An EMILIN1-negative microenvironment promotes tumor cell proliferation and lymph node invasion.

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Running title: EMILIN1 inhibits tumor growth and lymphatic spread

Keywords: EMILIN1, skin carcinogenesis, tumor growth, lymph node invasion, transmigration

Conflict of interest: No conflict of interest has to be disclosed.

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Word count: 5017

Figure number: 6
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ABSTRACT

The evidence that EMILIN1 deficiency in Emilin1-/− mice caused dermal and epidermal hyperproliferation and an abnormal lymphatic phenotype prompted us to hypothesize the involvement of this extracellular matrix component in tumor development and in lymphatic metastasis. Using the 12-dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol 13-acetate (DMBA/TPA) two-stage model of skin carcinogenesis, we found that Emilin1+/− mice presented an accelerated formation, a higher incidence and the development of a larger number of tumors compared to their wild type littersmates. EMILIN1 negative tumors showed more Ki67-positive proliferating cells and higher levels of pErk1/2. In these tumors PTEN expression was lower. Emilin1-/− mice displayed enhanced lymphangiogenesis both in the tumor and in the sentinel lymph nodes. Accordingly, tumor growth and lymph node metastasis of transplanted syngenic tumors also were increased in Emilin1+/− mice. In vitro transmigration assays through lymphatic endothelial cells showed that EMILIN1 deficiency greatly facilitated tumor cell trafficking. Overall these data established that EMILIN1 exerts a protective role in tumor growth, in tumor lymphatic vessel formation as well as in metastatic spread to lymph nodes and reinforced the importance of its presence in the microenvironment to determine the tumor phenotype.
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INTRODUCTION

The microenvironment in which a tumor originates plays a critical role in tumor development and progression (1). It consists of cells, mainly fibroblasts, immune and vascular cells, soluble molecules and extracellular matrix (ECM) constituents that co-evolve during tumorigenesis generating a complex crosstalk for both positive and negative influences on tumor cells (2). The cell-ECM interactions also are critical in determining tumor cell proliferation. The composition, the extent of stiffness, and the structural integrity of tumor ECM have a marked impact on tumor cell behavior and on the neoplasia natural history (2-4). The importance of ECM in modulating tumor cell motility and invasion, neo-angiogenesis and the consequent hematogenous dissemination have been widely investigated (5). Some ECM molecules, i.e. CCN1 (6), thrombospondin-1 (7), endostatin (8), SPARC (9), decorin (10) and EMILIN2 (11) impair cancer cell viability by increasing cell death and/or apoptosis. Other ECM molecules, i.e. fibronectin and laminin 10/11, promote survival and enhance tumor proliferation, progression and chemotherapy resistance (12-15). However, it is the extent of lymph node (LN) metastasis that represents a major determinant for the staging and the prognosis of most human malignancies and often guides therapeutic decisions (16). The VEGF-C/VEGF-D/VEGFR-3 lymphangiogenic signaling axis (17-20), VEGF-A (21), PDGF-BB (22), as well as HGF (23) actively promote formation of tumor lymphatics and metastatic spread of tumor cells to LNs and there is mounting evidence that also the tumor ECM constituents significantly affect lymphangiogenesis (24). For instance, endostatin inhibited lymphangiogenesis and LN metastasis in a skin carcinogenesis mouse model (25) and hyaluronic acid resulted in a dramatic increase of tumor lymphatic vessel density in breast cancer (26). Moreover, tumor-induced lymphangiogenesis as well as tumor metastasis to LNs was promoted through the interaction between fibronectin and the integrin α4β1 expressed on lymphatic endothelial cells (LECs) and (27). In a previous study we demonstrated that mice deprived of EMILIN1, an ECM multidomain glycoprotein, presented an abnormal lymphatic phenotype with a
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Significant reduction of anchoring filaments, lymphatic vessel hyperplasia and a mild lymphatic dysfunction (28). EMILIN1 (Elastic Microfibril Interface Located ProteIN) is associated with elastic fibers (29) and beside to be expressed in lymphatic capillaries it is particularly abundant in the walls of large blood vessels (30), in intestine, lung, LNs, and skin (28). EMILIN1 interacts with the α4β1 integrin through its gC1q domain and it has strong adhesive and migratory properties for different cell types (31-33). Emilin1−/− mice display dermal and epidermal hyperproliferation because of the lack of EMILIN1 engagement by α4β1 or α9β1 integrins and the consequent upregulation of pErk1/2 levels through PTEN (34).

