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Advances in Brief

TEM8 Interacts with the Cleaved C5 Domain of Collagen α3(VI)

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Abstract

Tumor endothelial marker (TEM)8 was uncovered as a gene expressed predominantly in tumor endothelium, and its protein product was recently identified as the receptor for anthrax toxin. Here, we demonstrate that TEM8 protein is preferentially expressed in endothelial cells of neoplastic tissue. We used the extracellular domain of TEM8 to search for ligands and identified the α3 subunit of collagen VI as an interacting partner. The TEM8-interacting region on collagen α3(VI) was mapped to its COOH-terminal C5 domain. Remarkably, collagen α3(VI) is also preferentially expressed in tumor endothelium in a pattern concordant with that of TEM8. These results suggest that the TEM8/C5 interaction may play an important biological role in tumor angiogenesis.

Introduction

Targeting the endothelial cells that line tumor vessels is a promising anticancer strategy that has generated widespread excitement among biologists and clinicians (1). To further our understanding of angiogenesis and identify new potential targets, we recently compared global gene expression patterns in human endothelial cells of normal and malignant colorectal tissues (2). This study revealed a series of genes preferentially expressed in tumor endothelium. Among these tumor endothelial markers (TEMs), TEM8 was of particular interest because of its cell–surface localization, conservation in mice, and unique pattern of expression (3). TEM8 mRNA expression was readily detected in tumor endothelium by *in situ* hybridization but was absent or barely detectable in normal adult endothelium and the proliferative endothelium of the corpus luteum (2, 3). In this study, we set out to determine whether this unique pattern of expression is preserved at the protein level. We have also used the extracellular domain of TEM8 to search for interacting partners to gain insight into the role of TEM8 in angiogenesis.

Materials and Methods

Antibodies. Anti-TEM8 monoclonal antibody clones SB5 and SB12 were made by immunizing mice with recombinant protein encompassing the extracellular region of TEM8 (Ivratech, Inc., Kyiv, Ukraine). The fragment encoding amino acids 30–302 was cloned into pIVEX2.4b, and protein was made by *in vitro* transcription/translation using the RTS-500 system (Roche). Hybridoma supernatants were screened by Western blotting. Clone SB20 (Imgenex) is a monoclonal antibody raised against the cytoplasmic peptide sequence CINFTRVKNNQPAKYPL.

Immunohistochemistry. Paraffin sections were deparaffinized, incubated with proteinase K (Invitrogen), heated at 95°C for 20 min in citrate buffer (pH 6.0), and treated with peroxidase blocking reagent (DAKO). Sections were incubated with a polyclonal antibody against vWF (DAKO) or a monoclonal antibody against TEM8 (clone SB12) followed by a biotin-conjugated secondary antibody (Pierce). A third layer consisting of horseradish peroxidase-conjugated antibiotin (DAKO) was then followed by diaminobenzidine (Sigma) staining. Sections were counterstained with hematoxylin.

Immunofluorescence. Dual-color immunofluorescence was performed on fresh-frozen sections fixed in Leukoperm (Serotec) and stained with anti-TEM8 clone SB12 and polyclonal anti-vWF (DAKO). vWF was detected with a FITC-conjugated antirabbit antibody (Jackson ImmunoResearch Laboratories), and TEM8 was detected using a biotin antimouse antibody (Jackson) followed by rhodamine–streptavidin (Vector). Sections were visualized by confocal microscopy.

***In Situ* Hybridization.** The *in situ* hybridization protocol described previously (3) was adapted for use on paraffin sections. A detailed protocol can be obtained from the authors on request.

Western Blotting. Samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Western blots were probed with an anti-TEM8 primary antibody, followed by a horseradish peroxidase-conjugated antimouse secondary antibody (Jackson), and visualized using the enhanced chemiluminescence plus system (Amersham) according to the supplier's instructions.

Immunoprecipitation. The TEM8 extracellular region (amino acids 28–320) and C5 deletion constructs (C5A, C5B, and C5C) were cloned into the myc-tagged AP-Tag5 vector (Genhunter) and sequence verified. Secreted fusion proteins from cotransfected 293 cells were incubated overnight with an anti-TEM8 antibody (clone SB5) or a nonspecific IgG control antibody. Precipitated proteins were eluted from protein G-agarose beads (Roche) and detected by Western blotting using an anti-myc monoclonal antibody (Clontech).

