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MT1-MMP–dependent neovessel formation within the confines of the three-dimensional extracellular matrix

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During angiogenesis, endothelial cells initiate a tissue-invasive program within an interstitial matrix comprised largely of type I collagen. Extracellular matrix–degradative enzymes, including the matrix metalloproteinases (MMPs) MMP-2 and MMP-9, are thought to play key roles in angiogenesis by binding to docking sites on the cell surface after activation by plasmin- and/or membrane-type (MT) 1-MMP–dependent processes. To identify proteinases critical to neovessel formation, an ex vivo model of angiogenesis has been established wherein tissue explants from gene-targeted mice are embedded within a three-dimensional, type I collagen matrix. Unexpectedly, neither MMP-2, MMP-9, their cognate cell-surface receptors (i.e., β_3 integrin and CD44), nor plasminogen are essential for collagenolytic activity, endothelial cell invasion, or neovessel formation. Instead, the membrane-anchored MMP, MT1-MMP, confers endothelial cells with the ability to express invasive and tubulogenic activity in a collagen-rich milieu, in vitro or in vivo, where it plays an indispensable role in driving neovessel formation.

Introduction

Angiogenesis is a specialized form of branching morphogenesis wherein endothelial cells detach themselves from the existing vasculature, invade surrounding tissues, and reorganize into patent tubules (Carmeliet and Jain, 2000; Pepper, 2001; Davis et al., 2002; Lubarsky and Krasnow, 2003). In the postnatal state, new blood vessels routinely traverse a dense connective tissue matrix largely comprised of type I collagen, the major extracellular protein found in mammals (Hay, 1991). Although the triple-helical molecule is resistant to almost all forms of proteolytic attack, endothelial cells engaged in the angiogenic process are believed to negotiate this structural barrier by mobilizing proteolytic cascades that converge on the matrix metalloproteinases (MMPs) progelatinase A (MMP-2) and progelatinase B (MMP-9; Pepper, 2001; Davis et al., 2002; Heissig et al., 2003).

Like all members of the MMP family, MMP-2 and MMP-9 are synthesized as latent enzymes (Egeblad and Werb, 2002, Seiki, 2002). However, coincident with the expression of tissue-invasive or morphogenic processes, secreted MMP-2 and MMP-9 are activated. With specific regard to angiogenesis, the serine proteinase plasmin has been proposed to act as an activator of both MMP-2 and MMP-9, whereas multiple members of the membrane-anchored family of MMPs (i.e., membrane-type [MT]1-, 2-, 3-, 4-, 5-, and 6-MMP) can process the MMP-2 zymogen to its active form (Pepper, 2001; Davis et al., 2002; Seiki, 2002). Surface localization of the activated metalloproteinases may be critical for allowing cells to migrate within dense connective tissues, as binding interactions between MMP-2 and the $\alpha_v\beta_3$ integrin, and between MMP-9 and the cell surface proteoglycan CD44, have been associated with invasive/angiogenic phenotypes (Brooks et al., 1996, 1998; Yu and Stamenkovic, 1999; Silletti et al., 2001).

Despite the wealth of evidence supporting a role for MMP-2, MMP-9, plasminogen, or MT1-MMP activity in angiogenic events in vivo (Brooks et al., 1998; Itoh et al., 1998; Bergers et al., 2000; Zhou et al., 2000; Heissig et al., 2003), the means by which these proteinases exert their effects remain largely undefined. Long thought to confine their activity to degrading ECM components, increasing evidence suggests that MMPs may modulate endothelial cell function indirectly by activating latent cytokines, cleaving membrane-anchored targets, releasing matrix-bound growth factors, or generating

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Abbreviations used in this paper: 3-D, three-dimensional; CAM, chorioallantoic membrane; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase; MT, membrane-type; TIMP, tissue inhibitor of metalloproteinase.

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bioactive neopeptides (Egeblad and Werb, 2002; Heissig et al., 2003). Consequently, it remains unclear whether MMP-2, MMP-9, their cognate receptors, or the upstream proteinases responsible for their activation, directly support the crucial collagen-remodeling events necessary to drive the morphogenic programs associated with angiogenesis.

To identify the proteolytic systems required for neovessel formation within a physiologically relevant interstitial matrix, three-dimensional (3-D) gels of cross-linked type I collagen were seeded with tissue explants or endothelial cells isolated from mice harboring inactivating mutations in either the plasminogen, *MMP-2*, *MMP-9*, *β_3 integrin*, *CD44*, or *MT1-MMP* genes. Unexpectedly, we demonstrate that neovessel formation proceeds in unperturbed fashion in the absence of either plasminogen, MMP-2, MMP-9, the β_3 integrin, or CD44. Instead, the membrane-anchored collagenase MT1-MMP plays a required role in conferring endothelial cells with the ability to both proteolytically remodel type I collagen and express a collagen-invasive phenotype critical to the tubulogenic process.

Results

3-D neovessel formation engages a collagenolytic phenotype

To determine whether neovessel formation is linked to a collagen remodeling process, mouse tissue explants were embedded in a 3-D type I collagen gel, stimulated with a growth factor cocktail, and monitored for vessel outgrowth as well as collagenolysis. After the egress of fibroblast-like cells during the first two days of ex vivo culture (Fig. 1 A, a), capillary sprouts emerge and coalesce to form an anastomosing network of PECAM-1–positive vessels surrounded by pericyte-like cells at day 7 (Fig. 1 A, b–f). Coincident with the expression of the tubulogenic program, type I collagen degradation products appear in the pericellular environment, as detected with antibodies specific for either (1) the COOH-terminal neopeptide of the three-quarter fragment of cleaved type I collagen or (2) denatured collagen products that have lost their triple-helical integrity (Fig. 1 B). Though the formation of capillary-like structures is associated with type I collagenolytic activity, the degradative phenotype is coordinated with the neodeposition of type IV collagen (Fig. 1 B) and laminin (not depicted) as maturing neovessels initiate basement membrane synthesis (Nicosia and Madri, 1987).

Although multiple proteinases have been implicated in the collagenolytic process (Davis et al., 2002; Heissig et al., 2003; Shi et al., 2003), the formation of patent vessels is unaffected by the broad-spectrum cysteine proteinase inhibitor E-64d or the aspartyl proteinase inhibitor pepstatin A (not depicted). In contrast, cellular outgrowth, as well as tubulogenesis, is almost completely inhibited by the peptidomimetic MMP inhibitor BB-94 (Fig. 1 C). As plasmin is thought to play a key role in type I collagen turnover by virtue of its ability to participate in the processing of latent MMPs to catalytically active forms (Pepper, 2001; Davis et al., 2002), neovessel outgrowth was monitored in tissue explants isolated from plasminogen-null mice that were cultured in plasminogen-null serum. Interestingly, in the absence of plasminogen, an angiogenic response indistinguishable from that displayed by wild-type controls is observed, as determined on morphologic grounds (Fig. 1 C) or by sprout density and length, respectively (6.0 ± 2.6 sprouts