In the light that EMILIN1 deficiency caused skin and lymphatic vessel hyperplasia and structural anomalies in lymphatic vasculature, Emilin1−/− mice could represent a useful model to study the involvement of this ECM molecule in tumor development and in lymphatic metastasis. Here, by different in vivo models, we demonstrated that EMILIN1 exerts a suppressive role in tumor growth, in tumor lymphatic vessel formation as well as in metastatic spread to LNs.
MATERIALS AND METHODS

Antibodies and reagents. For lymphatic and blood vessel detection a rabbit polyclonal anti mouse LYVE-1 (Abcam, Cambridge, UK) and a rat monoclonal anti mouse Multimerin2 (MMRN2, clone 2063E2A11) (28) were used, respectively. The rat monoclonal anti mouse EMILIN1 antibody (clone 1007C11A8) was developed in our laboratories (28). The rabbit polyclonal anti-Ki67 was purchased from Abcam (Cambridge, UK). Rabbit anti-pErk1/2, anti-Akt, anti-phospho-Akt (Ser 473), anti-PTEN, anti-PI3K p110α and anti-α4 integrin antibodies were from Cell Signaling Technology (Danvers, MA). Goat anti-Erk1/2 and anti-vinculin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit anti pan-cytokeratin antibody from DakoCytomation (Carpinteria, CA).

Cells and culture procedures. Mouse lymphangioma endothelial cells (LAECs) were isolated following the procedure previously described (28). The cells were cultured on 1% porcine skin gelatin (Sigma) coated plates in EGM-2 MV medium (Cambrex Bio Science, Verviers, Belgium) and immortalized by means of SV40 infection to minimize cellular variability and to assure a consistent cell number for all functional assays (Supplementary Fig. S1). Melanoma B16F10 Luc2 cells were purchased from Caliper Life Sciences (Hopkinton, MA); Lewis Lung Carcinoma (LLC), PC3 (prostate), MDA-MB-231 (breast), SKOV-3 (ovarian) carcinoma cells were from American Type Culture Collection and each cultured in RPMI medium supplemented with 10% FCS. All human cell lines were authenticated by BMR Genomics srl Padova, Italia, on December 2011 according Cell ID™ System (Promega) protocol and using Genemapper ID Ver 3.2.1 to identify DNA STR profiles.
Models of tumor growth and dissemination. *Chemically induced two-step skin carcinogenesis.* WT and *Emilin1*<sup>−/−</sup> mice (CD1 and C57BL/6 strains) were generated as previously described (30). Procedures involving animals and their care were conducted according to the institutional guidelines in compliance with national laws (D.Lgs. n° 116/92). For tumor initiation, 50 μg of 7,12-Dimethylbenz(a)anthracene (DMBA; Sigma) were topically applied to the shaved back skin of 7-week-old WT (n=23) and *Emilin1*<sup>−/−</sup> (n=24) CD1 mice, followed by weekly topical application of 5 μg of the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma) over a 20-week period. Mice were sacrificed starting at 30 weeks after the first treatment. *Transplanted tumors.* 6-8-week-old WT and *Emilin1*<sup>−/−</sup> mice (C57BL/6 strain) were subcutaneously (s.c.) injected with 5×10<sup>5</sup> firefly-expressing B16F10 Luc2 cells and bioluminescence was quantified by an in vivo imaging system (Xenogen IVIS-100; Caliper Life Sciences, Hopkinton, MA) at different time intervals. B16F10 Luc 2 LN metastasis was evaluated ex vivo after excising the draining inguinal and axillary LNs. LN metastasis also was studied by s.c. injection of 5×10<sup>5</sup> LLC cells in WT and *Emilin1*<sup>−/−</sup> mice of C57BL/6 strain. Animals were sacrificed 3 weeks later and inguinal LNs were analyzed by RT-PCR for the presence of tumor cells.

**RNA extraction and RT-PCR.** Total cellular RNA was isolated from mouse LNs using TRIZOL (Invitrogen, Milan, Italy) according to the manufacturer’s protocol. RT reactions were performed with 1μg of total RNA using AMV-ReverseTranscriptase (Promega Italia, Milan, Italy). RNA was reverse transcribed into first-strand cDNA using random hexamer primers. The primers for mouse keratin 14 (KRT14) and 8 (KRT8) were: 5’-GGCCAACACTGAACGGAGGTG-3’ and 5’-CAGCTCCTCCTTGGAGCTCT-3’ and 5’-AGGCTGAGCTTTGGCAACATC-3’ and 5’-GAGATCTGAGACTGCAACTCA-3’, respectively. The size of the amplification products were 229 (KRT14) and 234 (KRT8) bp. The primers for mouse β actin were: 5’-GGCATTGTGATGGACTCCG-3’ and 5’-GCTGGAAGGTGGACAGTGA-3’. PCR reactions were
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performed using Go Taq DNA Polymerase (Promega Italia). Amplification products were resolved on 1.2% agarose gels stained with ethidium bromide.