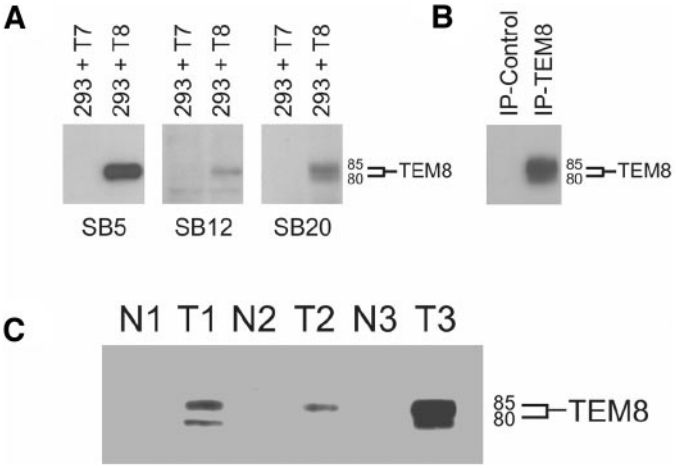

Fig. 1. Expression of tumor endothelial marker (TEM)8 by immunoblotting. In *A*, immunoblotting with TEM8 antibodies (clones *SB5*, *SB12*, and *SB20*) revealed an M_r 80–85,000 doublet in 293 cells transfected with full-length TEM8 (*293 + T8*). The doublet was not detected in TEM7-transfected controls (*293 + T7*). In *B*, TEM8 antibody (clone *SB5*) immunoprecipitated TEM8 from 293 cells transfected with TEM8. An equal amount of nonspecific mouse IgG was used as control. Immunoblotting was performed with clone SB20. In *C*, the same immunoprecipitation scheme was used on extracts prepared from colon cancer tissues (*T1*, *T2*, and *T3*) and patient-matched normal colonic mucosa (*N1*, *N2*, and *N3*).

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Note: A. Nanda and E. B. Carson-Walter contributed equally to this work.

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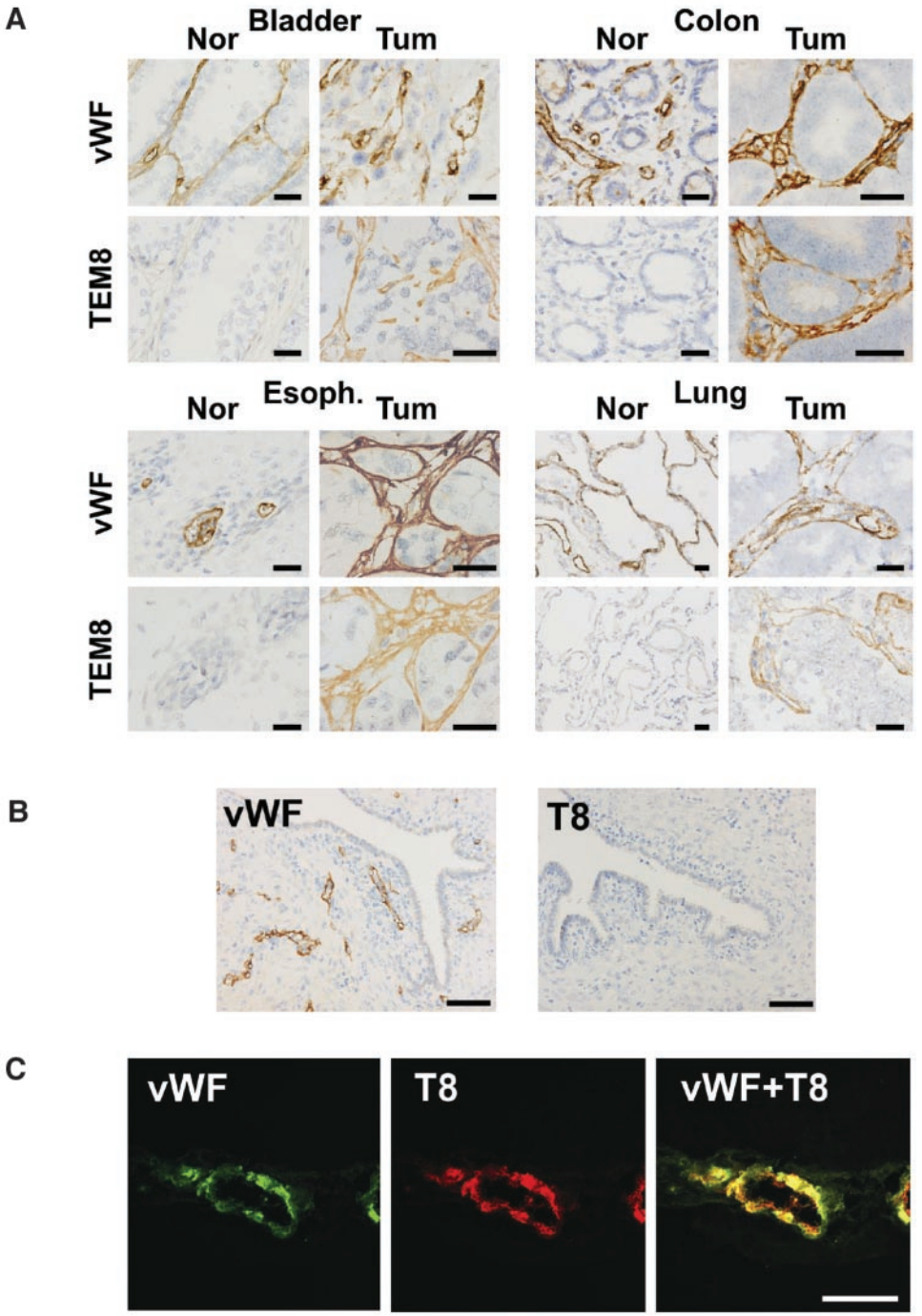

