, where it is located at the interface between the amorphous core and the surrounding microfibrils [105]. On the basis of this finding, the protein was named EMILIN1 (Elastin Microfibrillar Interface-Located proteIN-1).

EMILIN1 belongs to the EMILIN/multimerin family, constituted by glycoproteins that in addition to the shared C-terminus gC1q domain typical of the gC1q/TNF superfamily members contain a N-terminus unique cysteine-rich EMI domain [104-106]. EMILIN1 is the most extensively studied member both from the structural and functional point of view. The primary sequence shows that EMILIN1 comprises other domains [104] (Figure 1). Besides gC1q and EMI domains, it has a short collagenous domain that separates the gC1q domain from a long region with a high probability of coiled-coil conformation [104,107]. EMILIN1 is homotrimeric, assembles into high molecular weight multimers [107] and is particularly abundant in the walls of large blood vessels [108], in intestine, lung, lymph nodes, lymphatic capillaries and skin [109]. EMILIN1 exerts a diverse range of functions formally associated with the gC1q domain and others not directly gC1q-dependent.

Briefly, we will first summarize gC1q-independent functions. EMILIN1, which is intimately associated with elastic fibers and microfibrils in blood vessels [104,110], is implicated in elastogenesis and in maintenance of blood vascular cell morphology [108]. EMILIN1 binds elastin and fibulin-5 and the association of fibulin-5 with elastin is altered in the absence of EMILIN1. These interactions explain the localization of EMILIN1 between the amorphous core and microfibrils and the role of this protein that stabilizes elastic fibers through defined molecular interactions [108]. It controls blood pressure: *Emilin1*−/− mice display elevated systemic blood pressure associated to narrower arteries. Since EMILIN1 binds to proTGF-β1 prior to the cleavage of LAP and upstream of the furin convertase and prevents its processing [111], the increased TGF-β in the absence of EMILIN1 as it occurs in *Emilin1*−/− mice leads to reduced vascular cell proliferation, narrower blood vessels, and increased peripheral resistance. While the pathogenic mechanism of hypertension is largely explained by the interaction between the EMI-domain and proTGF-β1, EMILIN1 can exert a role also with additional mechanisms. We have hypothesized that EMILIN1 might contribute to affect the cell number and the size of smooth muscle cells in arterial wall through the gC1q domain and the consequent homeostatic control of cell proliferation as it will be clarified below for other cell types.

The importance of EMILIN1 in vasculature is not limited to blood district: the molecule is involved in the maintenance of the integrity of lymphatic vessels, a fundamental requirement for efficient function [109]. EMILIN1 deficiency results in hyperplasia, enlargement, and frequently in an irregular pattern of superficial and visceral lymphatic vessels and in a significant reduction of anchoring filaments [109]. Lymphatic vascular morphological alterations are accompanied by functional defects, such as mild lymphedema, a highly significant drop in lymph drainage, and enhanced lymph leakage [109]. The phenotype displayed by *Emilin1*−/− mice is the first abnormal lymphatic pattern associated with the deficiency of an ECM protein and identifies EMILIN1 as a local regulator of lymphangiogenesis. Experimental evidence for the involvement of a specific domain responsible for lymphangiogenic functions have not been provided yet; however, it could be suggested that the structural function associated with the anchoring filaments are linked to the whole molecule whereas the regulatory control of EMILIN1 on lymphatic endothelial cells could be associated to the functional domain gC1q. Recently, we have demonstrated that the defects of collecting lymphatic vessels in *Emilin1*−/− mice are due to the lack of interaction between gC1q and integrin α9 (Danussi et al., manuscript submitted).

Many EMILIN1 functions are regulated by the ligand-receptor interaction of the gC1q domain. The structure of gC1q of EMILIN1 solved by NMR highlights unique characteristics compared to other gC1q domains: an insertion of nine residues disrupts the ordered strand interaction of the gC1q domain. The structure of gC1q of EMILIN1, which is the first abnormal lymphatic phenotype displayed by *Emilin1*−/− mice is the first abnormal lymphatic pattern associated with the deficiency of an ECM protein and identifies EMILIN1 as a local regulator of lymphangiogenesis. Experimental evidence for the involvement of a specific domain responsible for lymphangiogenic functions have not been provided yet; however, it could be suggested that the structural function associated with the anchoring filaments are linked to the whole molecule whereas the regulatory control of EMILIN1 on lymphatic endothelial cells could be associated to the functional domain gC1q. Recently, we have demonstrated that the defects of collecting lymphatic vessels in *Emilin1*−/− mice are due to the lack of interaction between gC1q and integrin α9 (Danussi et al., manuscript submitted).

