Molecular Balance of Capillary Tube Formation *versus* Regression in Wound Repair: Role of Matrix Metalloproteinases and Their Inhibitors

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In this review, we discuss the identification of distinct matrix metalloproteinases (MMPs) and their inhibitors that differentially control the processes of capillary tube formation (morphogenesis) versus capillary tube regression in three-dimensional (3D) collagen matrices. This work directly relates to both granulation tissue formation and regression during wound repair. The membrane metalloproteinase, MT1-MMP (MMP-14), is required for endothelial cell (EC) tube formation using *in vitro* assays that mimic vasculogenesis or angiogenic sprouting in 3D collagen matrices. These events are markedly blocked by small interfering RNA (siRNA) suppression of MT1-MMP in ECs or by addition of tissue inhibitor of metalloproteinases (TIMPs)-2, -3, and -4 but not TIMP-1. In contrast, MMP-1 and MMP-10 are strongly induced during EC tube formation to regulate the process of tube regression (following activation by serine proteases) rather than formation. TIMP-1, which selectively inhibits soluble MMPs, blocks tube regression by inhibiting MMP-1 and MMP-10 while having no influence on EC tube formation. siRNA suppression of MMP-1 and MMP-10 markedly blocks tube regression without affecting tube formation. Furthermore, we discuss that pericyte-induced stabilization of EC tube networks in our model system appears to occur through EC-derived TIMP-2 and pericyte-derived TIMP-3 to block both the capillary tube formation and regression pathways.

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INTRODUCTION

The molecular and cellular events underlying wound repair are being elucidated using a variety of models (both *in vivo* and *in vitro*) (Clark *et al.*, 1982; Greiling and Clark, 1997; Smola *et al.*, 1998; Singer and Clark, 1999; Maas-Szabowski *et al.*, 2001; Werner and Grose, 2003; Stark *et al.*, 2004a, b) and technical strategies (Grose and Werner, 2003; Yang *et al.*, 2003; Martin and Parkhurst, 2004; Stramer and Martin, 2005; Stramer *et al.*, 2005). A major emphasis of many of the studies has been the utilization of skin as a model organ and keratinocytes as key cells to characterize these processes. An important event during wound repair of skin is the formation of granulation tissue, an admixture of endothelial cells (ECs), leukocytes, fibroblasts, and provisional extracellular matrix, which accumulates within the wound site (Clark *et al.*, 1982; Clark, 1985, 1993; Singer and Clark, 1999). Many studies have led to the identification of critical molecules that stimulate the process of angiogenesis (Conway *et al.*, 2001; Davis *et al.*, 2002; Jain, 2003, 2005; Carmeliet, 2005; Davis and Senger, 2005), a major step in wound repair, which regulates the formation of granulation tissue (Singer and Clark, 1999). Another important event during the healing phase of wound repair is the regression of granulation tissue, which involves removal of new blood vessels, provisional extracellular matrix as well as leukocytic infiltrates (Clark, 1985, 1993; Singer and Clark, 1999). The molecular control of this regression process is not well understood and is a significant area of investigation for future work.

The matrix metalloproteinases (MMPs) represent an important class of molecules that are clearly linked to the molecular control of wound repair (Saarialho-Kere *et al.*, 1994; Moses *et al.*, 1996; Kahari and Saarialho-Kere, 1997; Madlener *et al.*, 1998; Nwomeh *et al.*, 1998; Bullard *et al.*, 1999; Wu *et al.*, 2002, 2003; Krampert *et al.*, 2004; Davis and Senger, 2005). There are over 20 known members of this family and they have diverse functions in cellular events including wound repair, inflammation, and cancer (Parks, 1999; Parks and Shapiro, 2001; Pepper, 2001; Mott and Werb, 2004; Parks *et al.*, 2004; Handsley and Edwards, 2005; Rundhaug, 2005). These enzymes proteolytically cleave various substrates including extracellular matrix proteins, growth factors, growth factor binding and regulatory proteins,

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Abbreviations: 3D, three-dimensional; EC, endothelial cell; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase

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and membrane receptors (Sternlicht and Werb, 2001; Mott and Werb, 2004; Handsley and Edwards, 2005). While the majority of MMPs are secreted, some represent transmembrane proteins or glycophosphatidylinositol-linked proteinases tethered to the cell surface (Nagase and Woessner, 1999). Recent studies indicate that some of the secreted MMPs also become anchored to the cell surface through binding proteins such as integrins (Dumin *et al.*, 2001; Stricker *et al.*, 2001). MMPs can be specifically inhibited by a class of inhibitors termed tissue inhibitor of metalloproteinases (TIMPs) (Baker *et al.*, 2002). There are four members of this family, which are TIMPs 1–4. TIMP-1 appears to primarily inhibit soluble MMPs while TIMPs-2, -3, and -4 can inhibit both soluble and membrane-type MMPs (MT-MMPs) (Baker *et al.*, 2002). These inhibitors have been used as molecular tools to help elucidate the function of different classes of MMPs during the complex cellular and molecular events of wound repair.

The purpose and scope of this review is to discuss the emerging functional role of specific MMPs and their inhibitors during granulation tissue formation and regression using *in vitro* models that mimic these events. Our models mimic either the granulation tissue formation or regression phases of wound repair (Davis and Camarillo, 1996; Salazar *et al.*, 1999; Bayless *et al.*, 2000; Bell *et al.*, 2001; Davis *et al.*, 2001, 2002; Bayless and Davis, 2002; Davis and Senger, 2005; Saunders *et al.*, 2005), and we specifically focus on the molecular control of EC tubular network formation (i.e. morphogenesis) versus EC tube regression in three-dimensional (3D) collagen matrices. Our studies reveal that distinct MMPs are involved in these different steps and that specific TIMPs have differential effects on these events.