Fig. 2. Expression of tumor endothelial marker (TEM)8 in tissues. In *A*, immunohistochemical staining with TEM8 antibody (clone SB12) revealed TEM8 expression (*brown stain*) predominantly in the endothelial cells of various human cancer types, including colon, esophageal, lung, and urinary bladder. Note that TEM8 expression is weak or undetectable in normal tissues, but in tumors, TEM8 exhibits the same pattern of vessel staining as vWF, a pan-endothelial marker. Scale bar, 50 μm. In *B*, TEM8 expression was undetectable during the normal angiogenesis of human corpus luteum, whereas vWF was readily detected. Scale bar, 100 μm. Sections in *A* and *B* were counterstained with hematoxylin (*blue stain*). In *C*, immunofluorescence staining of colon cancer tissues demonstrated colocalization of TEM8 with vWF. Scale bar, 50 μm.

Two-Hybrid Analysis. The two-hybrid screen was performed using a fetal brain cDNA library and the Matchmaker II system (Clontech), according to the manufacturer's suggested procedure.

Results and Discussion

To explore the expression of TEM8 protein in tumor angiogenesis, we generated a panel of mouse monoclonal antibodies against its extracellular (clones SB5 and SB12) and cytoplasmic (clone SB20) domains. Each of these antibodies reacted with TEM8 when exogenously expressed in mammalian 293 cells (Fig. 1A). In these analyses, TEM8 appeared as a doublet of M_r 80,000 and 85,000, which is larger than expected. This difference is likely attributable to glycosylation, because treatment of cell extracts with a glycosidase cocktail reduced the size to M_r 70,000.[6]

To detect endogenous TEM8 protein in human tumors, wherein only a small fraction of the population (the endothelial cells) would be expected to express it, we used immunoprecipitation followed by Western blot analysis (IP-Western; Fig. 1*B*). Using IP-Western analysis, TEM8 protein levels were found to be markedly increased in malignant colorectal tissues compared with normal, adjacent colorectal tissues from the same patients (Fig. 1*C*). To determine the cellular source of this up-regulated protein and expand our analyses of tumors,

[6] A. Nanda and B. St. Croix, unpublished data.