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The EMILIN1 integrin receptors αβ1 and αβ1.

Integrins α4 and α9 share 39% amino acid identity, both bind the β1 subunit and exert distinct as well similar functions in vivo [114]. α4...
and α9 have several common ECM ligands, such as FN, osteopontin, TSP-1 and EMILIN1 [115]. Differently to many other integrins, α4 and α9 bind to the ligands in a RGD independent manner.

α4β1 is expressed on the surface of several cell types of the hematopoietic lineage, including lymphocytes, monocytes/ macrophages and eosinophils (but not neutrophils), in which it drives proliferation, survival and migration [116]. Contrary to common beliefs that consider α5β1 an exclusive leukocyte integrin, there is a compelling evidence that this receptor is widely expressed in several normal tissues including brain, heart, kidney, lung, muscle, liver, prostate, skin as their tumor counterparts [117]. α4β1-dependent interactions, extensively studied in hematopoietic cells, have shown that the initial and intermediate stages of cell adhesion, i.e. attachment and spreading, are supported, whereas focal adhesion and stress fiber formation, characteristic of strong cell adhesion, are rarely if ever observed [118]. Intermediate states of adhesion favor cell motility and cell migration is diminished in cells exhibiting strong adhesion [119]. Thus, whereas α4β1 in focal complexes mediates cell substratum adhesion stabilizing it [120], α4β1 promotes lamellipodia formation independent of focal adhesion complexes [121].

As stated before, the interaction between CS1 and integrin α4β1 plays an important role in adhesion and proliferation. This is particularly true in B-chronic lymphatic leukemia cells: when expressing both integrin α4 and CD38, the cells adhere to CS1 in a very efficient manner and are resistant to serum-deprivation-induced and spontaneous apoptosis [122]. When interacting with osteopontin, α4β1 can positively regulate macrophage survival and migration, suggesting an important role in the biology of these cells frequently associated with tumor microenvironment [123]. α5β1 is important in tumor progression because it indirectly helps metastatic cells to disseminate: its expression by proliferating lymphatic endothelial cells (LEC) and the ligand FN promote tumor-induced lymphangiogenesis as well as tumor metastasis to lymph nodes (LN) [124].

Integrin α9β1 is widely expressed in various cell types and has been shown to be important for a number of biological processes such as cell adhesion and migration, lung development and wound healing [115]. Since α9β1 null mice die around P12 of massive chylothorax and have severe defects in lymphatic valves [125], this integrin has reached the center stage in the field of the lymphatic vascular system development/function [125-128]. It is determinant during lymphatic valve morphogenesis: in primary human lymphatic endothelial cells, the integrin-α9-III A (EDA) interaction directly regulates FN fibril assembly, which is essential for the formation of the ECM core of valve leaflets [128]. In the field of tumor investigation, α9β1 is associated with reduced metastasis-free survival and reduced overall patient survival of breast cancer patients, identifying a novel cell-surface marker that promotes tumor cell invasion as demonstrated by in vitro assays using FN as migratory substrate for α9-integrin expressing cells [129]. Moreover, α9β1 is implicated in epithelial–mesenchymal transition, with a TGF-β-independent mechanism, favoring tumor growth and metastatic spread [130].

In general, there is a common propensity to assign a tumor promoting role for either α4 or α9 integrins when interacting with ECM ligands.

Adhesion to and migration on EMILIN1

EMILIN1 displays strong adhesive and migratory properties for different cell types [113,131,132]. The receptor responsible for these EMILIN1-mediated functions was initially identified as the integrin α4β1. The interaction between α4β1 and EMILIN1-αC1q is particularly efficient because even very low ligand concentrations provide very strong adhesion [131] and migration [132]. Accordingly to an α4-mediated adhesion model, the distribution pattern of actin and Paxillin of cells adhering to EMILIN1 leads to an accumulation of ruffles-inducing signals and a lack of stress fiber formation. The integrin α9β1 was first discovered as a novel receptor for EMILIN1 in keratinocytes, in epithelial cell lines and also in fibroblasts for which the adhesion pattern was identical to that observed in integrin α4β1-dependent attachment [113]. This finding was not surprising since α9 is highly homologous to α4 [114]. Very recently, we obtained supporting evidence that EMILIN1/α9 interaction is crucial also for LECs. In vitro LEC adhesion to and migration on EMILIN1 occur in a specifically integrin-α9-regulated manner (Danussi et al. submitted).