ROLE OF MMPS IN CAPILLARY TUBE MORPHOGENESIS

Work over the past two decades has shown that many MMPs are upregulated during angiogenesis and wound repair (Davis *et al.*, 2001, 2002; Pepper, 2001; Sternlicht and Werb, 2001; Mott and Werb, 2004; Handsley and Edwards, 2005; Rundhaug, 2005; Saunders *et al.*, 2005; Stetler-Stevenson and Seo, 2005). What has been lacking in many of the earlier studies is a convincing role for specific MMPs in particular events during these processes. In Figure 1, we schematically illustrate a time course of granulation tissue formation and regression during skin repair (Clark *et al.*, 1982; Clark, 1993, 1985; Singer and Clark, 1999) and predict the role of specific MMPs in these events based on our *in vitro* models (Davis and Camarillo, 1996; Davis *et al.*, 2001; Davis and Bayless, 2003; Davis and Senger, 2005; Saunders *et al.*, 2005). Our models show that MMPs function by either stimulating tube formation (e.g. MT1-MMP) or stimulating tube regression (e.g. MMP-1, MMP-10, and MMP-13), in 3D type I collagen matrices, the predominant ECM component of skin. This work supports the conclusion that distinct MMPs primarily function as promorphogenic (i.e. formation) or proregression agents (Figure 1 and later on). It remains to be determined how these events illustrated by our *in vitro* models of granulation tissue formation and regression directly relate to the events of human or rodent skin wound repair. For

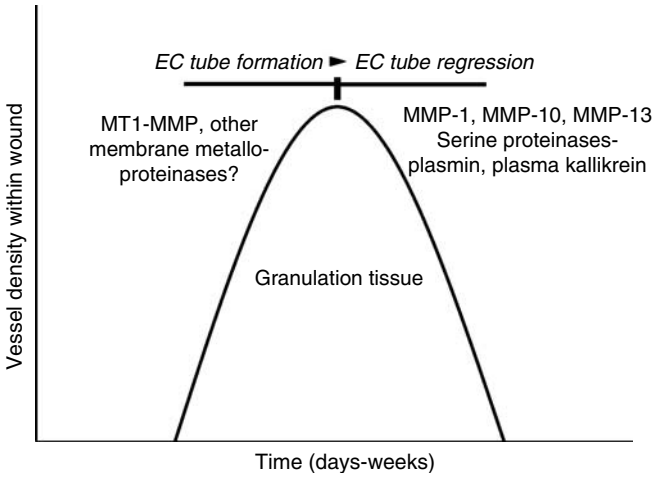

Figure 1. Potential role of different MMPs in granulation tissue formation and regression in human skin wound repair. The figure is based on previously described diagrams of granulation tissue formation and regression (Clark, 1993, 1985). The time frame on the *x*-axis is listed as days to weeks because of variability in the healing time courses of different wounds and species such as humans, mice, and pigs. The particular MMPs mentioned have been identified to play a critical role in *in vitro* models of granulation tissue formation and regression.

example, our studies have primarily focused on the EC population alone and have not until recently begun to address the potential paracrine interactions between different cell types such as ECs and pericytes during wound repair (Saunders *et al.*, in preparation). However, we believe our work illustrates new concepts that may explain previously reported data (see below for details) and poses new testable hypotheses.

In many prior studies, it was largely assumed that induction of an MMP during angiogenesis, for example, was indicative of a proangiogenic function. However, recent work indicates that MMPs induced by ECs during angiogenesis have complex functions and appear to contribute to two distinct processes, namely capillary tube formation (morphogenesis) and capillary tube regression (Zhu *et al.*, 2000; Davis *et al.*, 2002; Davis and Senger, 2005; Handsley and Edwards, 2005; Saunders *et al.*, 2005). Overall, the molecular basis for the latter process is not well known but a number of stimuli are clearly associated with the phenomenon. Such stimuli include vascular endothelial growth factor withdrawal (Benjamin *et al.*, 1998, 1999), influence of thrombospondins (Armstrong and Bornstein, 2003), influence of angiopoietin-2 (Gale *et al.*, 2002; Thurston, 2003; Carmeliet, 2005), treatment with microtubule disrupting agents (Bayless and Davis, 2004), integrin antagonists (Davis *et al.*, 2002; Davis and Senger, 2005), biologically active extracellular matrix fragments (i.e. matricryptins) (Davis *et al.*, 2000; Schenk and Quaranta, 2003), and activation of specific MMPs such as MMP-1 and MMP-10, which destroy the ECM scaffold in which a newly formed vascular network is embedded (Davis *et al.*, 2001; Saunders *et al.*, 2005). One unresolved and interesting question is whether the various treatments

mentioned above shift the proteolytic balance toward MMP-dependent vascular regression involving MMP-1, MMP-10, or other secreted collagenases such as human or murine MMP-13 (see below).

Since our new data reveal an independent role for MT-MMPs in vascular tube formation and MMP-1, MMP-10, and MMP-13 (see below) in vascular tube regression in 3D collagen matrices, these data should be considered in the context of previously reported therapeutic regimens using broad-spectrum MMP inhibitors to interfere with angiogenesis or cancer progression (Coussens *et al.*, 2002; Egeblad and Werb, 2002). There was much disappointment in that these MMP inhibitors had little clinical efficacy and for the most part did not work as expected (Coussens *et al.*, 2002; Egeblad and Werb, 2002). Our data suggest one possible reason for this failure to inhibit angiogenesis, since the added broad-spectrum inhibitors likely block both tube formation as well as tube regression in these *in vivo* contexts. Interference with the normal process of tube regression would be expected to increase vascularity within tissues (i.e. an unexpected consequence of these therapies). Thus, such therapeutic regimens were not specific enough to target individual molecules required for either capillary tube formation versus regression. Our new work raises the possibility that targeting individual MMPs or TIMPs might allow for a shift of the balance of vascularity toward more or less vessels (i.e. to independently regulate capillary tube formation versus regression) depending on the particular therapeutic application. Importantly, much continued work is required to identify the specific MMPs, TIMPs, or other molecules that regulate these distinct events. The work discussed below describes our recent attempts to delineate the role of specific MMPs and TIMPs in the molecular control of human capillary tube formation versus regression in 3D ECM environments.

