An EMILIN1-Negative Microenvironment Promotes Tumor Cell Proliferation and Lymph Node Invasion

Carla Danussi, Alessandra Petrucco, Bruna Wassermann, Teresa Maria Elisa Modica, Eliana Pivetta, Lisa Del Bel Belluz, Alfonso Colombatti, and Paola Spessotto

Abstract
The evidence that EMILIN1 (Elastic Microfibril Interface Located protein) 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 with their wild-type littermates. 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 were also 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. Cancer Prev Res; 1–13. ©2012 AACR.

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 coevolve during tumorigenesis generating a complex cross-talk for both positive and negative influences on tumor cells (2). The cell–ECM interactions are also critical in determining the tumor cell proliferation. The composition, the extent of stiffness, and the structural integrity of tumor ECM have a marked impact on the tumor cell behavior and on the neoplasia natural history (2–4). The importance of ECM in modulating tumor cell motility and invasion, neangiogenesis, and the consequent hematogenous dissemination have been widely investigated (5). Some ECM molecules, that is, 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, that is, 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 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), platelet-derived growth factor-BB (PDGF-BB; ref. 22), as well as hepatocyte growth factor (HGF; ref. 23) actively promote formation of tumor lymphatics and metastatic spread of tumor cells to lymph nodes and also there is mounting evidence that the tumor ECM constituents significantly affect lymphangiogenesis (24). For instance, endostatin inhibited lymphangiogenesis and lymph node 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 lymph nodes was...
promoted through the interaction between fibronectin and the integrin α4β1 expressed on lymphatic endothelial cells (LEC; ref. 27). In a previous study, we showed that mice deprived of EMILIN1, an ECM multidomain glycoprotein, presented an abnormal lymphatic phenotype with a 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 besides being expressed in lymphatic capillaries, it is particularly abundant in the walls of large blood vessels (30), intestine, lung, lymph nodes, 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). Emnilin1−/− mice display dermal and epidermal hyperplification 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, Emnilin1−/− 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 showed that EMILIN1 exerts a suppressive role in tumor growth, in tumor lymphatic vessel formation, as well as in metastatic spread to lymph nodes.

Materials and Methods

Antibodies and reagents

For lymphatic and blood vessel detection, a rabbit polyclonal anti-mouse LYVE-1 (Abcam) and a rat monoclonal anti-mouse Multimerin 2 (MRRN2, clone 2063E2A11; ref. 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. 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. Goat anti-Erk1/2 and anti-vinculin antibodies were from Santa Cruz Biotechnology and rabbit anti-pan-cytokeratin antibody from DakoCytomation.

Cells and culture procedures

Mouse lymphangioma endothelial cells (LAEC) 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) 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; Lewis lung carcinoma (LLC), PC3 (prostate), MDA-MB-231 (breast), and SKOV-3 (ovarian) carcinoma cells were from American Type Culture Collection and each were cultured in RPMI medium supplemented with 10% fetal calf serum. All human cell lines were authenticated by BMR Genomics srl Padova, Italy, on December 2011 according to the Cell ID System (Promega) protocol using Genemapper ID Ver 3.2.1 to identify DNA short tandem repeat profiles.

Models of tumor growth and dissemination

Chemically induced two-step skin carcinogenesis. Wild-type (WT) and Emnilin1−/− 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 the national laws (D.Lgs. n° 116/92). For tumor initiation, 50 μg of 7,12-dimethylbenz(α)anthracene (DMBA; Sigma) was topically applied to the shaved back skin of 7-week-old WT (n = 23) and Emnilin1−/− (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 at 30 weeks after the first treatment.

Transplanted tumors. Six- to 8-week-old WT and Emnilin1−/− mice (C57BL/6 strain) were subcutaneously injected with 5 × 105 firefly-expressing B16F10 Luc2 cells and bioluminescence was quantified by an in vivo imaging system (Xenogen IVIS-100; Caliper Life Sciences) at different time intervals. B16F10 Luc 2 lymph node metastasis was evaluated ex vivo after excising the draining inguinal and axillary lymph nodes. Lymph node metastasis also was studied by the subcutaneous injection of 5 × 105 LLC cells in WT and Emnilin1−/− mice of C57BL/6 strain. Animals were sacrificed 3 weeks later and inguinal lymph nodes were analyzed by reverse transcriptase (RT)-PCR for the presence of tumor cells.