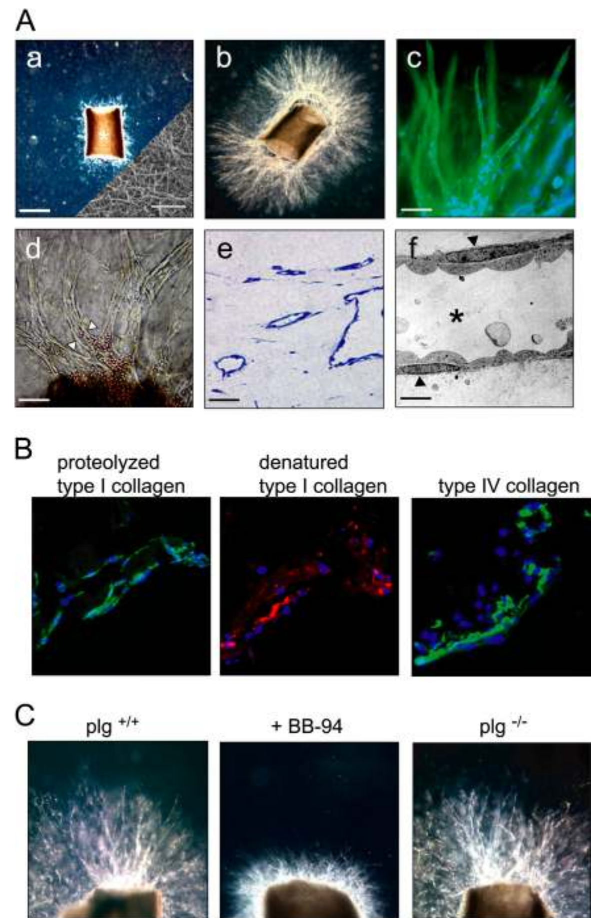

Figure 1. **ECM remodeling during ex vivo neovessel formation.** (A) Neovessel formation from mouse aortic ring explants. After culturing for 2 d in a 3-D type I collagen gel (2.2 mg/ml) in the presence of VEGF and HGF (50 ng/ml each) and 20% FBS, vessel wall–associated cells initiate migration from matrix-embedded aortic rings (a; bar, 500 µm). The asterisk indicates the position of the aortic explant and the inset displays a scanning electron micrograph of the type I collagen matrix that highlights the dense packing of the surrounding fibrillar gel (bar, 5 µm). Patent tubular structures form by day 5 (b) with PECAM-1–positive endothelial cells (c, green) comprising the wall of the formed neovessels (c). Cell nuclei are stained blue with DAPI (c; bar, 100 µm). By phase-contrast microscopy, the newly formed tubules (margins of a vessel are bordered by arrowheads) allow the passive outflow of red blood cells that remain in the aortic rings after isolation (d; bar, 50 µm). Cross sections of sprouting neovessels display tubular structures of varying diameters after a 7-d culture period (e; bar, 100 µm), whereas transmission electron micrographs show that endothelial cells line a patent lumen (f, asterisk) that is surrounded by pericyte-like accessory cells (arrowheads; bar, 5 µm). (B) Type I collagen degradation products surrounding egressing neovessels, as detected with antibodies directed against collagen neoepitopes generated after the proteolysis of collagen into three-quarter and one-quarter fragments, are shown on the left (green). Type I collagen denaturation products after proteolysis are shown in the middle (red). Coincident with the generation of type I collagen cleavage production, the endothelial cells deposit a matrix of type IV collagen (right; green). Cell nuclei are stained blue with DAPI. (C) Neovessel formation is blocked in the presence of 5 µm BB-94 (neovessel length decreases from 1,079 ± 20 µm to 271 ± 9 µm, *n* = 3), but proceeds in an unaffected manner from aortic rings isolated from *plg*$^{-/-}$ mice and cultured in *plg*$^{-/-}$ serum after a 7-d incubation period.

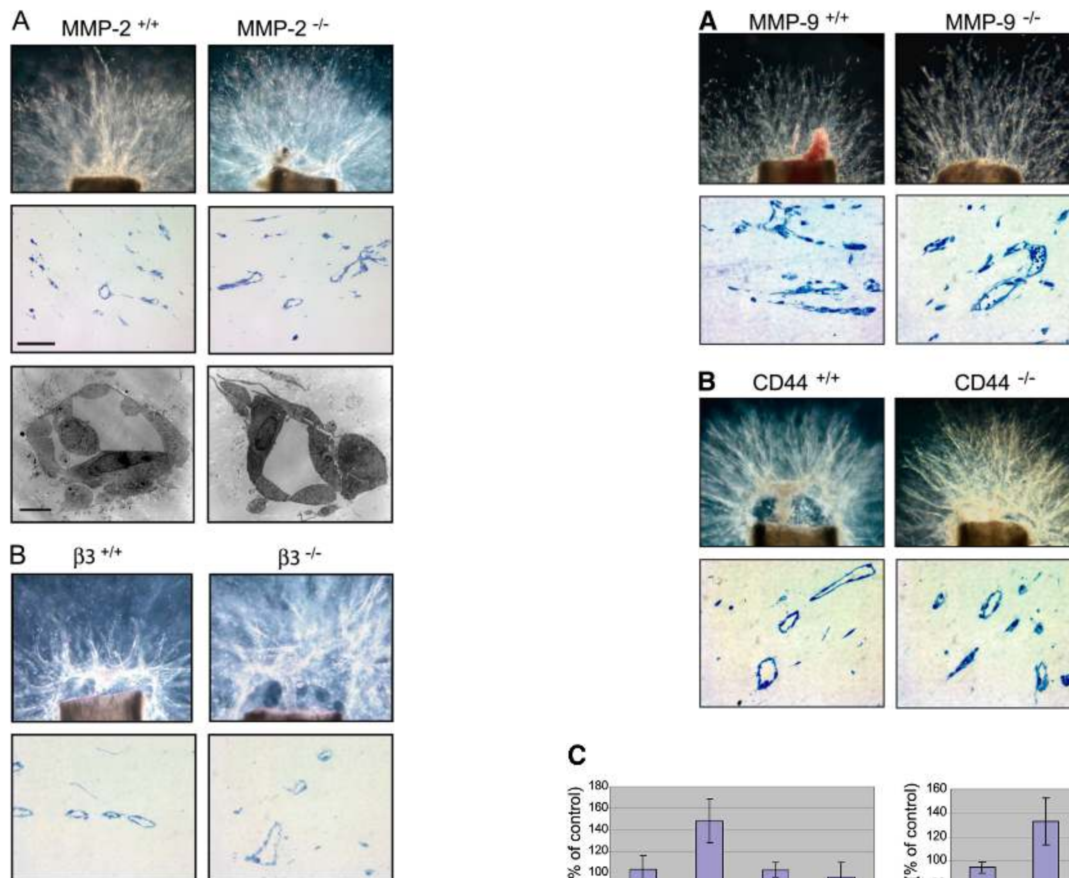

Figure 2. **The functional role of the MMP-2–β3 integrin axis in capillary sprout formation.** (A) Neovessel outgrowth from control littermate (left column) and MMP-2–null (right column) mouse aortic rings suspended in type I collagen gels and stimulated with VEGF–HGF in 5% wild-type or *MMP-2*$^{-/-}$ serum are shown, respectively, after a 7-d incubation period (top). Cross sections of mature neovessels highlight the nearly identical pattern of tubule formation assessed by either light microscopy (middle; bar, 100 μm) or transmission electron micrograph (TEM) analysis (bottom; bar, 5 μm). (B) Capillary sprouting from wild-type and β3 integrin–null mouse aortic rings as assessed by phase-contrast microscopy at day 7 is shown (top). Cross sections of the capillary sprouts from wild-type and β3-null aortic rings display similar morphology (bottom).