The lack of stress fibers and focal adhesions in cells attached to EMILIN1 indicates that cells, by binding via α4β1 to these ligands, are preferentially stimulated to migrate rather than to adhere firmly. Pro-migratory properties of EMILIN1 have been demonstrated for several cell types [104,132] but the finding that trophoblast cells attach and very efficiently migrate and haptotactically move on EMILIN1 is particularly important in the first phases of uterine wall invasion process [132]. Moreover, a cooperation of MMPs with integrin has been suggested in this process: membrane type I-matrix metalloproteinase (MT1-MMP) and MMP-2 are upregulated in co-cultures of trophoblast cells and stromal cells expressing EMILIN1, and enhance the haptotactic process towards EMILIN1 [132].

Regulation of cell proliferation

Beside the functional significance of adhesion and migration as the consequence of the interaction between EMILIN1 and α4/α9, the striking aspect of this ligand/receptor engagement is related to proliferation. It is generally known and explicitly suggested in this review that integrin engagement positively regulates cell growth [133]. The finding that EMILIN1 by the direct interaction with αC1q domain regulates skin cell proliferation points out a novel function of α4β1 as well as of α9β1 integrin [113]. At present targeted inactivation of the *Emilin1* gene in the mouse induces three phenotypes characterized by 1) systemic hypertension [111], 2) lymphatic alterations resulting in a mild lymphatic phenotype [109], and 3) increased thickness of epidermis and dermis [113]. The lack of integrin occupancy by EMILIN1 as occurs in *Emilin1−/−* mice leads to an increased number of Ki67-positive cells in epidermis and dermis. The molecular mechanism underlying the regulatory role of EMILIN1 in skin has been well defined, providing evidence that PTEN plays a central role in the cross talk between α4/α9β1 integrin and TGF-β signal pathways. We demonstrated that EMILIN1 binding to α4β1/α9β1 integrins empowers the down-regulation of proliferative cues induced by TGF-β through the upregulation of PTEN and the consequent inhibition of Erk [113]. The interaction between EMILIN1 with α4β1 (expressed on fibroblasts that secrete EMILIN1) and the closely related α9β1 (expressed on keratinocytes that do not secrete EMILIN1) provides an important external regulation for the maintenance of a correct homeostasis between proliferation and differentiation. Summarizing the role of EMILIN1 in skin, one can state that signals emitting from EMILIN1 engaged by α4/α9β1 integrins are anti proliferative.

The observations that *Emilin1*−/− mice display lymphatic hyperplasia associated with increased lymphatic vessel density are in line with the anti proliferative function of EMILIN1: three fold more Ki67-positive nuclei colocalizing with podoplanin-positive LECs are found...
in samples obtained from *Emilin1*−/− compared to wild-type (WT) mice [109]. The more recent findings of increased proliferation of human microvascular LECs in vitro and the increased number of proliferating Ki67-positive LECs in lymphatic collector valves have suggested a deregulated proliferation program of LECs as reported for basal keratinocytes of *Emilin1*−/− mice [113].

All these findings highlight the uniqueness of EMILIN1 for its integrin receptors: differently from what happens when other ECM ligands bind to α4 or α9 [65,122,123], the signal transduced by EMILIN1 has net antiproliferative effects (Figure 2).

**EMILIN1 and Cancer**

EMILIN1 is more than a scaffold molecule. Recent findings showing association between EMILIN1 and cancer suggest that the role played by this ECM glycoprotein in tumor microenvironment could be particularly crucial in providing regulation in cell growth and in metastatic spread.

The first analyses performed on lymphatic phenotype had already revealed that an EMILIN1-deficient microenvironment presents a clear propensity to develop tumors: *Emilin1*−/− mice develop larger lymphangiommas than WT mice [109]. Our recent studies have confirmed this property: tumor development in *Emilin1*−/− mice subjected to a skin carcinogenesis protocol was accelerated and the number and size of skin tumors was significantly increased compared to their WT littermates. *Emilin1*−/− skin tumors showed a dramatic increase of epidermal as well as dermal Ki67-positive cells compared to WT mice [134]. This suggested that aberrant skin homeostasis generated by EMILIN1 deficiency [113] also induced a pro-tumorigenic environment. Functional studies support the hypothesis that PTEN is a critical tumor suppressor for skin cancer in humans and in mice [135-137] by negatively regulating signal pathways involved in cell proliferation [138,139]. Accordingly, skin tumors of *Emilin1*−/− mice expressed less PTEN and higher levels of pErk 1/2, P13K and pAkt. Moreover, the higher lymphatic vessel density within the tumors as well as in the draining lymph nodes (LNs) in *Emilin1*−/− mice is likely the consequence of the lack of anti-proliferative effect of EMILIN1/α4-α9 integrin interaction.