RNA extraction and RT-PCR

Total cellular RNA was isolated from mouse lymph nodes using TRIZOL (Invitrogen) according to the manufacturer’s protocol. RT reactions were conducted with 1 μg of total RNA using AMV-ReverseTranscriptase (Promega Italia). RNA was reverse transcribed into the first-strand cDNA using random hexamer primers. The primers for mouse keratin 14 (KRT14) and 8 (KRT8) were: 5′-GGCCCAAACACT-GAAGCTGCCGGG-3′ and 5′-CAAGTCCCTCCTTGGCCTCGT-CT′ and 5′-AGGCTGAGGTGGACCAC-3′ and 5′-GA- GAATGACTGACTCCACTA-3′, respectively. The size of the amplification products was 229 (KRT14) and 234 (KRT8) bp. The primers for mouse β-actin were: 5′-GGCAATTTGCCAGCTGGTG-3′ and 5′-CTGGGAGGTGGACACTGA-3′. PCR reactions were conducted 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. The protein content of the samples was determined using Bradford protein assay reagent (Bio-Rad) and Western blot analysis was conducted as previously described (34).

Immunostaining
Mouse tissues were excised and processed as previously reported (34). In immunoperoxidase staining, horseradish peroxidase (HRP)-conjugated secondary antibodies (Amer- sham, GE Healthcare Europe GmbH) were used and visual- ization was achieved with a dianinobenzidine 3,3’-dia- minobenzidine (DAB) substrate (Vector Laboratories). Samples were counterstained with hematoxylin. For fluo- rescence detection, multiple staining was carried out using a combination of different secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 568 (Molecular Probes). Nuclei were visualized with ToPro (Molecular Probes). Images were acquired with a Leica TCS SP2 con- focal system (Leica Microsystems Heidelberg), 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 invasiveness and extravasation throughout LAEC monolayers was conducted growing 2 × 10^5 WT and Emilin1^−/− LAECs on the under- or upper-side of 1% gelatin-coated FluoroBlok inserts (8 μm pore size, BD Falcon). After 5 days, each insert was checked for the formation of an intact monolayer by adding fluor- escienc isothiocynate (FITC)-Dextran (MW 2000 KDa; 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). The inserts were used only when the intensity of fluorescence in the lower chamber was negligible. For chemotaxis experi- ments, LAECs were allowed to grow in 24-well plates until confluence and then the inserts were added to each well to obtain separate chambers. DiI-labeled (Molecular Probes) tumor cells (1 × 10^5 cells) 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 software. The same software was used to calculate the volume of tumor-bearing mouse lymph nodes 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 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 pro- motions with TPA. Emilin1^−/− mice showed accelerated formation of skin papillomas with an average latency period of 10 weeks after the first application of TPA, com- pared with 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 per mouse compared with 2.6 papillo- mas per 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 papillo- mas 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 with 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 2 genotypes. Histologic 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 identi- fied by morphologic 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 constituent (Fig. 2A and B). A dramatic increase of epider- mal as well as dermal Ki67-positive cells was evident in Emilin1^−/− 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^−/− tumors showed reduced PTEN levels with a homogenous distribution in the epidermal layer compared with the strong positivity and the gradient staining in the WT counterpart (Fig. 2J). Importantly, the higher Ki67 rate observed in Emilin1^−/− tumors was associated with an enhanced activation of proliferative signal pathway...
Figure 1. Enhanced skin tumorigenesis in Emilin1−/− mice. A, accelerated development of skin papillomas in Emilin1−/− mice (n = 24) compared with their WT littermates (n = 23). Incidence is 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 (>3 mm). C, development of large papillomas (>5 mm). D, frequency of large papilloma formation (>5 mm, P < 0.001). E, average volume (V = 0.5 × dL × dS; dL: larger distance; dS: smaller distance) of all WT and Emilin1−/− mouse papillomas (>5 mm, 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 hematoxylin and eosin 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’).
molecules, such as Erk1/2, phosphoinositiode-3-kinase (PI3K), and pAkt (Fig. 2K).