**Western blotting.** Papilloma extracts were prepared in T-PER lysis buffer supplemented with protease inhibitor cocktail, both from Pierce Biotechnology (Rockford; IL). The protein content of the samples was determined using Bradford protein assay reagent (Bio-Rad; Milan, Italy) and western blot analysis was performed as previously described (34).

**Immunostaining.** Mouse tissues were excised and processed as previously reported (34). In immunoperoxidase staining HRP-conjugated secondary antibodies (Amersham, GE Healthcare Europe GmbH, Milan, Italy) were used and visualization was achieved with diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA). Samples were counterstained with hematoxylin. For fluorescence detection multiple staining was performed using a combination of differently secondary antibodies conjugated with Alexa Fluor® 488 and Alexa Fluor® 568 (Molecular Probes, Eugene, OR, USA). Nuclei were visualized with ToPro (Molecular Probes). Images were acquired with a Leica TCS SP2 confocal system (Leica Microsystems Heidelberg, Mannheim, Germany), using the Leica Confocal Software (LCS).

**TUNEL assay.** Apoptotic cells were detected in tumor cryostat sections by In Situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer’s instructions. The apoptosis rate was calculated as the mean number of apoptotic cells per field (40× magnification).

**Transmigration assays.** Tumor cell intravasation and extravasation throughout LAEC monolayers was performed growing $2 \times 10^4$ WT and Emilin1−/− LAECs on the under- or upperside of 1% gelatin coated FluoroBlokTM inserts (8 μm pore size, BD Falcon, Milan, Italy). After 5 days, each insert was checked for the formation of an intact monolayer by adding FITC-Dextran (MW 2000 KDa;
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0.5 mg/ml; Molecular Probes) to the upper chamber and measuring the amount of labeled dextran passed into the lower chamber by a computer-interfaced Genios Plus microplate fluorometer (Tecan Italia, Milan, Italy). The inserts were used only when the intensity of fluorescence in the lower chamber was negligible. For chemotaxis experiments, LAECs were allowed to grow in 24-well plates until confluence and then inserts were added to each well to obtain separate chambers. DiI-labeled (Molecular Probes) tumor cells (1×10^5) were added into the apical chamber. Migration was monitored at different time-intervals by independent fluorescence detection from the top and bottom side of the membrane using GENios Plus reader (Tecan Italia).

**Computer-assisted morphometric analyses.** The images, acquired with a Leica TCS SP2 confocal system, were analyzed by an ImageJ (35) computer-assisted morphometric analysis. The same software was used to calculate the volume of tumor-bearing mouse LNs by measuring their large and small distance from digital camera-captured images.

**Statistical analysis.** Statistical significance of the results was determined by using the unpaired and paired Student’s *t* test. A value of *P* < 0.05 was considered significant.
RESULTS

Higher susceptibility to chemically induced skin carcinogenesis in *Emilin1*−/− mice. WT (*n*=23) and *Emilin1*−/− (*n*=24) CD1 mice were treated with DMBA as initiator followed by repeated promotions with TPA. *Emilin1*−/− mice showed accelerated formation of skin papillomas with an average latency period of 10 weeks after the first application of TPA, compared to 18 weeks for WT mice (Fig. 1A). By week 20, 100% of *Emilin1*−/− and only 60% of WT mice developed tumors. By week 25, *Emilin1*−/− mice developed an average of 10.4 papillomas/mouse, compared to 2.6 papilloma/mouse in their WT littermates (*P*<0.01; Fig. 1B). These differences were also maintained when only larger papillomas (diameter >3 mm) were evaluated: large papillomas developed 5 weeks earlier in *Emilin1*−/− than in WT mice (Fig. 1C) and by week 25, their average number was 23-fold (*P*<0.001; Fig. 1D). Accordingly, the volume of tumors was more than 8.7-fold in *Emilin1*−/− compared to WT mice (*P*<0.005; Fig. 1E). The general appearance of mouse back skin after a monitoring period of 30 weeks shows that larger and numerous papillomas were clearly detectable in *Emilin1*−/− mice (Fig. 1F).