CRITICAL ROLE FOR MEMBRANE-TYPE METALLOPROTEINASES IN VASCULAR TUBE FORMATION IN 3D COLLAGEN MATRICES

My laboratory has focused much attention on the development of models of capillary tube morphogenesis in 3D extracellular matrices including type I collagen or fibrin (Davis and Camarillo, 1996; Bayless *et al.*, 2000; Davis *et al.*, 2002) (Figure 2). This work has allowed us to identify critical regulatory molecules that regulate tube formation including integrins (Davis and Camarillo, 1996; Bayless *et al.*, 2000; Davis *et al.*, 2002; Davis and Bayless, 2003; Davis and Senger, 2005), cytoskeletal regulators such as Cdc42 and Rac1 (Bayless and Davis, 2002; Davis and Bayless, 2003; Davis and Senger, 2005), and provisional basement membrane matrix components such as collagen type IV (Bell *et al.*, 2001; Davis and Senger, 2005). The systems are excellent models to study the process of lumen formation (Davis and Camarillo, 1996; Bayless and Davis, 2002; Davis *et al.*, 2002; Davis and Bayless, 2003) in real-time where individual cells form 3D networks of EC-lined tubes within a 48–72 hour period. A time-lapse study is shown in Figure 2, whereby a group of ECs is observed to form a network of tubes over a period of 48 hours (Figure 2a). In Figure 2b, individual ECs

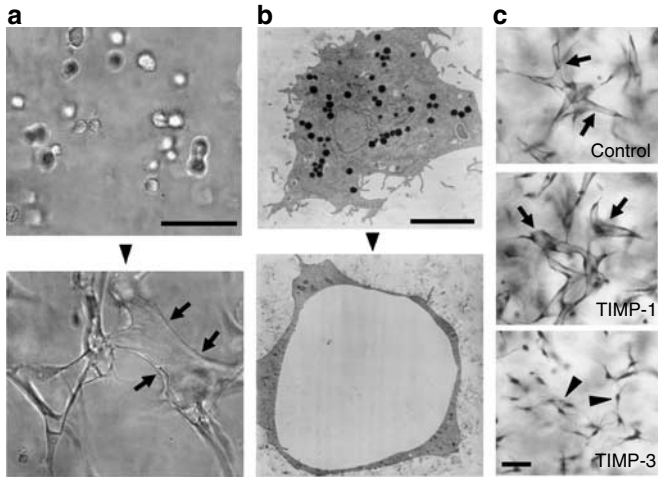

Figure 2. Formation of human capillary tubes in 3D collagen matrices. Human ECs are placed in collagen matrices as described (Davis and Camarillo, 1996) and were photographed using time-lapse microscopy (Saunders *et al.*, 2005), were processed for electron microscopy or were treated with purified TIMP-1 or TIMP-3 (5 μg/ml) (C). (**a**) Upper panel – 2 hours culture; lower panel – 48 hours culture of same field; bar = 100 μm. (**b**) Upper panel – 2 hours culture; lower panel – 48 hours culture; bar = 10 μm. (**c**) Arrows indicate capillary tube structures; arrowheads indicate ECs with fine processes with no lumenal structures; bar = 100 μm.

are examined by electron microscopy to form a matrix-free lumenal space over this time frame. Importantly, the addition of metalloproteinase inhibitors such as TIMP-3, which blocks membrane-type MMPs, completely blocks the ability of ECs to form tubular networks in 3D collagen matrices (Figure 2c). In contrast, TIMP-1, an inhibitor of soluble but not membrane-type MMPs, does not block capillary tubular network formation (Figure 2c). In this assay system, ECs are suspended as individual cells in collagen matrices, which mimics the process of vasculogenesis (Davis *et al.*, 2002). The influence of TIMPs is observed in a different assay system, which mimics the process of angiogenesis (Figure 3a). In this assay, ECs invade and undergo tubular morphogenesis by sprouting from a pre-existing monolayer of ECs on the surface of a collagen matrix (Bayless and Davis, 2003). As shown in Figure 3a, the addition of recombinant TIMPs including either TIMP-2 or TIMP-3 markedly block invasion and tube formation. As in the vasculogenic assay, TIMP-1 has no effect when compared to control. TIMP-2 and TIMP-3 have in common the ability to block the cell surface expressed MT-MMP family of proteinases, while TIMP-1 does not block these enzymes. Chemical inhibitors such as GM6001 are also known to interfere with the activity of MT-MMPs. EC tubular morphogenesis is completely blocked by GM6001 addition in either the ''vasculogenic'' or ''angiogenic'' systems (not shown). A newly developed variation of these assay systems is shown in Figure 3b. In this system, human ECs are preaggregated for 2 hours prior to attaching them to collagen-coated plastic surfaces and overlaying them with a 3D collagen matrix. In this system, EC–EC aggregates of about 10 cells form dramatic lumenal structures and undergo sprouting