**Increased lymphangiogenesis within skin tumors and sentinel lymph nodes in Emilin1−/− 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 in vessel density was observed in both genotypes, an accurate quantitative analysis showed that Emilin1−/− tumors displayed more LYVE-1–positive vessels compared with WT littermates (Fig. 3B). In contrast, no significant differences were found in MMRN2-positive blood vessels (Fig. 3B). A similar analysis was
conducted on cryostat sections of inguinal and axillary lymph nodes of chemically treated mice. We found a highly significant (P = 1 × 10^-8) increase in lymphatic vessel density in draining lymph nodes of Emilin1^-/- compared with 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 2 genotypes (data not shown). Together, these findings indicated that the lack of EMILIN1 determines increased lymphangiogenesis in skin tumors as well as within sentinel lymph nodes likely generating a lymphatic pre-metastatic environment.

Increased lymph node metastasis in Emilin1^-/- mice

When tumor-bearing mice were sacrificed, we noticed that the lymph nodes of Emilin1^-/- mice presented a severely altered gross morphology (Fig. 3G and H) when compared with their WT littermates (Fig. 3E and F). Emilin1^-/- lymph nodes were characterized by a larger size (Fig. 4I; P < 4.5 × 10^-8) and the frequent presence of wide hemorrhagic foci (Fig 3G and H). Metastatic cells in lymph nodes were detected by the PCR analysis of the tumor markers Keratins 8 and 14 (K8 and K14; ref. 36). Emilin1^-/- mice presented about 30% K8- and K14-positive lymph nodes, whereas in WT mice, this percentage was 5% for K14 and 15% for K8 (Fig. 3I). A pan-cytokeratin antibody was used to visualize metastatic cells in lymph node cryostat sections. The staining confirmed a higher presence of micrometastases in Emilin1^-/- than in WT lymph nodes (Supplementary Fig. S2). The 5.5-fold increased percentage of K14-positive lymph nodes 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 cell dissemination to lymph nodes.

Enhanced tumor growth and lymph node metastasis of transplanted tumors in Emilin1^-/- mice

It is possible that the increased lymph node 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 subcutaneously injected with 5 × 10^5 B16F10 Luc2 cells and the 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 2 mouse genotypes (Fig. 4A and B). Starting from day 7 postinoculation, B16F10 Luc2 grew at a significantly higher rate in Emilin1^-/- mice compared with their WT littermates (Fig. 4A and B). After 9 days, B16F10 Luc2 tumors began to 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 as melanoma cells express α4 (Fig. 4C) and very weakly α9-integrin (data not shown): the lack of EMILIN1-α4/α9 integrin promotes proliferation (34), and this might be the reason for which B16F10 Luc2 cells gain a growth advantage in an EMILIN1-negative microenvironment.

To investigate lymphatic metastasis, WT and Emilin1^-/- mice bearing B16F10 Luc2 tumors were sacrificed at 16 to 17 days postinoculation; inguinal and axillary lymph nodes were excised and analyzed ex vivo for the presence of luciferase signal. Notably, only 25% WT and almost 75% Emilin1^-/- lymph node s were metastatic (Fig. 4D). Brown metastatic foci of B16F10 Luc2 melanoma cells were clearly visible under the dissecting microscope and were more numerous and larger in Emilin1^-/- than in WT lymph nodes (Fig. 4E). The extent of lymph node metastases was actually higher in Emilin1^-/- mice, as evidenced by a 2.8-fold increase of the average luciferase signal detected in the lymph nodes (Fig. 4F and G). To confirm that the increased lymph node metastasis was linked to lymphatic anomalies present in Emilin1^-/- 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 subcutaneously injected in WT (n = 9) and Emilin1^-/- (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^-/- mice (Fig. 5A). The hematoxylin and eosin staining of cryostat inguinal lymph node sections revealed the presence of more metastatic cells, positive for a pan-cytokeratin antibody used in corresponding serial sections, in Emilin1^-/- lymph nodes compared with 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 lymph nodes was 2-fold higher in Emilin1^-/- mice, compared with their WT
Figure 4. B16F10 Luc2 metastases in WT and Emilin1−/− lymph nodes. A, WT (n = 10) and Emilin1−/− (n = 10) mice were implanted subcutaneously in the right flank with 5 × 10⁵ B16F10 Luc2 melanoma cells. Tumor development was quantified by luciferase signal, measured by in vivo optical imaging at days 0, 2, 5, 7, and 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−/− lymph nodes. The presence of brown melanoma cell foci in the excised lymph nodes was evaluated at the dissection microscope. Altogether, 20 lymph nodes were analyzed per each mouse genotype. E, representative images from dissection microscope of excised WT and Emilin1−/− lymph nodes of B16F10 Luc2–bearing mice. Highlighted areas indicate the presence of brown foci. F, Mean ± SE luciferase signal detected by ex vivo optical imaging in the excised lymph nodes of B16F10 Luc2–bearing mice. G, representative images and color scale of the luciferase signal emitted by lymph nodes of B16F10 Luc2–bearing mice. *: P < 0.05; **: P < 0.01.
Overall, the data obtained from the syngenic tumor models showed that EMILIN1 deficiency promoted lymphatic metastasis.