Our supporting evidence for a tumor suppressor role played by EMILIN1 in the microenvironment is in line with other studies. Edlunger et al. have recently demonstrated that among stromal genes candidate for a role in non-small lung cancer the expression of EMILIN1 resulted altered [140]. While increased expression levels of genes such as PDLIM5, SPARC and TAGLN were associated with a high proliferation rate in the tumor, an increased expression levels of EMILIN1 and FBN1 were associated with low proliferation (lower fraction of Ki67-positive tumor cells) [140].

In a gene expression profile associated with response to doxorubicin-based therapy in breast cancer, Folgueira et al. searched for predictors of clinical response or no response, and found that EMILIN1 was upregulated in responsive tumors [141]. Even if the expression pattern of EMILIN1 did not significantly separate samples from the validation set according to response to chemotherapy, these data suggest that EMILIN1 would act as a protective microenvironment element against cancer growth.

This hypothesis seems not to be supported by two different studies of gene expression and proteomic analysis related to matrix protein profiles in ovarian carcinomas and soft tissue osteosarcomas where EMILIN1 was upregulated [142,143]. A pro- or anti-tumor action could be exerted by EMILIN1 in a tissue-specific manner. Another explanation for the EMILIN1 upregulation in tumors is that there is increased gene expression but the protein is not functional. Under appropriate conditions, specific proteolytic enzymes released by tumor cells and/or cells of the microenvironment could degrade EMILIN1 and its loss results in a condition similar to that of the ablated molecule in KO mice leading to uncontrolled cell proliferation. The use of KO model has been a very useful tool to discover most EMILIN1 functions and in particular to provide new insights in its role in proliferation and tumor growth. A question arises spontaneously: is there a situation in human life resembling EMILIN1 deficiency? Our very recent experience supports the following hypothesis: EMILIN1 can be digested in vitro by proteolytic enzymes, including neutrophil elastase. This finding is interesting since elastase profoundly influences cancer growth and development [144] and the presence of infiltrating inflammatory cells such as neutrophils is a peculiar feature of many tumor microenvironments [145,146]. Accordingly, EMILIN1 is digested in vivo: its degradation can be a relevant aspect of inflammatory and degenerative processes occurring in humans and can be important in tumor growth and metastatic process.

In conclusion these findings reinforce the idea that EMILIN1 structural integrity may be crucial to determine the tumor phenotype and may represent a regulator of fundamental processes such as
tumor dormancy and metastatic niche formation. Likely, this is what happens during the metastatic process in an EMILIN1-negative microenvironment. An important step in tumor progression is represented by LN metastases and by the role played in this process by soluble factors, ECM constituents and integrins on tumor or endothelial cells [147-149]. In skin tumor bearing-mice or in syngenic tumor cell transplantation models (B16F10 Luc2 and LLC cells), Emilin1−/− mice displayed more metastatic LNs compared to their WT littermates. A working hypothesis, taking into account that EMILIN deficiency severely affects the structural integrity of LECs due to a reduction of anchoring filaments and the presence of abnormal intercellular junctions [109], is that the structural defects of LECs in Emilin1−/− mice facilitate tumor cell passage and favor the metastatic spread.

An EMILIN1-negative or EMILIN1-unfunctional microenvironment promotes tumor cell proliferation (direct mechanism) as well as dissemination to LNs (indirect mechanism) (Figure 3). The lack of EMILIN1 expression may lead to alteration in cell-ECM molecular architecture and provide enhanced opportunity for tumor cell proliferation and migration through the disrupted barriers of the altered morpho-functional lymphatic vessels. Thus,

Figure 3: EMILIN1 and tumor microenvironment. EMILIN1 exerts a direct role in tumor growth through the specific interaction with α4/α9β1 integrins. When EMILIN1 is missing or degraded by proteolytic enzymes secreted by tumor microenvironment cells (stromal or inflammatory cells), the PTEN-mediated tumor growth suppressor function is lost. The dissemination of cancer cells in an EMILIN1-negative microenvironment (loss of function or protein deficiency) is indirectly favored by the structural defects of lymphatic vessels that display an irregular pattern, an increased vessel diameter, and a reduced number of anchoring filaments. These structural alterations determine higher lymphatic vessel permeability and render lymphatic vessels more permissive to the entry and exit of cancer cells. In addition, the lack of EMILIN1/integrin interaction results in an increased proliferation of LECs. Thus, there are more and not functional lymphatic vessels to facilitate cancer spreading.
taking into account that EMILIN1/α4-α9 integrin engagement seems to be crucial not only to directly suppress tumor cell growth but also to control lymphangiogenesis and tumor cell transmigration through LECs, we suggest that the suppressive role of EMILIN1 is associated to “structural” and “signaling-mediated” functions.

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