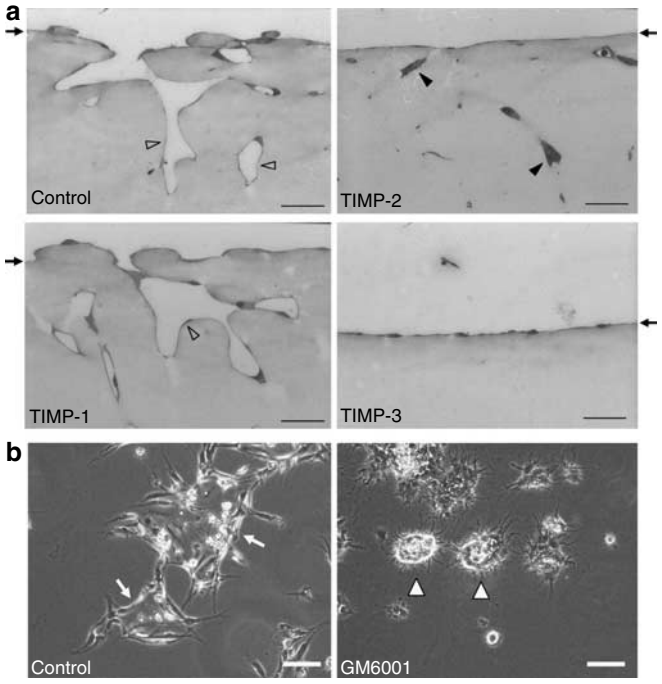

Figure 3. Membrane-type MMPs are required for EC invasion and tube morphogenesis in 3D collagen matrices. Human ECs are placed onto the surface of collagen matrices as described which contain 1 μM sphingosine-1-phosphate (Bayless and Davis, 2003). Cultures were treated with purified TIMPs each at 5 μg/ml and the cultures were fixed and processed for thin plastic sectioning after 48 hours. (**a**) Arrows indicate EC monolayer; open arrowhead indicates invading EC sprouts with open lumenal structures; closed arrowhead indicates invading ECs with no lumenal structures. Bar = 40 μm. (**b**) ECs were preaggregated in solution (1×10^7 cells/ml) at 37°C in serum-free medium for 2 hours and were then gently added to collagen coated plastic wells (50 μg/ml collagen type I in phosphate-buffered saline) in 24-well plates for 20 minutes. Unattached aggregates were washed off and the remaining attached aggregates were covered with collagen type I gel which is polymerized at 3.75 mg/ml as described (Davis and Camarillo, 1996). Culture media was prepared as described (Davis and Camarillo, 1996) and contained GM6001 (5 μM) or DMSO carrier control. Cultures were fixed and photographed after 24 hours of culture. Arrows indicate multicellular EC lumenal structures while arrowheads indicate EC–EC aggregates with fine process extensions but without lumen structures. Bar = 100 μm.

over a 24 hour period (Figure 3b, left panel). In the right panel of Figure 3b, aggregates treated with GM6001 are shown at 24 hours and are of similar appearance to the starting aggregates (open arrowheads) except that fine EC processes are observed projecting from the aggregates. Thus, addition of GM6001 completely interferes with the ability of the aggregates to undergo tube formation and lumenal morphogenesis. Interestingly, these EC-derived fine processes can project into the collagen matrix even with inhibition of MT-MMPs suggesting that these enzymes are not required for EC process extension in 3D collagen matrices. Overall, these data strongly indicate the MT-MMPs are critical regulators of human capillary tubular morphogenesis in 3D collagen matrices. Previously, we showed that invasion and tubular morphogenesis in fibrin matrices was similarly blocked by proteinase inhibitors that block MT-MMPs (Bayless and Davis, 2003). In support of our studies, other work has shown the importance of MT-MMPs in vascular morphogenesis (Hotary *et al.*, 2000; Davis *et al.*, 2002; Lafleur *et al.*, 2002; Bayless and Davis, 2003; Chun *et al.*, 2004; Davis and Senger, 2005).

MT1-MMP IS REQUIRED FOR HUMAN CAPILLARY TUBE FORMATION IN 3D COLLAGEN MATRICES

To address the specific role of individual surface metalloproteinases in our vasculogenic and angiogenic morphogenic systems, we suppressed expression of various MMPs in small interfering RNA (siRNA) experiments. In a recent report, we have successfully adapted siRNA technology for use in our 3D systems whereby ECs can be pretreated with siRNAs and then placed in 3D tube morphogenesis and regression assays (Saunders *et al.*, 2005). We have considerable experience with this technique in our systems and have observed only a small number of siRNAs that have a profound blocking influence on EC tube morphogenesis. One such siRNA is directed to the α2 integrin subunit (Mareth *et al.*, in preparation), a collagen receptor component known to regulate tube morphogenesis in these models (Davis and Camarillo, 1996; Davis and Senger, 2005). In Figure 4, we show that an siRNA directed to MT1-MMP markedly blocks EC tube formation, lumen development, and invasion of ECs in vasculogenic or angiogenic assays. We have documented that MT1-MMP protein expression is strongly suppressed by this treatment (Saunders *et al.*, in preparation). Importantly, an MT3-MMP siRNA and a control luciferase siRNA have no effect in either assay system (Figure 4). An examination of the MT1-MMP siRNA-treated ECs in the middle right panel of Figure 4 reveals that the ECs are sending out fine processes just like the processes observed in the GM6001 treated cells in Figure 3b (right panel). These processes are also clearly observed in the TIMP-3-blocked ECs in Figure 2c (lower right panel). Essentially identical results are observed when EC cultures are treated with either purified TIMP-2 or TIMP-4 (Saunders *et al.*, in preparation). It should also be noted that EC lumen development is completely inhibited by these treatments (Saunders *et al.*, in preparation). These data strongly indicate that TIMPs-2-4 and GM6001 are likely targeting MT1-MMP since ECs treated with MT1-MMP siRNA strongly recapitulate the phenotype observed following treatment with TIMPs-2-4 or GM6001. Overall, our data and the work of others indicate a critical role for MT1-MMP in EC tube assembly and network formation in 3D ECM environments.