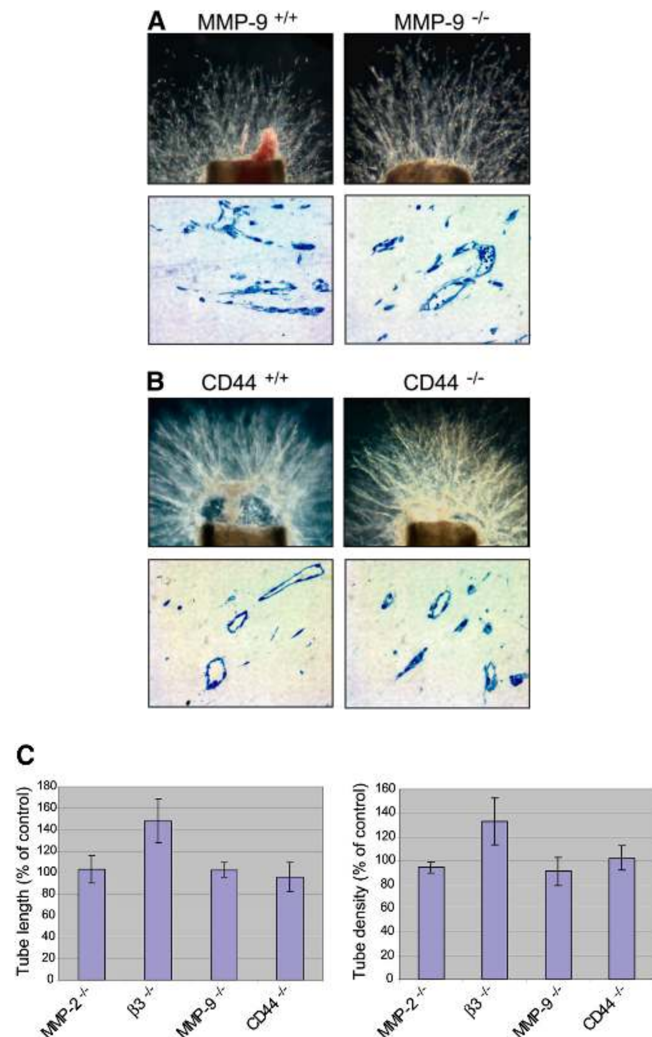

Figure 3. **Neovessel outgrowth from MMP-9– and CD44-null explants.** (A) Capillary outgrowth from control littermate (left) and MMP-9–null mouse aortic rings (right) suspended in type I collagen gels and stimulated with VEGF–HGF in 5% autologous serum do not exhibit obvious differences in capillary number, length, or morphology, as assessed by phase-contrast microscopy (top) or in hematoxylin and eosin (H&E)–stained cross sections (bottom) at day 7. (B) Capillary sprouting from wild-type and CD44-null aortic explants prepared as in A, as assessed by phase-contrast microscopy (top) or in H&E–stained cross sections (bottom) after 7 d. (C) Mean neovessel length and density from aortic explants isolated from *MMP-2*$^{-/-}$, *β3 integrin*$^{-/-}$, *MMP-9*$^{-/-}$, and *CD44*$^{-/-}$ mice and incubated in type I collagen gels for 7 d with VEGF–HGF in 5% autologous serum are expressed as percent control of the respective wild-type littermate (mean ± 1 SD, $n = 4$).

per field with a mean sprout length of 1,272 ± 83 μm in wild-type explants vs. 6.7 ± 2.1 sprouts per field and a sprout length of 1,359 ± 22 μm for plasminogen-null explants; $n = 3$).

Capillary morphogenesis proceeds independently of the MMP-2–$\alpha_v\beta_3$ or MMP-9–CD44 axes

The association of catalytically active MMP-2 with the $\alpha_v\beta_3$ integrin has been reported to regulate the angiogenic response (Brooks et al., 1996, 1998; Silletti et al., 2001), but the role that this complex plays in directing invasive/tubulogenic programs has not been defined. To determine the relative roles of MMP-2 and $\alpha_v\beta_3$ in neovessel formation, we monitored vessel outgrowth and morphology in explants isolated from MMP-2–null or $\alpha_v\beta_3$-null mice. Although wild-type explants expressed both MMP-2 and β_3, as assessed by RT-PCR (not depicted), MMP-2–null and wild-type littermate explants mounted an indistinguishable tubulogenic response, without significant differences in mean capillary length or density (Figs. 2 A and 3 C). Furthermore, morphogenesis proceeded in normal fashion, with a ring of endothelial cells circumscribing a patent lumen (Fig. 2 A). Consistent with an MMP-2–independent tubulogenic program, neither vessel outgrowth nor vessel morphology was inhibited in the absence of the β_3 integrin (Figs. 2 B and 3 C).

In a fashion similar to that described for MMP-2, MMP-9 has also been linked to tissue-invasive events and angiogenesis, in part, by associating with its cell surface–binding partner, CD44 (Yu and Stamenkovic, 1999; Davis et al., 2002). However, capillary outgrowth and vessel morphogenesis were not affected by the deletion of either MMP-9 or CD44 (Fig. 3).

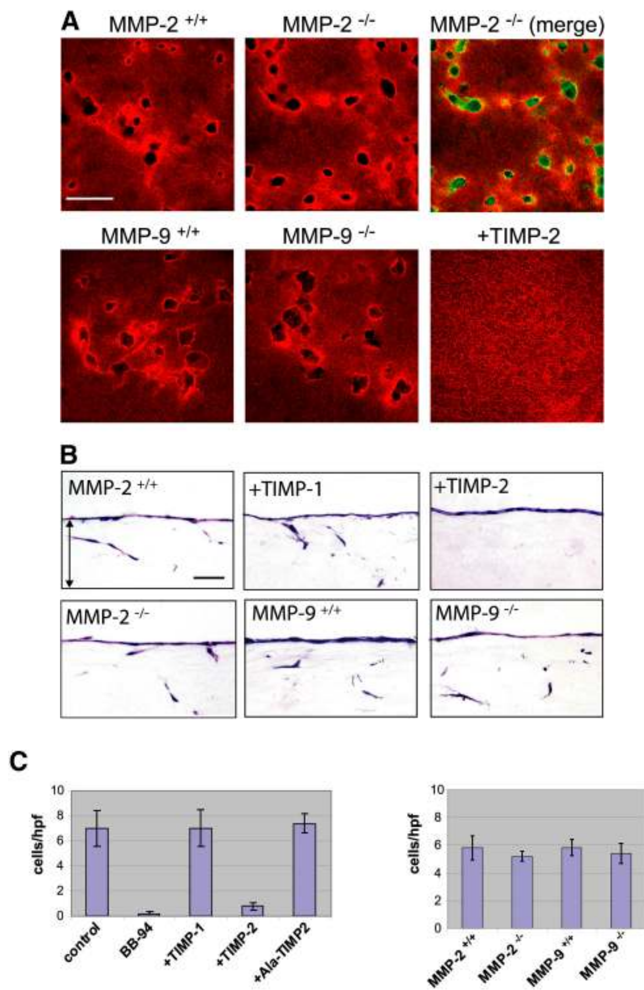

Figure 4. **A nonessential role of MMP-2 and MMP-9 in regulating the collagenolytic and collagen-invasive activities of microvascular endothelial cells.** (A) Littermate control, *MMP-2*$^{-/-}$, or *MMP-9*$^{-/-}$ endothelial cells focally degrade a subjacent film (~100 μg) of fluorescently labeled type I collagen after a 7-d culture period in the presence of VEGF–HGF and 5% autologous wild-type or knockout sera. Areas of collagenolytic activity expressed by *MMP-2*$^{+/+}$, *MMP-2*$^{-/-}$, *MMP-9*$^{+/+}$, and *MMP-9*$^{-/-}$ correspond precisely with sites subjacent to overlying endothelial cells as shown in merged images of MMP-2–null, phalloidin-stained cells (top, right). Collagenolytic activity by control littermate and null cells is completely blocked by 3 μg/ml TIMP-2 (bottom, right). Bar, 50 μm. (B) Invasion of 3-D collagen gels (2.2 mg/ml) by microvascular endothelial cells isolated from wild-type (*MMP-2*$^{+/+}$/C57BL6 and *MMP-9*$^{+/+}$/129SvEv), MMP-2–null, or MMP-9–null mice are shown after a 5-d incubation period with VEGF–HGF in the presence of 5% autologous serum. Endothelial cell invasion was insensitive to 3 μg/ml TIMP-1, but inhibited completely by 3 μg/ml TIMP-2. Bar, 50 μm. (C) The invasive activity of isolated wild-type, *MMP-2*$^{-/-}$, or *MMP-9*$^{-/-}$ endothelial cells was quantified after a 5-d incubation period in the absence or presence of BB-94, TIMP-1, TIMP-2, or Ala-TIMP-2 mutant. Results are shown as the mean number of invading cells per high powered field (hpf) in two experiments.