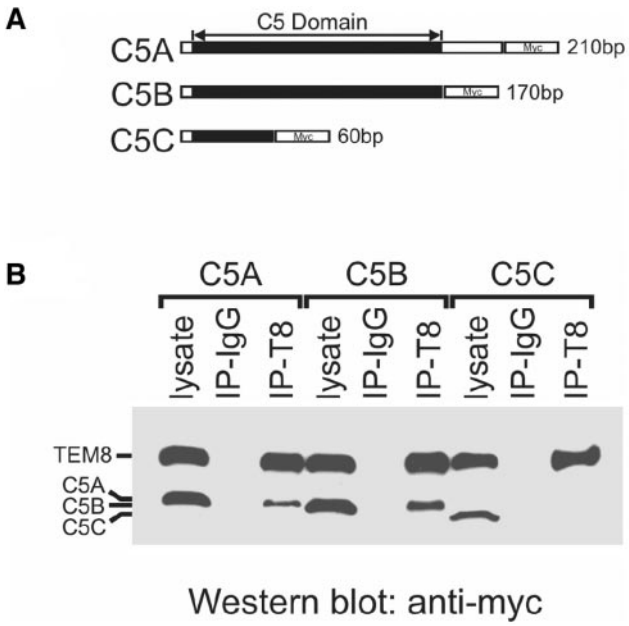

Fig. 3. The C5 domain of collagen α3(VI) interacts with tumor endothelial marker (TEM)8. In *A*, clone C5A contains the 210-bp cDNA derived from the yeast two-hybrid screen, encompassing the entire C5 domain and extending to the COOH-terminal end of collagen α3(VI); C5B contains just the C5 domain; C5C contains only the first 60 bp of the C5 domain. All constructs were fused to a COOH-terminal myc-tag and contained signal peptides at the NH_2 terminus. In *B*, supernatants were harvested from 293 cells cotransfected with myc-tagged secreted extracellular TEM8 and either C5A, C5B, or C5C and then immunoprecipitated with either nonspecific IgG (*IP-IgG*) or anti-TEM8 antibody clone SB5 (*IP-T8*). Supernatants or immunoprecipitates were immunoblotted with an anti-myc antibody. Note that the protein encoded by the C5C construct containing the partial C5 domain is undetectable in TEM8 immunoprecipitates.

an immunohistochemical survey of human tumors was performed. Staining of TEM8 with the monoclonal antibodies described above revealed a vessel-like pattern in all cases of human colorectal cancers analyzed but not in matched normal colonic mucosa (Fig. 2*A*). Moreover, evaluation of other cancer types, including bladder, esophageal, and lung cancer revealed similarly strong staining of tumor endothelium. Importantly, TEM8 protein was either absent or barely detectable in the endothelium of every normal tissue analyzed and proliferative endothelium of the corpus luteum (Fig. 2, *A* and *B*). The lack of detectable TEM8 protein in the corpus luteum is consistent with TEM8 mRNA expression patterns and appears to be unique for this particular TEM, because other TEMs (TEMs 1, 4, 5, 7, and 9) were readily detected in the corpus luteum (2). To ensure that the antibodies were predominantly staining endothelial cells, colocalization studies were performed. As shown in Fig. 2*C*, staining of TEM8 colocalized with that of von Willebrand Factor, a classic endothelial marker. Taken together, these studies clearly demonstrate preferential expression of TEM8 protein in tumor endothelium.

Identification of natural ligands for TEM8 could provide important clues about its function. We therefore searched for TEM8-binding proteins using the complete extracellular domain of TEM8 as bait in a yeast two-hybrid screen. A screen of 6 million clones representing 3.5 million distinct cDNAs from human fetal brain revealed two clones that were of particular interest. These clones encoded overlapping inserts from the COOH terminus of collagen α3(VI). To confirm the interaction between TEM8 and collagen α3(VI), we tested the ability of TEM8 and collagen α3(VI) to coprecipitate when expressed in mammalian cells. Soluble extracellular TEM8 containing its I-domain (a.k.a. vWF-A domain) was expressed in 293 cells, along with myc-tagged proteins containing either the longest collagen α3(VI) insert from the two-hybrid experiment, a subfragment of this insert containing the entire C5 domain, or a truncated C5 domain. TEM8 antibodies readily coimmunoprecipitated those collagen α3(VI) proteins containing an intact C5 domain, whereas control IgG did not (Fig. 3). These results demonstrate that TEM8 binds to the C5 domain of collagen α3(VI).

To examine the *in vivo* relationship between collagen α3(VI) and TEM8, we performed *in situ* hybridization analyses to localize expression of these genes in normal and malignant colonic tissues.