ROLE OF MMPS IN CAPILLARY TUBE REGRESSION EVENTS

Capillary tube regression is a well known response during wound repair (Clark, 1993, 1985) (see Figure 1), hyaloid vessel regression in the developing retina (Saint-Geniez and D'Amore, 2004), and the menstrual cycle in both the ovary and uterus in humans and selectively in the ovary in a variety of other species (Marbaix *et al.*, 1995, 1996; Galant *et al.*, 2004). In humans, there is ample evidence for hormonally regulated expression of MMPs, such as MMP-1, during the menstrual cycle, which coincidently peak with vascular

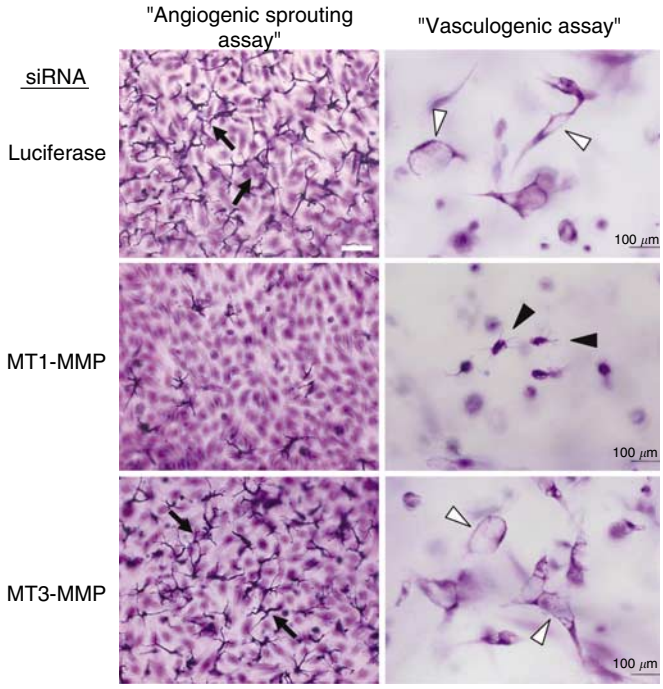

Figure 4. MT1-MMP is required for human endothelial capillary tube formation in 3D collagen matrices. ECs were transfected with MT1-MMP, MT3-MMP, or luciferase (control) siRNA duplexes using methods previously described (Saunders *et al.*, 2005) prior to placement on the surface of 3D collagen gels in the ''angiogenic sprouting assay'' (left panels) or suspended within 3D collagen gels in the ''vasculogenic assay'' (right panels). Cultures were allowed to undergo capillary tube formation for 24 hours prior to fixation, staining with 0.1% toluidine blue, and photography at original magnification $\times 10$ (left panels) or $\times 20$ (right panels). (**a**) In the angiogenic assay, ECs from a confluent monolayer (shown in background) are induced to form sprouts into the 3D collagen matrix, which over time form lumenal structures (capillary tube morphogenesis). ECs transfected with luciferase or MT3-MMP siRNA undergo normal tube formation, while MT1-MMP treated ECs are unable to undergo tube formation. Bar $= 50\,\mu$m. (**b**) In the vasculogenic assay, ECs suspended as individual cells throughout the 3D collagen gel form pinocytic vacuoles, which coalesce into lumenal structures over time. Eventually, individual lumen-containing cells unite to form multi-cellular 3D tubular networks. As in the angiogenic assay, ECs transfected with luciferase or MT3-MMP siRNA undergo normal vasculogenesis, while MT1-MMP treated ECs are unable to undergo morphogenesis; however, these cells are capable of forming sprouts (arrowheads), similar to treatment with TIMP-2,-3, or GM6001. Bar $= 100\,\mu$m.

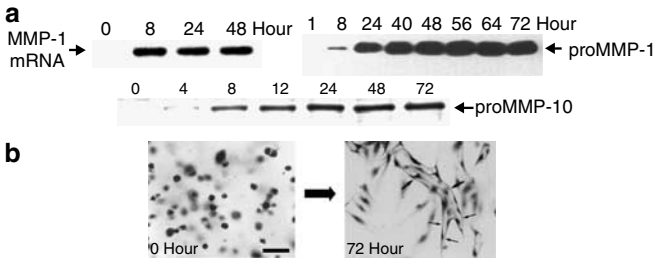

Figure 5. Human endothelial cells induce proMMP-1 and proMMP-10 in 3D collagen matrices to regulate capillary tube regression, and not capillary tube formation (morphogenesis). ECs were suspended as described in 3D collagen matrices (Davis and Camarillo, 1996) and allowed to undergo capillary tube formation (morphogenesis) in the absence of serine proteases in serum-free media. (**a**) At the indicated time points, conditioned media were collected and cultures were extracted for reverse transcriptase-PCR or Western blot analysis. (**b**) Alternatively, cultures were fixed in 3% glutaraldehyde and stained with 0.1% toluidine blue for photography, bar $= 50\,\mu$m. As demonstrated, ECs induce both proMMP-1 mRNA and protein zymogen. Additionally, ECs induce proMMP-10 as detected via Western blot. (**b**) In the absence of serine proteases, MMP-1 and MMP-10 remain as zymogens. Importantly, ECs form capillary tubular networks over 72 hours in the absence of serine proteases or activated MMP-1 and MMP-10. Arrows indicate the borders of an EC sprout and tube structure.

regression and the onset of menses (Marbaix *et al.*, 1995, 1996). These reports argue for a potential role for MMP-1 as a vascular regression factor in humans. Also, MMP-1 was demonstrated to be present in or directly adjacent to blood vessels within granulation tissue in both human and pig skin wounds, and thus was observed to be present in a location and time that is consistent with a role in vascular regression during wound repair (Stricklin *et al.*, 1993, 1994; Stricklin and Nanney, 1994).