Next, we compared the rate of malignant conversion in the two genotypes. Histological examination did not reveal any major difference(s) in WT and *Emilin1*−/− tumors of similar size, all of which displayed a clear cut border in many cases and projecting above the surrounding tissue (Fig. 1G and H). A similar percentage of carcinomas were clearly identified by morphological examination in both genotypes by the loss of an orderly basal layer, keratin pearls, clusters of tumor cells in the dermis, as well as microinvasions (Fig. 1G and H). Thus, EMILIN1 was unlikely to play a major role in malignant conversion.

EMILIN1 deficiency promotes tumor cell hyperplasia. EMILIN1 was abundantly expressed in the mesenchymal stroma (i.e. the dermal layer) of the papillomas, whereas it was almost completely absent in the epidermal layer, where only the basal keratinocytes took contact with this ECM
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constituent (Fig. 2A and B). A dramatic increase of epidermal as well as dermal Ki67 positive cells was evident in Emilin1<sup>−/−</sup> tumors compared with their WT littermates (Fig. 2A-F). A quantitative analysis revealed that these differences were highly significant (Fig. 2G and H). In contrast, the rate of apoptotic cells was similar in both genotypes (Fig. 2I).

Emilin1<sup>−/−</sup> tumors showed reduced PTEN levels with a homogenous distribution in the epidermal layer compared to the strong positivity and the gradient staining in the WT counterpart (Fig. 2J). Importantly, the higher Ki67-rate observed in Emilin1<sup>−/−</sup> tumors was associated with an enhanced activation of proliferative signal pathway molecules, such as Erk1/2, PI3K and pAkt (Fig. 2K).

**Increased lymphangiogenesis within skin tumors and sentinel LNs in Emilin1<sup>−/−</sup> mice.** To determine if EMILIN1 modulated the extent of tumor lymphangiogenesis in skin papillomas, we analyzed lymphatic and blood vessel density (Fig. 3A and B). While great variability vessel density in both genotypes was observed, an accurate quantitative analysis demonstrated that Emilin1<sup>−/−</sup> tumors displayed more LYVE-1–positive vessels compared to WT littermates (Fig. 3B). In contrast, no significant differences were found in MMRN2-positive blood vessels (Fig. 3B). A similar analysis was performed also on cryostat sections of inguinal and axillary LNs of chemically treated mice. We found a highly significant ($P=1\times10^{-8}$) increase in lymphatic vessel density in draining LNs of Emilin1<sup>−/−</sup> compared to those of WT mice (Fig. 3C and D). In accord with the results in the tumor stroma, no quantitative differences were found in blood vessels of the two genotypes (data not shown). Together, these findings indicated that the lack of EMILIN1 determines increased lymphangiogenesis in skin tumors as well as within sentinel LNs likely generating a lymphatic pro-metastatic environment.

**Increased LN metastasis in Emilin1<sup>−/−</sup> mice.** When tumor-bearing mice were sacrificed, we noticed that the LNs of Emilin1<sup>−/−</sup> mice presented a severely altered gross morphology (Fig. 3G and H) compared to their WT littermates (Fig. 3E and F). Emilin1<sup>−/−</sup> LNs were characterized by a larger size
EMILIN1 inhibits tumor growth and lymphatic spread (Fig. 4I; \( P<4.5\times10^{-6} \)) and the frequent presence of wide hemorrhagic foci (Fig 3G and H). Metastatic cells in LNs were detected by a PCR analysis of the tumor markers Keratins 8 and 14 (K8 and K14) (36). \( Emilin1^{-/-} \) mice presented about 30% K8 and K14 positive LNs, whereas in WT mice this percentage was 5% for K14 and 15% for K8 (Fig. 3J). A pan-cytokeratin antibody was used to visualize metastatic cells in LN cryostat sections. The staining confirmed a higher presence of micrometastases in \( Emilin1^{-/-} \) than in WT LNs (Supplementary Fig. S2). The 5.5-fold increased percentage of K14-positive LNs detected in \( Emilin1^{-/-} \) mice together with the notion of the structural alterations of lymphatic vasculature in \( Emilin1^{-/-} \) mice (28), prompted us to hypothesize that EMILIN1 deficiency could play an important role not only in increasing lymphangiogenesis, but also in facilitating tumor cells dissemination to LNs.