The tubulogenic response mounted by tissue explants involves multiple cell types (e.g., Nicosia and Madri, 1987) that might conceivably mask or compensate for an endothelial cell–specific defect in collagenolytic or invasive activity. Hence, microvascular endothelial cells were isolated from MMP-2 and MMP-9–null mice, and their ability to degrade a subjacent bed of type I collagen fibrils or invade 3-D type I collagen gels was assessed. As shown in Fig. 4 A, wild-type endothelial cells stimulated with a VEGF–hepatocyte growth factor (HGF) mixture proteolyzed a subjacent film of rhodamine-labeled collagen in an area confined to the boundaries of the overlying cell by a process sensitive to either tissue inhibitor of MP (TIMP) 2 or BB-94 (not depicted). Of note, subjacent collagenolytic activity was not affected by targeting either *MMP-2* or *MMP-9* (Fig. 4 A). Furthermore, consistent with the collagen-degradative phenotypes displayed by either population of null-endothelial cells, *MMP-2* or *MMP-9* knockout cells retained the wild-type capability to invade 3-D collagen gels (Fig. 4, B and C). Invasion, like collagen degradation, was also blocked completely by both BB-94 and wild-type TIMP-2, but was not affected by TIMP-1, a potent inhibitor of MMP-2 and MMP-9 (Hotary et al., 2003; Fig. 4 C). Although TIMP-2 can affect cell function independently of its ability to block MMP activity (Seo et al., 2003), endothelial cell invasion was not inhibited by an Ala–TIMP-2 mutant devoid of anti-MMP activity (Fig. 4 C).

MT1-MMP regulates capillary sprouting, collagenolysis, and endothelial cell invasion in a type I collagen-specific fashion

Although neovessel formation, endothelial cell invasion, and collagenolytic activity proceeded in an unperturbed manner in the absence of either MMP-2 or MMP-9, recent studies indicate that MT1-MMP can display collagenolytic activity directly (Ohuchi et al., 1997; Atkinson et al., 2001). To determine whether MT1-MMP controls endothelial cell–collagen interactions, MT1-MMP–null aortic explants or microvascular endothelial cells were recovered from the knockout mice in order to assess their ability to (1) mount a tubulogenic program, (2) mediate subjacent collagenolysis, and (3) express a collagen-invasive phenotype.

In contrast with wild-type explants, tissues isolated from *MT1-MMP*$^{-/-}$ mice are completely unable to generate neovessels during a 7-d culture period (Fig. 5 A). Co-cultures of MT1-MMP–null explants with wild-type aorta rings demonstrate that soluble inhibitors of capillary formation are not released from the knockout tissues and that wild-type tissues do not generate soluble factors that are able to rescue the null phenotype (Fig. 5 A). Similar, if not identical, results are obtained when explants of lung, myocardium, or skin are recovered from MT1-MMP–null mice and tested ex vivo (unpublished data). Furthermore, although neovessel formation by control aortic explants resulted in the release of 6.2 ± 1.1 μg hydroxyproline, *MT1-MMP*$^{-/-}$ explants released only 1.2 ± 0.6 μg hydroxyproline in the course of a 7-d culture period. In the presence of TIMP-2, collagenolysis by wild-type and *MT1-MMP*$^{-/-}$ explants was inhibited completely (0.4 ± 0.3 μg and 0 ± 0 μg hydroxyproline released, respectively; $n = 3$). Though MT1-MMP has been posited to regulate cell function by activating latent TGFβ or generating denatured collagen products that mediate integrin signaling (Heissig et al., 2003), neither the addition of active TGFβ nor that of proteolyzed collagen affected the *MT1-MMP*$^{-/-}$ phenotype (unpublished data).

Consistent with the inability of *MT1-MMP*$^{-/-}$ tissue explants to mount a tubulogenic program, isolated *MT1-MMP*$^{-/-}$ endothelial cells were unable to degrade subjacent collagen

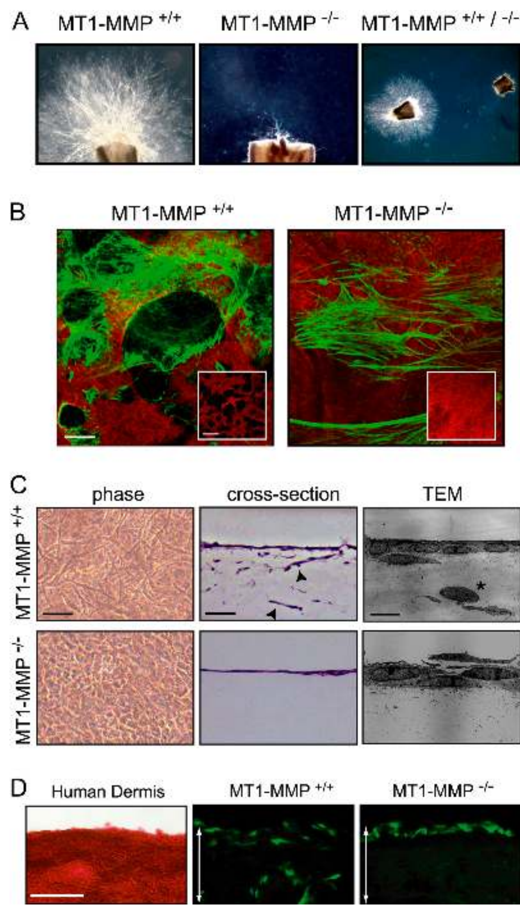

Figure 5. **A required role for MT1-MMP in neovessel formation, endothelial cell invasion, and collagenolytic activity.** (A) Aortic vessel explants isolated from MT1-MMP–null mice display defective capillary sprouting (middle) compared with wild-type tissue, after a 7-d culture period in type I collagen gels in the presence of VEGF–HGF and 20% FBS (left) or 5% autologous serum (not depicted). The co-culture of wild-type and MT1-MMP–null aortic rings (left and right, respectively, of the right panel) does not affect the sprouting behavior of wild-type or MT1-MMP–null explants. (B) Phalloidin-stained endothelial cells (green) circumscribe zones of collagen degradation generated by wild-type, but not *MT1-MMP*$^{-/-}$, cells cultured atop a type I collagen film in the presence of VEGF–HGF in 20% FBS for 7 d. Bar, 10 μm. Insets show a low magnification image of multiple sites of collagenolysis relative to the complete absence of degradation observed when MT1-MMP–null endothelial cells are cultured atop fluorescently labeled collagen gels (bar, 50 μm). (C) In contrast with wild-type endothelial cells, *MT1-MMP*$^{-/-}$ cells do not invade 3-D type I collagen gels (2.2 mg/ml), as assessed by phase-contrast microscopy (bar, 50 μm), in H&E–stained cross sections (bar, 50 μm), or by TEM analysis (bar, 5 μm) after a 7-d culture period with VEGF–HGF in the presence of 20% FBS. Arrowheads and asterisk indicate the positions of invading *MT1-MMP*$^{+/+}$ endothelial cells. (D) An acellular explant of type I/III collagen–rich human dermis stained with Sirius red is infiltrated by GFP-labeled control endothelial cells, but not by MT1-MMP–null endothelial cells. The double-headed arrow marks the boundary of the explant tissue beneath the monolayer of seeded endothelial cells. Bar, 100 μm.