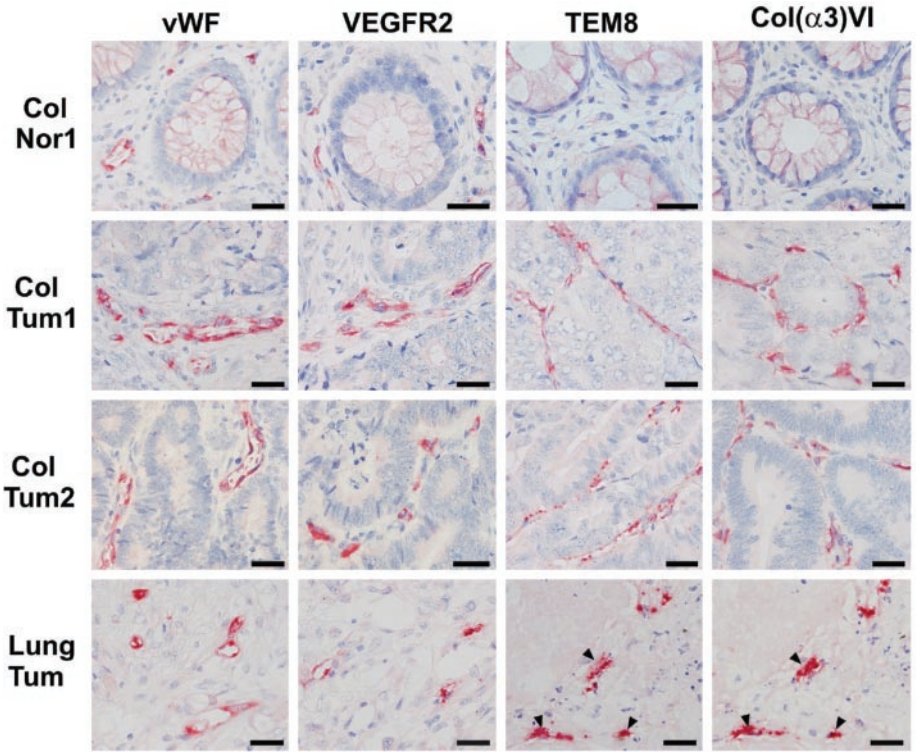

Fig. 4. Coordinate expression of collagen α3(VI) and tumor endothelial marker (TEM)8 transcripts in tumor endothelium. The mRNA for both TEM8 and collagen α3(VI) (*red stain*) was readily detected in the ECs of every colon cancer tumor analyzed but was absent or barely detectable in the ECs of the corresponding normal colonic mucosa. Three of these cases contained adjacent normal and tumor tissue on the same section (*e.g., Nor1* and *Tum1*). TEM8 and collagen α3(VI) staining could also be colocalized on serial sections of lung (data shown), colon, and esophageal cancer where the same vessel-like structures were observed (*arrowheads*). The endothelial markers vWF and VEGFR2 exhibited a similar pattern of staining to that of TEM8 and collagen α3(VI) in tumor tissues and confirmed the mRNA integrity of normal colonic mucosa. The faint, red extracellular staining around the crypts represents nonspecific binding of the *in situ* hybridization reagents to mucous. Sections were counterstained with hematoxylin (*blue*). Scale bar, 50 μm.

Staining for collagen α3(VI) was strong in the endothelium of each colorectal tumor analyzed but was weak or undetectable in the corresponding matched normal colonic mucosa, a pattern of expression strikingly similar to that of TEM8 (Fig. 4). Analysis of lung and esophageal cancer also revealed colocalization of TEM8 and collagen α3(VI) in these tissues (Fig. 4 and data not shown). Importantly, when adjacent serial sections from the same tissue were evaluated, similar vessel-like patterns of staining were observed (see arrowheads in Fig. 4). These results suggest that expression of TEM8 and collagen α3(VI) in tumor endothelium is coordinately regulated.

The ability of extracellular matrix molecules or cleaved extracellular matrix fragments to regulate angiogenesis is well established (4–7). With this in mind, several specific observations suggest that the interaction between TEM8 and collagen α3(VI) is important for angiogenesis: (*a*) the closest known homologue of TEM8, capillary morphogenic protein 2, has been shown to interact with the extracellular matrix via its I-domain (8); (*b*) collagen α3(VI) has been reported to be up-regulated in healing wounds, which are known to be rich in neovasculature (9); and (*c*) collagen α3(VI) was identified in our previous unbiased screen as one of a limited number of transcripts preferentially expressed in tumor endothelium among 32,500 total transcripts analyzed (2). Indeed, in the same experiments wherein TEM8 was identified as the 19th most preferentially expressed transcript, collagen α3(VI) was identified as the 12th. Although the cells producing collagen α3(VI) in tumors have not been well characterized previously, the serial analysis of gene expression data along with the *in situ* hybridization data strongly suggest that endothelial cells are a primary source (Fig. 4).