**Enhanced tumor growth and LN metastasis of transplanted tumors in \( Emilin1^{-/-} \) mice.** It is possible that the increased LN metastatic spread in \( Emilin1^{-/-} \) mice (Fig 3J) was the consequence of the considerably larger tumor burden in \( Emilin1^{-/-} \) mice. To circumvent this confounding element we further investigated the role of EMILIN1 in lymphatic metastasis using syngenic transplanted tumor models B16F10 Luc2 and LLC cells. WT (n=10) and \( Emilin1^{-/-} \) (n=10) mice were s.c. injected with \( 5\times10^5 \) B16F10 Luc2 cells and tumor growth was monitored by in vivo bioluminescence imaging at different time intervals (Fig. 4A). Until day 5, the growth of B16F10 Luc2 tumors was comparable in the two mouse genotypes (Fig. 4A and B). Starting from day 7 post-inoculation, B16F10 Luc2 grew at a significantly higher rate in \( Emilin1^{-/-} \) mice compared to their WT littermates (Fig. 4A and B). After 9 days B16F10 Luc2 tumors began to be necrotic and to avoid analytical bias the luciferase signal was no longer acquired. The higher growth of B16F10 Luc2 cells in \( Emilin1^{-/-} \) mice was expected since melanoma cells express \( \alpha 4 \) (Fig. 4C) and very weakly \( \alpha 9 \)-integrin (data not shown): the lack of EMILIN1-\( \alpha 4/\alpha 9 \) integrin promotes proliferation (34) and this might be the reason for which B16F10 Luc2 cells gain a growth advantage in an EMILIN1-negative microenvironment.
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To investigate lymphatic metastasis WT and *Emilin1<sup>−/−</sup>* mice bearing B16F10 Luc2 tumors were sacrificed at 16-17 days post-inoculation; inguinal and axillary LNs were excised and analyzed ex vivo for the presence of luciferase signal. Notably, only 25% WT and almost 75% *Emilin1<sup>−/−</sup>* LNs were metastatic (Fig. 4D). Brown metastatic foci of B16F10 Luc2 melanoma cells were clearly visible under dissecting microscope and were more numerous and larger in *Emilin1<sup>−/−</sup>* than in WT LNs (Fig. 4E). The extent of LN metastases was actually higher in *Emilin1<sup>−/−</sup>* mice, as evidenced by a 2.8-fold increase of the average luciferase signal detected in the LNs (Fig. 4F and G). To confirm that the increased LN metastasis was linked to lymphatic anomalies present in *Emilin1<sup>−/−</sup>* mice rather than to an enhanced proliferation effect, we assayed LLC cells as an α4 and α9-integrin lacking cellular model (Fig. 4C and data not shown) for tumor transplant studies. LLC cells were s.c. injected in WT (n=9) and *Emilin1<sup>−/−</sup>* (n=9) mice. After 3 weeks, mice were sacrificed and tumor volume was calculated. Unexpectedly, the primary tumors were significantly larger in WT than in *Emilin1<sup>−/−</sup>* mice (Fig. 5A). The H/E staining of cryostat inguinal LN sections revealed the presence of more metastatic cells, positive for a pan-cytokeratin antibody used in corresponding serial sections, in *Emilin1<sup>−/−</sup>* LNs compared to their WT littermates (Fig. 5B). The presence of metastatic LLC cells that are positive for K8 (data not shown) was assayed by RT-PCR analysis for K8 expression. The percentage of K8-positive LNs was 2-fold higher in *Emilin1<sup>−/−</sup>* mice, compared to their WT littermates (Fig. 5B). Overall, the data obtained from the syngenic tumor models demonstrated that EMILIN1 deficiency promoted lymphatic metastasis.

**EMILIN1 deficiency favors intra- and extravasation of tumor cells through LAEC monolayers.** It has been previously demonstrated that EMILIN1 deposition is required for the structural integrity of lymphatic vessels (28). LECs isolated from both *Emilin1<sup>−/−</sup>* and WT mice expressed specific lymphatic markers and did not differ for the expression of other ECM molecules such as fibronectin (Supplementary Fig. S1, A and B). To verify if LECs were differently permissive for tumor cell passage a comparative transmigration assay was performed growing WT
and *Emilin1*−/− LAECs on the lower-side or on the upper-side of transwell filters to reproduce lymphatic intra- and extravasation, respectively. The capability of PC3, MD-MBA-231 and SKOV-3 carcinoma cell lines to migrate through WT or *Emilin1*−/− LAEC monolayer was evaluated at different time intervals. The percentage of tumor cell types migrated through *Emilin1*−/− LAECs at 6 hours was higher both for the intra- as well as for the extravasation process (Fig. 6A and 6B). Since expression of α4β1 integrin on B16F1 cells correlated with tumor cell spreading to draining LNs (37) we checked if also under our experimental model the migratory ability of cancer cells was limited to α4 integrin expression: FACS analysis revealed a weak positivity only for SKOV-3 cells (data not shown) and function blocking anti α4 integrin antibodies did not alter transmigration process (Supplementary Fig. S1). This suggested that the integrity of the LAEC layer lining lymphatic vessels required EMILIN1 deposition and that the lack of EMILIN1 promoted tumor cell entry and/or exit from the lymphatics. The fact that both carcinoma cell lines did not migrate under chemotactic conditions further reinforced the importance of the lymphatic endothelium integrity in tumor cell transmigration (Supplementary Fig. S1).