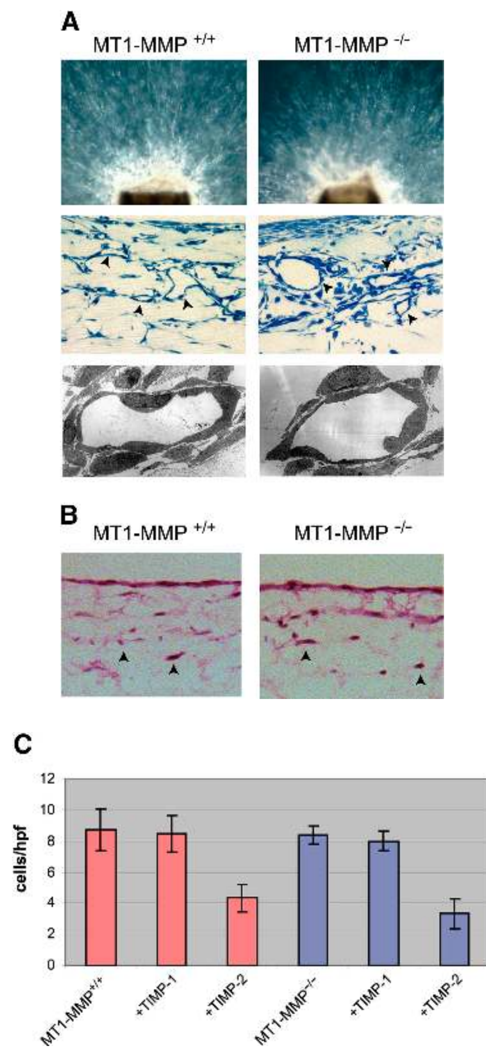

Figure 6. **The fibrin-invasive activity of MT1-MMP–null explants and endothelial cells.** (A) Wild-type and MT1-MMP–null explants display comparable neovessel outgrowth and tubulogenic activity when embedded in a 3-D gel of cross-linked fibrin (3.0 mg/ml) and cultured for 7 d with VEGF–HGF in the presence of 5% mouse serum as assessed by phase-contrast microscopy, in H&E–stained cross sections (representative vessels marked with arrowheads), or by TEM analysis (middle and bottom, respectively). (B) Isolated endothelial cells from wild-type or MT1-MMP–null mice invade fibrin matrices (3.0 mg/ml) in comparable fashion in the course of a 5-d incubation period in the presence of VEGF–HGF and 20% FBS. Representative invading cells are marked with arrowheads. (C) Fibrin invasion by isolated endothelial cells from wild-type and MT1-MMP–null mice are comparable, and inhibited by 1 μg/ml TIMP-2 but not 1 μg/ml TIMP-1 after a 5-d culture period under the conditions described above. Results are shown as the number of invading cells per hpf (mean ± 1 SD of 10 randomly chosen fields in two experiments).

(Fig. 5 B) or invade 3-D collagen gels (Fig. 5 C). A requirement for MT1-MMP during tissue-invasive activity was further confirmed by the inability of MT1-MMP–null endothelial cells to penetrate an explant of collagen-rich human dermal tissue ex vivo (Fig. 5 D). Despite the fact that MT1-MMP has been posited to regulate cell adhesion or migration by proteolyzing integrins, cadherins, or surface enzymes (Gálvez et al., 2002; Seiki, 2002), *MT1-MMP*$^{-/-}$ endothelial cells did not express defects in their two-dimensional interactions with collagen, as the cells migrated across type I collagen–coated surfaces at a rate indistinguishable from that of controls (i.e., 29 ± 1 μm/24 h vs. 29 ± 1 μm/24 h; $n = 3$). Likewise, although MT1-MMP serves as a necessary collagenolysin in endothelial cells, fibrin-invasive and -degradative activity can be conferred by other members of the membrane-anchored MMP family, including MT3-MMP (Hotary et al., 2002). Given the ability of vessel explants as well as isolated endothelial cells to express MT3-MMP (unpublished data), morphogenesis and invasion through 3-D fibrin

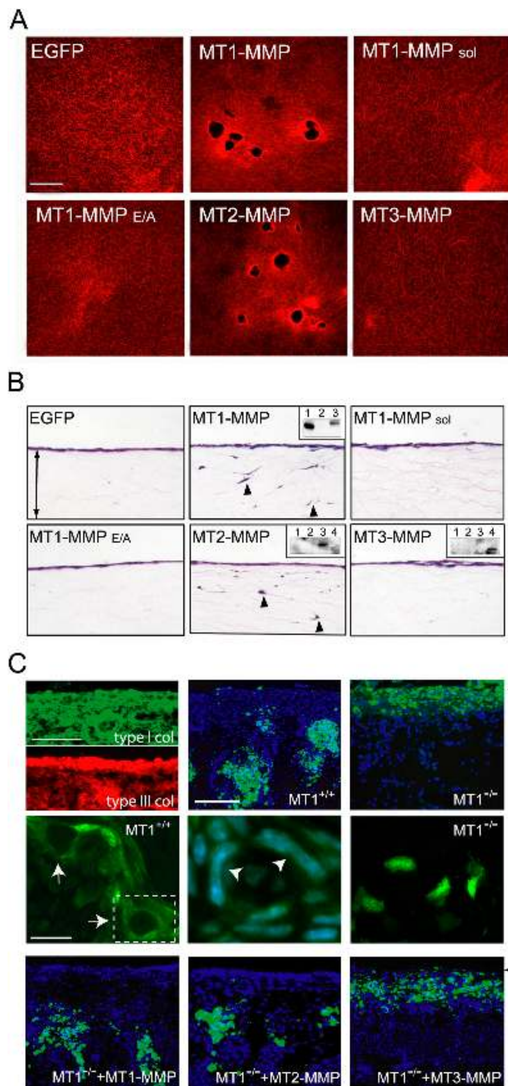

Figure 7. **Retroviral reconstitution of the collagenolytic and type I collagen–invasive activity of MT1-MMP–null endothelial cells.** (A) Although *MT1-MMP*$^{-/-}$ endothelial cells transduced with a control cDNA (top, left) were unable to degrade a subjacent collagen film after a 7-d culture period with VEGF–HGF in the presence of 5% mouse serum, retroviral reconstitution of active MT1-MMP restores the collagenolytic activity of *MT1-MMP*$^{-/-}$ endothelial cells (top, middle). Cells transduced with a catalytically inactive MT1-MMP$_{E/A}$ mutant or a transmembrane-deleted, soluble form of MT1-MMP (MT1-MMP$_{sol}$) do not display collagen degradative activity. Whereas the collagenolytic activity of *MT1-MMP*$^{-/-}$ endothelial cells could be rescued by expressing MT2-MMP, MT3-MMP–transduced cells did not display a collagenolytic phenotype. Bar, 50 μm. (B) Collagen-invasive activity of *MT1-MMP*$^{-/-}$ cells cultured atop a 3-D gel of type I collagen (2.2 mg/ml) for 7 d in the presence of VEGF–HGF and 20% FBS is rescued after transduction with MT1-MMP or MT2-MMP, but not EGFP, MT1-MMP$_{sol}$, MT1-MMP$_{E/A}$, or MT3-MMP. The inset for MT1-MMP shows the protein level of endogenous MT1-MMP in wild-type cells (lane 1), MT1-MMP–null endothelial cells (lane 2), and MT1-MMP–transduced-null cells (lane 3). The insets for MT2-MMP and MT3-MMP show the expression of MT2-MMP and MT3-MMP, respectively, in wild-type cells (lane 1), and MT1-MMP–null endothelial cells after retroviral transduction with MT1-MMP, MT2-MMP, or MT3-MMP (lanes 2–4). (C) Immunofluorescent micrographs of 11-d-old CAM cross sections depict a dense interstitial matrix composed of types I and III collagen (top, left; bar, 50 μm). *MT1-MMP*$^{+/+}$ or *MT1-MMP*$^{-/-}$ endothelial cells labeled with fluorescent microbeads (green) invaded 144 ± 55 μm and 53 ± 13 μm, respectively, into the CAM interstitium after a 3-d incubation (the CAM surface is marked by the black arrowheads to the right; top, middle and right, and bottom; bar, 100 μm). Retroviral gene transfer of GFP highlights the morphology of a group of invading *MT1-MMP*$^{+/+}$ cells (*MT1*$^{+/+}$; middle row, left and center; bar, 20 μm), which form tubular structures (left, arrows). The section of the micrograph bounded by the dashed square is enlarged (middle) and shows a group of elongated endothelial cells (arrowheads) forming a tubule with the nuclei highlighted by Hoechst staining. Morphology of GFP-labeled *MT1-MMP*$^{-/-}$ endothelial cells (*MT1*$^{-/-}$) is shown (middle row, right). After retroviral gene transfer of MT1-MMP or MT2-MMP into *MT1-MMP*$^{-/-}$ endothelial cells, invasion increased to 147 ± 46 μm and 123 ± 34 μm, respectively. After retroviral gene transfer of MT1-MMP or MT2-MMP into *MT1-MMP*$^{-/-}$ endothelial cells, invasion increased to 147 ± 46 μm and 123 ± 34 μm, respectively (mean ± 1 SD, $n = 3$). The invasive activity of MT3-MMP–transduced cells was not affected relative to the *MT1-MMP*$^{-/-}$ cells (i.e., 47 ± 22 μm, $n = 3$).