The studies described here implicate collagen α3(VI) as a ligand for TEM8. The interaction of these proteins and their coordinate expression in tumor endothelial cells suggests a carefully orchestrated role in angiogenesis. In this regard, recent studies of the dynamics of the C5 domain of collagen α3(VI) are of interest. The C5 domain of collagen α3(VI) is initially incorporated into the newly forming type VI collagen fibrils but immediately after secretion is cut off and not present in mature collagen VI-containing matrix (10). Antibodies specific to the C5 domain have shown reactivity with both the cytoplasm and immediate pericellular region of cells actively producing collagen α3(VI) (10, 11).

The C5 domain provides a new reagent for the selective targeting of tumor vasculature through TEM8. The validity of TEM8 as a target is supported by several recent studies using anthrax toxin as an antitumor agent. The expression pattern of TEM8 may help to explain the tumor regressions observed when anthrax toxin is injected into tumor-bearing mice at nontoxic doses (12–14). Before the identification of TEM8 as the anthrax toxin receptor, inhibition of angiogenesis was postulated to mediate the antineoplastic effects of anthrax toxin, because the treated tumors appeared "white" and were found to be deficient in CD31-positive blood vessels (12). Given the TEM8 protein expression patterns in tumor endothelium described here, it seems likely that TEM8 is responsible for this effect.

References

1. Kerbel, R., and Folkman, J. Clinical translation of angiogenesis inhibitors. Nat. Rev. Cancer, *2:* 727–739, 2002.
2. St. Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B., and Kinzler, K. W. Genes expressed in human tumor endothelium. Science (Wash. DC), *289:* 1197–1202, 2000.
3. Carson-Walter, E. B., Watkins, D. N., Nanda, A., Vogelstein, B., Kinzler, K. W., and St. Croix, B. Cell surface tumor endothelial markers are conserved in mice and humans. Cancer Res., *61:* 6649–6655, 2001.
4. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, *88:* 277–285, 1997.
5. Grant, D. S., and Kleinman, H. K. Regulation of capillary formation by laminin and other components of the extracellular matrix. EXS, *79:* 317–333, 1997.
6. Marneros, A. G., and Olsen, B. R. The role of collagen-derived proteolytic fragments in angiogenesis. Matrix Biol., *20:* 337–345, 2001.
7. Kalluri, R. Angiogenesis: basement membranes: structure, assembly and role in tumour angiogenesis. Nat. Rev. Cancer, *3:* 422–433, 2003.
8. Bell, S. E., Mavila, A., Salazar, R., Bayless, K. J., Kanagala, S., Maxwell, S. A., and Davis, G. E. Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signaling. J. Cell Sci., *114:* 2755–2773, 2001.
9. Oono, T., Specks, U., Eckes, B., Majewski, S., Hunzelmann, N., Timpl, R., and Krieg, T. Expression of type VI collagen mRNA during wound healing. J. Investig. Dermatol., *100:* 329–334, 1993.
10. Aigner, T., Hambach, L., Soder, S., Schlotzer-Schrehardt, U., and Poschl, E. The C5 domain of Col6A3 is cleaved off from the Col6 fibrils immediately after secretion. Biochem. Biophys. Res. Commun., *290:* 743–748, 2002.
11. Mayer, U., Poschl, E., Nischt, R., Specks, U., Pan, T. C., Chu, M. L., and Timpl, R. Recombinant expression and properties of the Kunitz-type protease-inhibitor module from human type VI collagen alpha 3(VI) chain. Eur. J. Biochem., *225:* 573–580, 1994.
12. Duesbery, N. S., Resau, J., Webb, C. P., Koochekpour, S., Koo, H. M., Leppla, S. H., and Vande Woude, G. F. Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. Proc. Natl. Acad. Sci. USA, *98:* 4089–4094, 2001.
13. Liu, S., Aaronson, H., Mitola, D. J., Leppla, S. H., and Bugge, T. H. Potent antitumor activity of a urokinase-activated engineered anthrax toxin. Proc. Natl. Acad. Sci. USA, *100:* 657–662, 2003.
14. Koo, H. M., VanBrocklin, M., McWilliams, M. J., Leppla, S. H., Duesbery, N. S., and Woude, G. F. Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen-activated protein kinase kinase. Proc. Natl. Acad. Sci. USA, *99:* 3052–3057, 2002.

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