**DISCUSSION**

The ECM microenvironment constitutes an integral part of the tumor anatomy and pathophysiology, and is functionally interconnected with cell proliferation and metastatic dissemination (38). However, only very few ECM proteins exert a tumor suppressor function: for instance, Trombospondin-1 (TSP-1), Trombospondin-2 (TSP-2) and Fibulin-2 mainly counteract tumor growth and impact angiogenesis (39;40); LTBP-2 impairs migration and invasion ability of neoplastic cells (41). Here, we demonstrated that EMILIN1, an ECM glycoprotein diffusely expressed in several tissues, exerted a suppressive function on tumor growth, tumor lymphatic vessel formation as well as tumor cell spread to LNs. Tumor development in *Emilin1*−/− mice subjected to a skin carcinogenesis protocol was accelerated and the number and size of skin tumors
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was significantly increased compared to their WT littermates. We have recently shown that EMILIN1 directly inhibited cell proliferation by engaging integrin α4β1 expressed on dermal fibroblasts or α9β1 expressed on basal keratinocytes. The lack of EMILIN1-α4/α9 integrin interaction in Emilin1-/- mice promoted normal skin cell hyperplasia because of the reduction of PTEN, activation of PI3K/Akt and Erk1/2 pathways and hence increased proliferation (34). Here, Emilin1-/- skin tumors showed a dramatic increase of epidermal as well as dermal Ki67 positive cells compared to WT mice. This suggested that aberrant skin homeostasis generated by EMILIN1 deficiency (34) 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 as well (42-44) by negatively regulating signal pathways involved in cell proliferation (45;46). Notably, skin tumors of Emilin1-/- mice display less PTEN, demonstrating that the presence of EMILIN1 in tumor microenvironment can regulate the expression of this important tumor suppressor. A potential mechanism was previously described (34), suggesting that PTEN deregulation in Emilin1-/- is the result of a complex cross-talk between TGF-β and α4/α9 integrin signal pathways. In Emilin1-/- mice the PTEN/Erk/Akt signaling accelerated an imbalance in homeostasis and progression but further studies will be necessary to fully clarify all the steps that allow the altered tissue homeostasis program to endow the cells mutated by DMBA to abruptly respond to the tumor promoter TPA.

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 (24;47;48). Here, in all the in vivo models analyzed, either skin tumor bearing-mice or syngenic tumor cell transplantation (B16F10 Luc2 and LLC cells), Emilin1-/- mice presented a statistically significant increase in the percentage of metastatic LNs, compared to their WT littermates. Integrins control LEC adhesion, migration, and survival, promote lymphangiogenesis and favor metastatic spread (49). For instance, as the expression of α4β1 integrin on B16F1 increased, the likelihood of tumor cell spreading to regional LNs significantly increased because α4β1 expressed by tumor cells
interacted with VCAM-1 expressed on LECs (37). In another study, a different role for α4β1 was proposed that its expression by proliferating LECs and the ligand fibronectin directly promote tumor-induced lymphangiogenesis as well as tumor metastasis to LNs (27). The higher lymphatic vessel density within the tumors as well as in the draining LNs in Emilin1−/− mice is likely the consequence of the lack of anti-proliferative effect of EMILIN1/α4-α9 integrin interaction. The structural properties of EMILIN1 in lymphatic spread were better disclosed and evident in the extra- or intravasation phases of the metastatic process: the finding that expression of α4β1 on transplanted tumor cells did not correlate with in vitro transmigration assays suggested that this integrin was not playing a primary role in tumor cell dissemination at least in our system. 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 (28), the structural properties of LECs in Emilin1−/− mice very likely facilitated tumor cell passage and favored the metastatic spread.