barriers was assessed in wild-type versus *MT1-MMP*$^{-/-}$ tissues. In contrast with their defect in collagen-invasive activity, MT1-MMP–null explants mounted a tubulogenic phenotype in fibrin gels that was comparable to that mounted by wild-type controls (Fig. 6 A). Furthermore, both *MT1-MMP*$^{+/+}$ and *MT1-MMP*$^{-/-}$ endothelial cells were able to invade fibrin gels via a TIMP-2-inhibitable process (Fig. 6, B and C). Hence, we conclude that (1) a requirement for MT1-MMP in tubulogenesis and invasion is restricted to the type I collagen matrix; and (2) although MT1-MMP has been reported to cleave a multiplicity of cell surface–associated substrates (Seiki, 2002), invasion and tubulogenesis can proceed independently of MT1-MMP in a physiologically relevant, but noncollagenous, matrix.

Structural requirements for MT1-MMP–dependent endothelial cell collagenolysis and invasion in vitro and in vivo

To define the structural features that underlie the ability of MT1-MMP to confer endothelial cells with collagen-degradative and -invasive activity, MT1-MMP–null endothelial cells were transduced with wild-type MT1-MMP or MT1-MMP variants harboring either an inactivating point mutation in the catalytic domain or a deletion of the transmembrane domain (Hotary et al., 2003). Although full-length MT1-MMP bestowed *MT1-MMP*$^{-/-}$ endothelial cells with both collagenolytic and invasive activity, catalytically inactive MT1-MMP did not rescue the null phenotype (Fig. 7, A and B). Similarly, transduction of MT1-MMP–null cells with a catalytically active, but soluble, form of MT1-MMP failed to confer collagenolytic or invasive activity, presumably because the recipient cell loses its ability to focus proteolytic activity to the subjacent compartment (Fig. 7, A and B).

Together, these results suggest that pericellular collagenolysis, as well as invasive activity, minimally requires the membrane display of a tethered collagenase. As such, MT1-MMP activity could conceivably be compensated by other members of the MT-MMP family that likewise express type I collagen–degradative activity. Interestingly, recent studies raise the possibility that MT2-MMP, though not normally expressed in mouse endothelial cells (unpublished data), can act as an alternate membrane-anchored collagenolysin (Hotary et al., 2000). Indeed, when *MT1-MMP*$^{-/-}$ endothelial cells were transduced with an MT2-MMP expression vector, subjacent proteolysis was restored fully and in tandem with collagen-invasive activity (Fig. 7, A and B). In contrast, a third member

of the MT-MMP family that displays little if any collagenolytic activity, MT3-MMP (Hotary et al., 2000), was unable to rescue the invasion-null phenotype (Fig. 7, A and B).

Finally, we sought to determine whether the ability of MT1-MMP or MT2-MMP to confer invasive activity to *MT1-MMP*$^{-/-}$ endothelial cells could be extended from homogenous 3-D constructs of type I collagen in the in vitro setting to a more complex interstitial barrier in vivo. Hence, wild-type or MT1-MMP–null endothelial cells transduced with control, MT1-MMP, MT2-MMP, or MT3-MMP retroviral expression vectors were labeled with either fluorescent microbeads or GFP and cultured atop the type I/III collagen–rich chick chorioallantoic membrane (CAM; Fig. 7 C). Consistent with the results obtained in vitro, wild-type, but not MT1-MMP–null endothelial cells, were able to penetrate deeply into the CAM interstitium. Furthermore, although GFP-labeled wild-type endothelial cells adopted an elongated phenotype and displayed morphogenic properties by generating tubular structures in the in vivo setting, MT1-MMP–null endothelial cells remained spherical in shape and did not form tubules (Fig. 7 C). Though the *MT1-MMP*$^{-/-}$ phenotype was not rescued by overexpressing MT3-MMP, tissue-invasive activity was restored fully by expressing either MT1-MMP or MT2-MMP (Fig. 7 C). Thus, membrane-anchored collagenases uniquely confer expressing cells with the proteolytic machinery necessary to drive the invasive phenotype critical to morphogenic programs in collagen-rich environments in vitro and in vivo.

Discussion

Angiogenesis, like the more generalized program of branching morphogenesis, requires tissue-infiltrating cells to negotiate an ECM rich in cross-linked type I collagen (Fisher et al., 1994; Pepper, 2001; Seandel et al., 2001; Davis et al., 2002; Lubarsky and Krasnow, 2003). Proteolytic remodeling of type I collagen has been posited to play a necessary role in allowing numerous cell types to generate a passageway through the matrix, migrate across the newly exposed collagen substratum, and then engage tubulogenic programs (Fisher et al., 1994; Tournier et al., 1994; Lelongt et al., 1997; Haas et al., 1998; Miralles et al., 1998; Zhu et al., 2000; Davis et al., 2001; Seandel et al., 2001; Kheradmand et al., 2002). Although multiple proteolytic systems have been implicated in angiogenic events in vivo (Carmeliet and Jain, 2000; Pepper, 2001; Davis et al., 2002), attempts to identify enzymes critical to tissue remodeling per se have been complicated by the more recent appreciation that the substrate repertoire of MMPs extends to a diverse array of growth factors, cytokines, chemokines, and cell adhesion molecules (Egeblad and Werb, 2002).