We have developed a series of models of capillary tube regression, which mimic the regression phase of granulation tissue removal during wound repair (Figure 1). Interestingly, we have clear evidence for a role of MMP-1 (collagenase-1) and MMP-10 (stromelysin-2) as vascular regression factors during these events (Davis *et al.*, 2001; Saunders *et al.*, 2005). Furthermore, both MMP-1 and MMP-10 are expressed by human ECs derived from endometrial tissue (Krikun *et al.*, 2005), which supports our conclusions and suggests that they could participate in vascular regression events of endometrial vessels during the shedding phase of the menstrual cycle.

Both pro-MMP-1 and pro-MMP-10 are markedly induced during capillary tubular morphogenesis in our serum-free model of vascular morphogenesis (Figure 5) (Davis *et al.*, 2001; Saunders *et al.*, 2005). We have found no evidence that these MMP proenzymes are activated during morphogenesis in either the vasculogenic or angiogenic models, despite a marked induction of these two proteins. Western blots show a marked predominance of the proenzyme forms of both MMP-1 and MMP-10 (Figure 5). This result is obtained using our standard assay conditions (i.e. serum-free conditions), which were used to demonstrate a role for membrane-type metalloproteinases in EC tube morphogenesis as illustrated in earlier Figures 2–4 and our previous publications (Davis and Camarillo, 1996; Davis *et al.*, 2001; Saunders *et al.*, 2005). These data suggest that activated forms of MMP-1 and MMP-10 are not necessary for EC tube formation or invasion. Also, addition of TIMP-1 or alpha2-macroglobulin, which represent strong inhibitors of both of the activated forms of these enzymes, has no influence on tube formation (see Figures 2 and 3).

MMP-1 AND MMP-10 ARE VASCULAR REGRESSION FACTORS FOR HUMAN CAPILLARY TUBE NETWORKS

We have reported in several studies that MMP-1 and MMP-10 are vascular regression factors in our granulation tissue-like assay system (Davis *et al.*, 2001; Saunders *et al.*, 2005). When pro-MMP-1 was activated by the addition of plasminogen

(converted to plasmin by EC-derived plasminogen activators), there was a marked tube regression response coincident with degradation of the type I collagen matrix and collagen gel contraction, which is a convenient measure of the regression event (Davis *et al*., 2001; Saunders *et al*., 2005). Also, there was a time-dependent EC apoptotic response with pro-caspase 3 activation and cleavage of a known caspase substrate, gelsolin (an induced gene during tube morphogenesis) (Salazar *et al*., 1999) following the morphologic tube regression response (Davis *et al*., 2001). Interestingly, there is a known temporal overlap of wound contraction with granulation tissue regression during human skin wound repair *in vivo*, suggesting the possibility that ECs could participate directly in wound contraction along with myofibroblasts (Clark, 1993, 1985). During tube regression it is evident that ECs exert considerable mechanical force on the proteolyzed collagen matrix, which directly contributes to the collapse of EC-lined tubes (Davis *et al*., 2001; Saunders *et al*., 2005). Importantly, the addition of plasminogen or plasmin, which induces the activation of MMP-1 and MMP-10, has no influence of capillary tube formation, which proceeds normally even with activation of these soluble MMPs. Thus, our data suggest that MMP-1 and MMP-10 act as EC tube regression agents and do not appear to play a role in EC tube formation.

Similar types of EC-contractile phenomena occur during EC tube network collapse following the addition of microtubule disrupting agents (Bayless and Davis, 2004). The MMP-dependent and microtubule-depolymerizing mechanisms of tube collapse that we are investigating appear to occur through distinct but related processes. For example, it is clear that the mechanical strength of the ECM resists the normal tendency of EC tube networks to collapse due to mechanical forces generated by the ECs on the 3D ECM (Davis *et al*., 2002; Davis and Senger, 2005). When the integrity of this ECM scaffold is compromised by proteolysis through MMP-1 and MMP-10 activation, this scaffold can no longer resist these forces and the tube structures collapse (Davis *et al*., 2001; Saunders *et al*., 2005). This is likely to occur during wound repair processes whereby ECM remodeling and removal occurs coincident with regression of granulation tissue and wound contraction (Clark 1993, 1985). Importantly, in the case of MMP-dependent tube regression, we have shown that these tube collapse events occur prior to the eventual collagen gel contraction event (Saunders *et al*., 2005). In contrast, microtubule disrupting agents rapidly collapse tubes because ECs appear to lose contact with the collagen ECM in a manner which depends on the small GTPase, RhoA (Bayless and Davis, 2004). In this case, the ECM scaffold is not significantly changed but the connection of the ECs with the collagen ECM is clearly altered to direct the collapse event. Thus, alterations of the ECM scaffold or the underlying and connected EC cytoskeleton (downstream of integrins) results in similar types of morphologic tube collapse which in both cases leads to later EC apoptosis (Davis *et al*., 2001; Bayless and Davis, 2004). Both of our models result in the development of apoptosis, which is a characteristic feature of regressing tissues *in vivo* (Dimmeler and Zeiher, 2000; Zuzarte-Luis and Hurle, 2005; Baffert *et al*., 2006; Simian *et al*., 2006).

To further delineate the key features and characteristics of our MMP-1-dependent regression system, we investigated and reported that plasminogen/plasmin was only one serine protease that could activate MMP-1 and that six additional serine proteases were shown to similarly activate pro-MMP-1 to induce capillary tube regression (Saunders *et al*., 2005). The most important of these is likely plasma prekallikrein since like plasminogen, it is strongly activated by the EC cell surface (Colman, 1999; Rojkjaer and Schmaier, 1999; Schmaier, 2000). Plasma kallikrein was found to activate pro-MMP-1 and to markedly induce capillary tube regression (Figure 6a). Interestingly, recent studies indicate that plasminogen and plasma kallikrein can synergistically act to facilitate mammary gland regression following the cessation of lactation (Selvarajan *et al*., 2001). We have also found that a combination of plasminogen and plasma kallikrein induces a more rapid capillary tube regression response than either protease alone. Thus, MMPs and their activating serine proteinases are not only implicated in capillary tube regression, but also another type of tissue regression that occurs during mammary gland involution (Green and Lund, 2005).