An EMILIN1-negative microenvironment promotes tumor cell proliferation as well as dissemination to LNs. 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, taking into account that EMILIN1/α4 integrin engagement seems to be crucial not only to directly suppress tumor cell growth but also to control lymphangiogenesis and that structural defects in Emilin1−/− LECs are responsible for tumor cell transmigration, we suggest that the suppressive role of EMILIN1 is associated to both “structural” and “signaling-mediated” functions. Considering that proteolytic degradation of ECM has long been associated with tumor aggressiveness (50), our study indicated 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.
Acknowledgements. We wish to thank Prof. G.M. Bressan for providing Emilin1−/− mice.

Grant support. This work was supported by grants to P. Spessotto from AIRC (IG 10119) and to A. Colombatti from AIRC, PRIN (20074S758W_002), FIRB (RBRN07BMCT) and Progetto di ricerca finalizzata 5%/anno Anno 2007-Intramural bench to bedside. C. Danussi is a recipient of a Federazione Italiana Ricerca sul Cancro fellowship.

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(35) http://rsb.info.nih.gov


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FIGURE LEGENDS

Figure 1. Enhanced skin tumorigenesis in Emilin1−/− mice. A, Accelerated development of skin papillomas in Emilin1−/− mice (n=24), compared to their WT littermates (n=23). Incidence is
EMILIN1 inhibits tumor growth and lymphatic spread expressed as the percentage of mice with detectable papillomas (>1 mm). B, Frequency of papilloma formation, expressed as the average number of papillomas per mouse (*P<0.01). C, Development of large papillomas (>3 mm). D, Frequency of large papillomas formation (*P<0.001). E, Average volume (V= 0.5xdLxdS^2; dL: larger distance; dS: smaller distance) of all WT and Emilin1^+/− mouse papillomas (*P<0.005). F, General appearance of skin tumors of WT and Emilin1^+/− mice after 30 weeks from the beginning of treatment. G and H, Representative H&E staining of WT (G) and Emilin1^+/− (H) mouse papillomas. Black arrows indicate clear cut border, black arrowheads clusters of tumors cells in the dermis, white arrow keratin pearls and white arrowheads tumor cells projecting above the surrounding tissue. Scale bars: 200 μm (a, b and c) and 50 μm (a’, b’ and c’).

Figure 2. Increased cell proliferation in Emilin1^+/− skin tumors is correlated with increased Erk1/2, PI3K and Akt activity and impaired PTEN expression. A-B, Representative images of WT papilloma cryostat sections stained for EMILIN1 (green) and for the proliferation marker Ki67 (red). C, Zoomed image of B (boxed area). D-F, Representative images of Emilin1^+/− papilloma cryostat sections stained for Ki67. e: epidermis; d: dermis; dashed lines denote dermal-epidermal border. Scale bar: 75 μm. G and H, Quantitative analysis of the average number (± SD) of Ki67 positive cells in the epidermis and dermis of WT (n=11) and Emilin1^+/− (n=30) papillomas. *P=0.03; **P=0.009. I, Quantitative analysis of the average number of apoptotic cells per field (40×) in TUNEL stained skin cryostat sections of WT (n=6) and Emilin1^+/− (n=15) papillomas.

J, Representative images of PTEN immunostaining of WT (a, a’) and Emilin1^+/− (b, b’) papilloma cryostat sections. a’ and b’ are higher magnification of boxed area in a and b, respectively. Scale bars: 200 μm (a, b) and 50 μm (a’, b’). K, Representative western blot analysis of WT and Emilin1^+/− papilloma extracts and corresponding quantification by QuantityOne software (Bio-Rad; Milan, Italy).
Figure 3. Enhanced tumor and sentinel lymph node lymphangiogenesis in tumor-bearing
Emilin1+/− mice. A, Immunofluorescence analysis of cryostat sections of WT and Emilin1+/−
papillomas. Lymphatic vessels are LYVE-1-positive (red) and blood vessels are MMRN2-positive
(green). Nuclei are labeled by ToPro (blue). Scale bar: 300 μm. B, Computer-assisted morphometric
analysis of tumor-associated lymphatic and blood vessels. Three representative images of
papillomas (n=6) for each genotype were analyzed. The values represent the mean number ± SD of
vessels per unit area (mm²). *P = 0.003. C, Representative immunofluorescence images of inguinal
lymph node cryostat sections stained for LYVE-1 (red) and MMRN2 (green) excised from
papilloma-bearing WT and Emilin1+/− mice. Scale bar: 300 μm. D, Computer-assisted morphometric
analysis of lymph node lymphatic and blood vessels. **P=1x10⁻⁸. E and G, Representative images
of inguinal LNs (black arrows) of papilloma-bearing WT and Emilin1+/− mice. F and H, Images of
excised inguinal and axillary lymph nodes. Scale bar: 5 mm. I, Volume mean values ± SD of WT
(n=34) and Emilin1+/− (n=38) inguinal and axillary LNs. Larger (dL) and smaller distance (dS) of
LNs were measured with ImageJ software and volume was calculated by the formula V =
0.5xdLxdS². *P<4.5x10⁻⁶. J, Number of K8 and K14-positive or negative lymph nodes evaluated by
RT-PCR analysis.