Accumulating evidence supports an angiogenic scheme in which endothelial cell–associated MMP-2 and MMP-9 drive endothelial migration, invasion, or tubulogenesis (Haas et al., 1998; Koivunen et al., 1999; Lyden et al., 1999; Xu et al., 2001). Because MT1-MMP proteolyzes the MMP-2 zymogen to its active form which, in turn, can activate MMP-9 (Seiki, 2002; Toth et al., 2003), we speculated initially that all three MMPs would form a collaborative network to drive the angiogenic response. Likewise, reports documenting the ability of $\alpha_v\beta_3$ and CD44 to localize MMP-2 and MMP-9, respectively, to the migrating front of tissue-invasive cells are consistent with a proteolytic model in which integrins and transmembrane glycoproteins act as docking sites for the assembled proteinases (Brooks et al., 1996; Yu and Stamenkovic, 1999; Rolli et al., 2003). Nonetheless, despite the appeal of such schemes, neither MMP-2, $\alpha_v\beta_3$, MMP-9, nor CD44 played a required role in neovessel formation, endothelial cell collagen invasion, or collagenolytic activity ex vivo. Likewise, although plasmin has been linked to the angiogenic process (Carmeliet and Jain, 2000; Pepper, 2001), plasminogen-null explants displayed no defects in our ex vivo model. In contrast, tissue explants recovered from *MT1-MMP*$^{-/-}$ mice were completely unable to mount a tubulogenic response when suspended in collagen gels. Furthermore, consistent with the proposition that collagenolytic activity is required to support endothelial cell invasion within a type I collagen–rich environment (Fisher et al., 1994; Haas et al., 1998; Seandel et al., 2001; Davis et al., 2002), MT1-MMP–null endothelial cells were unable to degrade subjacent collagen in a serum-containing milieu. Apparently, MT1-MMP is the major, if not sole, collagenolysin operative in mouse endothelial cells that is capable of mediating the pericellular dissolution of type I collagen under physiologic conditions. Given that the MT1-MMP zymogen undergoes efficient processing to its active form via a proprotein convertase–dependent process (Yana and Weiss, 2000), plasmin-mediated processing of MT1-MMP does not play a required role in this system. Although mouse endothelial cells express soluble collagenases (e.g., MMP-13, MMP-2, and MMP-8) and can use plasmin to activate these proteinases, these MMPs were unable to mount a focal collagenolytic effect in the presence of serum antiproteinases (unpublished data). Hence, in the absence of MT1-MMP activity, mouse endothelial cells fail to degrade collagen or negotiate collagenous barriers. As human endothelial cell tubulogenesis is also sensitive to TIMP-2, but not TIMP-1 (Lafleur et al., 2002; Collen et al., 2003), we posit that MT1-MMP and/or MT2-MMP play(s) a dominant role in directing angiogenesis in humans as well.

Despite the correlation between collagenolytic and invasive activity, MT1-MMP hydrolyzes not only type I collagen, but also a variety of noncollagenous targets (Seiki, 2002; Egeblad and Werb, 2002). Consequently, we cannot eliminate the possibility that MT1-MMP cleaves type I collagen in tandem with other substrates in a fashion necessary to affect an invasive and/or tubulogenic program. Our data do, however, rule out recently proposed models wherein MT1-MMP drives invasion by either processing the $\alpha_v\beta_3$ integrin or cleaving CD44 (Deryugina et al., 2000; Kajita et al., 2001; Mori et al., 2002). Furthermore, a more generalized defect in cell adhesion, migration, or invasion that might be consistent with MT1-MMP–dependent proteolysis of integrins, cadherins, growth factors, or surface enzymes is not supported by our observations that *MT1-MMP*$^{-/-}$ cells migrate at normal rates across collagen-coated surfaces and invade cross-linked fibrin barriers. Though recent studies have suggested that MT1-MMP can regulate endothelial cell migration (Gálvez et al., 2002), these conclusions are founded

on the use of neutralizing antibodies directed against MT1-MMP. Because these antibodies do not recapitulate the *MT1-MMP*$^{-/-}$ phenotype and, in independent studies, affected the activity of multiple membrane-anchored as well as secreted MMPs (unpublished data), caution should be exercised in assuming their specificity or utility as MT1-MMP inhibitors.

The striking defects in collagenolytic and invasive activity, as well as in neovessel formation, displayed by *MT1-MMP*$^{-/-}$ tissues seem at odds with the fact that the null animals develop normally (Holmbeck et al., 1999; Zhou et al., 2000). In this regard, it is interesting to note that during embryogenesis, as well as perinatally, the type I collagen content of most tissues is low relative to the content in the postnatal state (Van Exan and Hardy, 1984; Carver et al., 1993). As a possible consequence, newborn null animals appear normal and only begin to display serious skeletal and connective tissue abnormalities after the first week of birth (Holmbeck et al., 1999; Zhou et al., 2000). Hence, although the ECM composition of the developing animal may afford *MT1-MMP*$^{-/-}$ animals a protected status, we posit that the increased deposition of type I collagen that arises as a consequence of the mechanical demands of adult life initiates the onset of the pathologic states observed in the knockout mice. Indeed, though null animals display an apparently normal vasculature at birth, Zhou et al. (2000) have reported that angiogenic responses in 15-d-old *MT1-MMP*$^{-/-}$ animals are abrogated in collagen-rich corneal tissues. Nonetheless, even in collagen-replete tissues, our results suggest that angiogenesis in fibrin-rich fields (e.g., wounds) may proceed normally in MT1-MMP–null mice because MT3-MMP, though largely devoid of collagenolytic activity, can function as an efficient fibrinolysin (Hotary et al., 2002).

Together, our observations appear to be at variance with other in vitro or in vivo studies concluding that plasminogen, MMP-2, or MMP-9 plays a required role in the angiogenic process (Carmeliet and Jain, 2000; Brodsky et al., 2001; Pepper, 2001). However, it should be noted that defects in angiogenesis have not been observed uniformly in either *plasminogen*$^{-/-}$, *MMP-2*$^{-/-}$, or *MMP-9*$^{-/-}$ mice in response to wounding (Romer et al., 1996; Itoh et al., 1998) or tumor growth (Bergers et al., 2000; Hamano et al., 2003). Furthermore, $\alpha_v\beta_3{}^{-/-}$ mice mount an exaggerated angiogenic response in a range of pathophysiologic settings (Reynolds et al., 2002). In cases where MMP-2 deficiency has been shown to affect neovascularization, a partial reduction in the angiogenic response (~30%) has been most frequently described (Itoh et al., 1998; Berglin et al., 2003; Guedez et al., 2003). In contrast, MMP-9–deficient mice can, in some cases, display more significant defects in angiogenesis in vivo, but this effect has been ascribed largely to the ability of the metalloproteinase to release matrix-bound forms of VEGF (Bergers et al., 2000). Indeed, vascular defects in these animals are reversed by the exogenous application of VEGF, despite the continued absence of MMP-9 (Engsig et al., 2000; Heissig et al., 2003). Nonetheless, under defined circumstances, plasminogen, MMP-2, MMP-9, $\alpha_v\beta_3$, or CD44 can play an important role in modifying angiogenic events in vivo (Carmeliet and Jain, 2000; Pepper, 2001; Davis et al., 2002; Heissig et al., 2003; Oh et al., 2004), but based on our results, these effects are more likely exerted in a fashion independent of the matrix remodeling events associated strictly with invasion or tubulogenesis within the 3-D interstitium. As the ex vivo models used herein largely obviate a required role for endogenous growth factors, chemokines, or immune cell populations, it may well develop that these latter players serve as preferred targets for plasminogen, MMP-2, or MMP-9 in vivo. Similarly, the ex vivo model may circumvent a requirement for basement membrane proteolysis, a key step in the initiation of the angiogenic process, though it should be noted that angiogenesis can proceed in a normal fashion in vivo even in the combined absence of MMP-2 and MMP-9 (Baluk et al., 2004). Clearly, a mounting number of observations describing normal, or near normal, tubulogenic programs in MMP-2– or MMP-9–null animals (Andrews et al., 2000; Bergers et al., 2000; Wiseman et al., 2003) strongly suggest that tubulogenic programs—including angiogenesis—can proceed in the absence of these downstream MMPs. As such, the rules we have established for generating patent neovessels in collagen-rich tissues raise the possibility that MT1-MMP and perhaps MT2-MMP play dominant roles in driving a variety of tubulogenic programs in the in vivo, postnatal setting.