To further characterize these systems, we recently developed a 384 well assay system that facilitates our ability to quantitate the capillary tube regression response, which is assessed using collagen gel contraction over time (Saunders *et al*., 2005). This assay system is illustrated in Figure 6b whereby a dose response of plasmin is shown to directly affect the timing of the vascular regression response. Increasing concentrations of plasmin are shown to increase the speed by which tube regression occurs. The regression response also directly correlates with proenzyme activation of both MMP-1 and MMP-10 (Figure 6b, lower panel). Like MMP-1, MMP-10 was similarly activated by all seven of the serine proteases that induced capillary tube regression (Saunders *et al*., 2005).

The MMP-1 and MMP-10-dependent capillary tube regression pathway can be inhibited by multiple TIMPs including TIMP-1 (Davis *et al*., 2001; Saunders *et al*., 2005). Thus, TIMP-1 selectively blocks capillary tube regression without blocking EC tube morphogenesis (see Figures 2 and 3), indicating that soluble MMPs such as MMP-1 and -10 are regulating regression, while membrane-bound MMPs such as MT1-MMP are required for morphogenesis. Interestingly, the tumor microenvironment has been described as a ''wound that does not heal'' (Dvorak, 1986) and many tumor cell types overexpress TIMP-1 (Wurtz *et al*., 2005). Thus, TIMP-1 can interfere with capillary tube regression without affecting tube formation, which might result in a net increase in vessel density. Many abnormal vessels in a tumor wound bed, which in a normal context would undergo regression, may not regress due to elevated levels of tumor-derived TIMP-1. Consistent with such conclusions are reports showing that TIMP-1 knockout animals show decreased vessel density during angiogenesis, while the opposite is true of TIMP-1 overexpressing transgenic mice (Yamada *et al*., 2001).

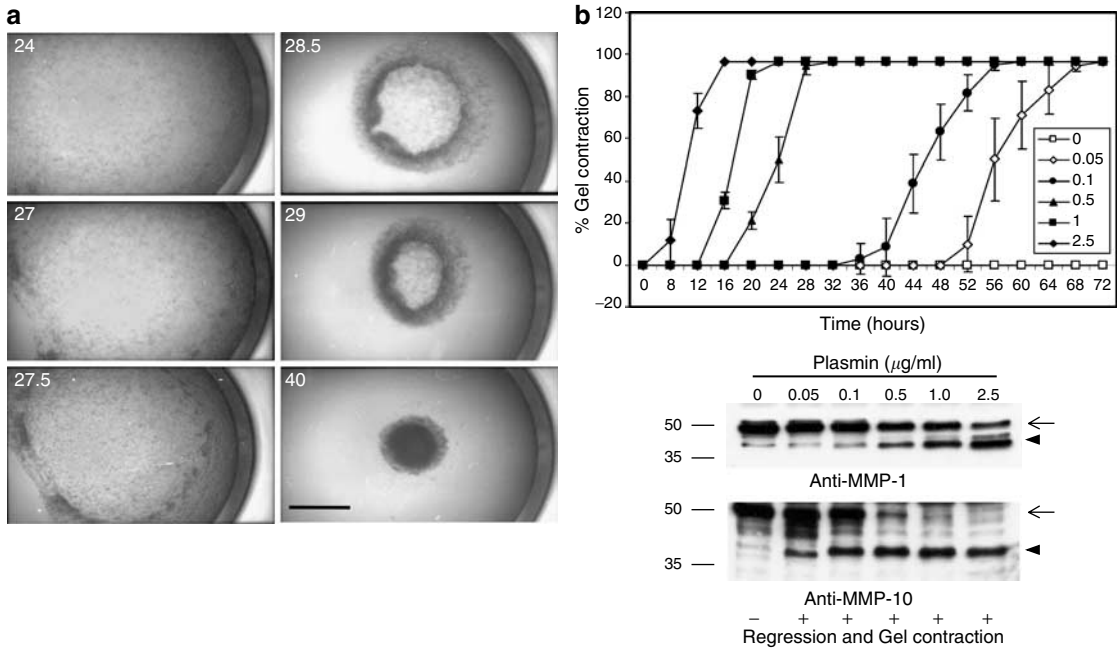

Figure 6. Serine proteinase induced activation of EC-derived MMP-1 and MMP-10 results in capillary tubular network regression and collagen gel contraction. (**a**) ECs were suspended in collagen matrices, plated in 96-well tissue culture plates (4.5 mm diameter), and cultured in the presence of plasma kallikrein at 1 μg/ml. Serial time-lapse photography of a single well was performed at the indicated time points (in hours) during the capillary tube regression and collagen gel contraction process (Saunders *et al.*, 2005). Bar = 500 μm. (**b**) ECs were suspended in a quantifiable 384 micro-well regression assay as previously described. ECs were cultured in the absence (0) or in the presence of increasing concentrations of plasmin (μg/ml). Cultures were monitored every 4 hours for capillary tube regression and gel contraction, and percentage gel contraction was recorded for each dose of plasmin at each time point. Data are reported as the percent collagen gel contraction over time ($\pm$SD). At 72 hours, conditioned media were collected and analyzed for MMP-1 and MMP-10 expression and activation via Western blot. Capillary tube regression and collagen gel contraction directly correlated with the activation of MMP-1 and MMP-10 from latent (arrows) to activated (arrowheads) forms on Western blots.

siRNA SUPPRESSION OF MMP-1 AND MMP-10 MARKEDLY BLOCKS CAPILLARY TUBE REGRESSION

To further address the function and importance of both MMP-1 and MMP-10 in the tube regression response, we performed siRNA knockdown experiments (Saunders *et al.*, 2005). These experiments demonstrate a selective ability of MMP-1 (Figure 7) and MMP-10 to strongly regulate capillary tube regression while siRNAs to MMP-2, MMP-9, and MMP-3 had no effect (Saunders *et al.*, 2005). In addition, we find no evidence for protein expression of either pro-MMP-13 or pro-MMP-8 (i.e. other known interstitial collagenases) by ECs during tube formation (Saunders and Davis, unpublished observations).