Figure 4. B16F10 Luc2 metastases in WT and Emilin1+/− LNs. A, WT (n=10) and Emilin1+/−
(n=10) mice were implanted s.c. in the right flank with 5x10⁵ B16F10 Luc2 melanoma cells. Tumor
development was quantified by luciferase signal, measured by in vivo optical imaging at days 0, 2,
5, 7, 9 after inoculation. The color scale indicates bioluminescence intensity. B, Tumor growth
curve of B16F10 Luc2 tumors expressed as increased percentage of luciferase signal (mean ± SE).
C, Western blotting analysis of α4 integrin expression in B16F10 Luc2 and LLC cells. D,
Percentage of B16F10 Luc2 metastatic WT and Emilin1+/− LNs. The presence of brown melanoma
cell foci in the excised LNs was evaluated at the dissection microscope. Altogether, 20 LNs were
analyzed per each mouse genotype. E, Representative images from dissection microscope of
EMILIN1 inhibits tumor growth and lymphatic spread

excised WT and Emilin1−/− LNs of B16F10 Luc 2-bearing mice. Highlighted areas indicate the presence of brown foci. F, Mean ± SE luciferase signal detected by ex vivo optical imaging in the excised LNs of B16F10 Luc 2-bearing mice. G, Representative images and color scale of the luciferase signal emitted by LNs of B16F10 Luc 2-bearing mice. *P<0.05; **P<0.01.

**Figure 5. LLC metastases in WT and Emilin1−/− LNs.** A, WT (n=9) and Emilin1−/− (n=9) mice were implanted s.c. in the right flank with 5×10^5 LLC cells. Animals were sacrificed 3 weeks later and tumor volume was calculated (V= 0.5xdLxdS^2; dL: larger distance; dS: smaller distance). Representative WT (n=4, T1-T4) and Emilin1−/− (n=3, T1-T3) tumors and average volume of all tumors (n=9) are reported. B, Excised inguinal LNs were stained with H&E (a, b); serial cryostat sections were immunostained with a pan-cytokeratin antibody (b’, b’’). White arrows point micrometastases and boxed areas correspond to serial sections enlarged and stained for cytokeratin. Inguinal LNs were analyzed by RT-PCR for the presence of tumor cells and percentage of K8-positive LNs of LLC-bearing WT (n=9) and Emilin1−/− (n=9) mice is reported. Scale bars: 50 μm (a, b) and 20 μm (b’, b’’). *P<0.05.

**Figure 6. Tumor cell transmigration.** A and B, Transmigration assays of PC3, MDA-MB-231 and SKOV-3 cells were performed growing WT and Emilin1−/− LAECs on the lower-side or on the upper-side of transwell filters to mimic lymphatic intra- (A) and extravasation (B). The values report the mean migration percentages ± SD obtained in three separate experiments performed in triplicate. *P<0.05.
Figure 2
**Figure 3**

A-B. Papilloma analysis showing differences between WT and Emilin1−/− mice. The number of lymphatic vessels is significantly reduced in Emilin1−/− mice compared to WT mice. (B) Bar graph illustrating the quantification of lymphatic vessels and blood vessels in WT and Emilin1−/− mice.

C-D. Lymph node (LN) analysis showing lymphatic vessel density. The density of lymphatic vessels in Emilin1−/− mice is significantly increased compared to WT mice. (D) Bar graph illustrating the comparison of lymphatic vessel density between WT and Emilin1−/− mice.

E-G. Female mammary gland analysis showing differences in vascularization. Arrows indicate areas of interest in the tumor and surrounding vasculature. (G) Bar graph illustrating the comparison of tumor volume between WT and Emilin1−/− mice.

H-J. Analysis of positive LN samples using K8 and K14 markers. Emilin1−/− mice show a higher percentage of positive LN samples compared to WT mice. (J) Bar graph illustrating the comparison of positive LN samples between WT and Emilin1−/− mice.
Figure 4
Figure 5
Figure 6
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An EMILIN1-negative microenvironment promotes tumor cell proliferation and lymph node invasion.

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Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-12-0076-T

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