**EMILIN1 deficiency favors intra- and extravasation of tumor cells through LAEC monolayers**

It has been previously shown that EMILIN1 deposition is required for the structural integrity of lymphatic vessels (28). LECs isolated from both Emilin1−/− and WT mice expressed specific lymphatic markers and did not differ for the expression of other ECM molecules such as fibronectin (Supplementary Fig. S1A and S1B). To verify if LECs were differently permissive for tumor cell passage, a comparative transmigration assay was conducted 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 the WT or \textit{Emilin1}\textsuperscript{-/-} LAEC monolayer was evaluated at different time intervals. The percentage of tumor cell types migrated through \textit{Emilin1}\textsuperscript{-/-} LAECs at 6 hours was higher both for the intra- as well as for the extravasation process (Fig. 6A and B). Because expression of \(\alpha_4\beta_1\) integrin on B16F1 cells correlated with tumor cells spreading to draining lymph nodes (37), we also checked whether under our experimental model, the migratory ability of cancer cells was limited to \(\alpha_4\) integrin expression: fluorescence-activated cell-sorting analysis revealed a weak positivity only for SKOV-3 cells (data not shown), and function blocking anti-\(\alpha_4\) 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 showed 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 lymph nodes. 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 with their WT littermates. We have recently shown that EMILIN1 directly inhibited cell proliferation by engaging integrin αβ1 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 with WT mice. This suggested that aberrant skin homeostasis generated by EMILIN1 deficiency (34) induced a protumorigenic 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, showing 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/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 lymph node 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 lymph nodes, compared with their WT littermates. Integrins control LEC adhesion, migration and survival, promote lymphangiogenesis, and favor metastatic spread (49). For instance, as the expression of αβ1 integrin on B16F1 increased, the likelihood of tumor cell spreading to regional lymph nodes 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 lymph nodes (27). The higher lymphatic vessel density within the tumors as well as in the draining lymph nodes in Emilin1−/− mice is likely the consequence of the lack of antiproliferative effect of EMILIN1/α4 or 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 αβ1 on transplanted tumor cells did not correlate with in vitro transmigration assays suggested that this integrin was not playing a primary role in the 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 lymph nodes. 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 morphofunctional 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: C. Danussi, P. Spessotto
Development of methodology: C. Danussi, A. Petrucco, T.M.E. Modica, E. Pivetta, L. Di Bel Belhar
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Colombatti
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Danussi, E. Pivetta, P. Spessotto
Writing, review, and/or revision of the manuscript: C. Danussi, A. Colombatti, P. Spessotto
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Study supervision: A. Colombatti, P. Spessotto

Acknowledgments
The authors thank Prof. G.M. Bressan for providing Emilin1−/− mice.

Grant Support
This work was supported by grants from AIRC (IG 10119; to P. Spessotto) and from AIRC, PRIN (2007IS758W_002), FIRB (RBIN07BMC7) and Progetto di ricerca finalizzata 5% Anno 2007-Intramural bench to bedside (to A. Colombatti). C. Danussi is a recipient of a Federazione Italiana Ricerca sul Cancro fellowship.

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Received February 17, 2012; revised June 14, 2012; accepted July 2, 2012; published OnlineFirst July 24, 2012.
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