Materials and methods

Ex vivo angiogenesis assay

Mouse aortic ring assays were performed with minor modifications of protocols described previously (Nicosia and Madri, 1987; Hiraoka et al., 1998). Mouse aortas were isolated from 4- to 5-wk-old male mice deficient in either plasminogen, MMP-2, MMP-9, MT1-MMP, β3 integrin, or CD44, or their respective littermate controls. Mice carrying null mutations for plasminogen (provided by T. Bugge, National Institutes of Health, Bethesda, MD), β3 (provided by S. Teitelbaum [Washington University, St. Louis, MO] and R. Hynes [Massachusetts Institute of Technology, Cambridge, MA]), MMP-2 (provided by S. Itohara, RIKEN Brain Science Institute, Saitama, Japan), or CD44 (provided by C. Doerschuk [Rainbow Babies and Children's Hospital, Case Western Reserve University, Cleveland, OH] and T. Mak [Ontario Cancer Institute, University of Toronto, Toronto, Ontario, Canada]) were backcrossed into the C57BL7 background for at least 10 generations. MT1-MMP– and MMP-9–null mice were backcrossed 10 or more generations into the Black Swiss or 129SvEv backgrounds, respectively (Romer et al., 1996; Itoh et al., 1998; Holmbeck et al., 1999; Andrews et al., 2000; Reynolds et al., 2002; Wang et al., 2002). Isolated aortas were sectioned (in 1-mm square fragments) and embedded in 2.2 mg/ml reconstituted type I collagen gels or 3.0 mg/ml cross-linked fibrin (Hotary et al., 2000, 2002) in 24-well plates and cultured in RPMI 1640 (GIBCO BRL) supplemented with either 20% FBS or 5% mouse sera (prepared from wild-type littermate or null animals) in the presence of 100 ng/ml recombinant human VEGF-165, 50 ng/ml recombinant HGF (both provided by Genentech, Inc., San Francisco, CA), and 100 pg/ml human TGF-β (R&D Systems). In selected experiments, fragments were cultured in the presence of 100 μg/ml aprotinin (Sigma-Aldrich), 10 μM E64d (Sigma-Aldrich), 5 μM pepstatin (Sigma-Aldrich), 5 μM BB94 (British Biotechnology), endotoxin-free recombinant human TIMP-1 or TIMP-2 (both manufactured by Fujii), or Ala–TIMP-2 (provided by W. Stetler-Stevenson, National Cancer Institute, Bethesda, MD).

After 7 d in culture, the collagen gel–embedded fragments were fixed, the endothelial cells were immunostained with anti–mouse CD31 antibody (BD Biosciences) followed with Alexa Fluor 488 anti–rat IgG secondary antibody (Molecular Probes), and the nuclei were visualized with DAPI (Vector Laboratories). Samples were mounted in Vector Shield (Vector Laboratories) and fluorescent or light images were captured by a microscope (DMLB; Leica; 5×/0.7 NA or 10×/0.7 NA objective lens) equipped with a SPOT RT camera (Diagnostic Instruments). Neovessel length was determined with SPOT software (Diagnostic Instruments) after calibration, using an objective micrometer. For light and transmission electron microscopic studies, gels were fixed with 2% glutaraldehyde and

1.5% PFA in 0.1 M sodium cacodylate, pH 7.4 (Hotary et al., 2002). Neovessel density was quantified by counting the number of tubules in six or more cross sections taken 5 mm from the edge of the cultured aortic rings in all four quadrants surrounding the embedded explants, after capturing the images with a microscope (DMLB; Leica; 20×/0.7 NA objective lens) fitted with a SPOT RT camera. To visualize sites of collagen matrix remodeling, we stained frozen sections with either the polyclonal antibody against cleaved type I collagen (gift of A.R. Poole, McGill University, Montreal, Canada) or a monoclonal antibody directed against denatured type I collagen (Xu et al., 2001). Type IV collagen and laminin were identified with polyclonal anti–type IV collagen (Oncogene Research Products) and monoclonal antilaminin (Sigma-Aldrich) antibodies. Chicken types I and III collagen were localized using anti–chicken types I and III collagen antibodies, respectively (CHEMICON International). Alexa Fluor 488– or 594–conjugated anti–mouse or anti–rabbit antibodies (Molecular Probes) were used as secondary antibodies. Sirius red staining was performed as described by Canham et al. (1999). Type I collagen degradation during neovessel formation was quantified by hydroxyproline assay (Creemers et al., 1997).

Endothelial cell invasive activity

Mouse dermal endothelial cells were isolated as described by Murphy et al. (1998). The isolated cells were cultured on gelatin-coated dishes in RPMI 1640 media with 5% mouse serum and supplemented with 100 μg/ml endothelial cell growth supplement (BD Biosciences). The endothelial characteristics of the isolated cells were confirmed by von Willebrand factor, CD31, and VE-cadherin staining (Murphy et al., 1998). DiI-Ac-LDL uptake was >99%. To assess invasive activity, confluent monolayers of microvascular endothelial cells were cultured atop type I collagen gels (2.2 mg/ml), fibrin gels (3.0 mg/ml), or acellular explants of human dermis (Alloderm; Life Cell) in the upper compartment of Transwell dishes (Costar) and exposed to a chemotactic gradient of VEGF (100 ng/ml) and HGF (50 ng/ml).

Collagen film degradation assay

Type I collagen gel films (~100 μg) prepared on glass coverslips were labeled with tetramethylrhodamine isocyanate (Molecular Probes) for 45 min. Microvascular endothelial cells were cultured atop the collagen gels in the presence of 100 ng/ml VEGF, 50 ng/ml HGF, and 5% autologous serum. After 7 d, the samples were fixed, polymerized actin was visualized with Alexa Fluor 488 phalloidin (Molecular Probes), and fluorescent images were obtained by a laser scanning fluorescent microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.) equipped with acquisition software (Version 3.2; Service Pck 2) using either 40× or 63× C-Apochromat (1.2 NA) water immersion objective lenses at 25°C. Samples were mounted in Vector Shield (Vector Laboratories).

Chick CAM assays

Endothelial cell invasion, before or after retroviral transduction, was determined in vivo using 11-d-old chick embryos wherein wild-type or MT1-MMP–null endothelial cells were seeded atop the CAM for 3 d (Cameron et al., 2000). To visualize endothelial cells, 10^5 cells were either labeled with 0.05 μm fluoresbrite carboxylate microspheres (Polysciences) or transduced with a GFP retroviral vector. The mean depth of endothelial invasion was determined by measuring the distance from the CAM surface to the leading front of two or more invading endothelial cells in 10 or more randomly selected fields, as assessed with a microscope (DMLB; Leica)/SPOT RT camera system in at least two experiments.

Retroviral gene transfer

HA-tagged human MT1-MMP cDNA; soluble MT1-MMP (Met^1-Gly^{535} that lacks the COOH-terminal transmembrane and cytosolic domains of the wild-type proteinase, and $MT1\text{-}MMP_{sol}$), a catalytically inactive full-length form of MT1-MMP that harbors an E^{240} to A substitution in its catalytic domain ($MT1\text{-}MMP_{E/A}$); or mouse cDNA clones for MT2-MMP and MT3-MMP (provided by M. Seiki, University of Tokyo, Tokyo, Japan) were subcloned into pRET2 retroviral vector derived from the Moloney murine leukemia virus–based MFG backbone (Morita et al., 2001). The control pRET vector carried an EGFP cDNA. Subconfluent monolayers of the isolated endothelial cells were cultured in the retroviral supernatant for 12 h in the presence of 100 μg/ml endothelial cell growth supplement and collagen invasion and degradation assays were performed 24 h later. The expression of MT1-MMP, MT2-MMP, and MT3-MMP was confirmed by Western blot analysis using polyclonal anti–MT1-MMP antibody (Yana and Weiss, 2000) and monoclonal anti–mouse MT2-MMP and MT3-MMP antibodies (Calbiochem), respectively.

RT-PCR analysis

Endothelial cells were cultured atop type I collagen gels in the presence of 100 ng/ml VEGF and 50 ng/ml HGF in 10% serum, and total RNA was isolated using TRIzol reagent (GIBCO BRL). RT-PCR was performed using One-Step RT-PCR System reagent (Life Technologies). The identities of the PCR products were confirmed by sequence analysis.

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