Suppression of MMP-1 and MMP-10 protein expression by siRNA treatment does not influence EC tube formation as illustrated for MMP-1 in Figure 7b and c. In contrast, a marked delay in EC tube regression resulted from the knockdown of MMP-1 protein (Figure 7a). Furthermore, we developed adenoviral vectors producing either MMP-1 or MMP-10. Increased expression of both MMP-1 and MMP-10 strongly induces capillary tube regression without having any influence on tube formation (Saunders *et al.*, 2005). The mechanism of action of MMP-10 in this context appears to be in part the facilitation of MMP-1 activation since it is known that stromelysins (e.g. MMP-3 or MMP-10) and serine proteases such as plasmin can act together to create a ''superactive'' MMP-1 molecule (Suzuki *et al.*, 1990). Western blot analysis shows that increased MMP-1 activation occurs with increased MMP-10 expression while a strong decrease in MMP-1 activation occurs with siRNA knockdown of MMP-10 (Saunders *et al.*, 2005). Overall, our data indicate that MMP-1 and MMP-10 synergize together to regulate capillary tube regression responses (Saunders *et al.*, 2005). Furthermore, MMP-1 targets collagen matrices while MMP-10 (and the related MMP-3) can target basement membrane matrices (Bejarano *et al.*, 1988), which represent the two key ECM scaffolds that are responsible for formation and maintenance of capillary tubular networks (Vernon and Sage, 1995; Davis *et al.*, 2002; Davis and Senger, 2005).

MURINE MMP-13 CAN INDUCE CAPILLARY TUBE REGRESSION

In the mouse genome, there is no homologue for MMP-1 which shows a similar expression pattern to that of human MMP-1 mRNA or protein. Many studies have focused on murine MMP-13 (Henriet *et al.*, 1992), which has interstitial collagenase activity (Wu *et al.*, 2002, 2003) and may substitute for the collagenase activity of human MMP-1. Interestingly, murine MMP-13 is induced in a temporal time frame during mouse skin wound repair in a manner consistent with a role in regression of granulation tissue (i.e. vascular

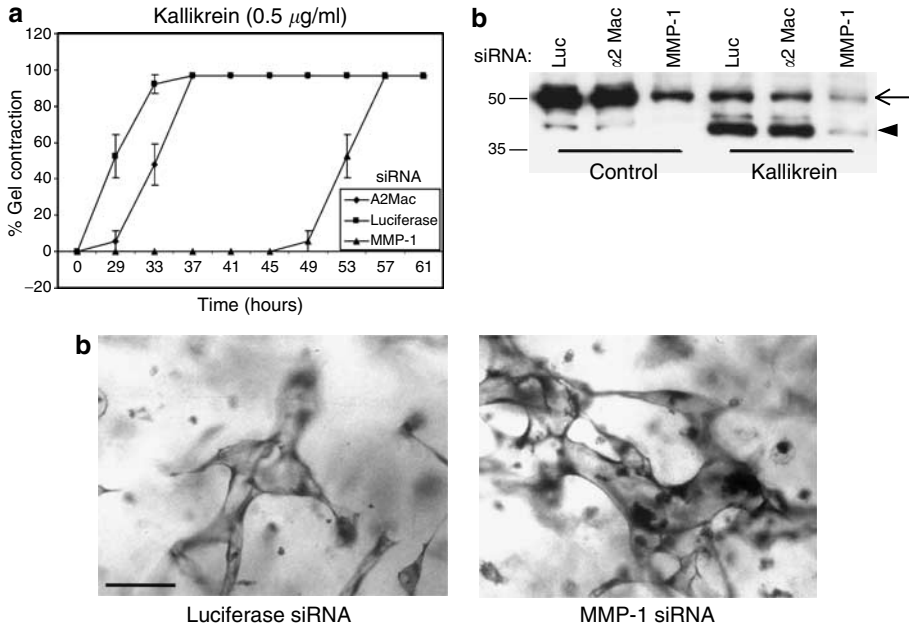

Figure 7. Treatment of ECs with an MMP-1 siRNA decreases MMP-1 protein expression and delays capillary tube regression and collagen gel contraction, but does not affect EC tube morphogenesis. ECs were transfected with MMP-1, α2macroglobulin (α2Mac), or a luciferase control (Luc) siRNA duplexes, suspended in collagen matrices, placed in the 384 micro-well regression assay, and cultured in the absence (control) or presence of 0.5 μg/ml plasma kallikrein. (**a**) Cultures were monitored for tube regression and gel contraction as described (Saunders *et al.*, 2005). Data are reported as the percent collagen gel contraction over time (±SD). MMP-1 siRNA markedly delayed the regression response, while luciferase and α2macroglobulin siRNAs regressed normally. (**b**) At 72 hours, conditioned media from control cultures (no kallikrein) and kallikrein-containing cultures were collected and analyzed for MMP-1 expression and activation. As displayed, ECs transfected with MMP-1 siRNA and cultured in the presence of kallikrein show low levels of activated MMP-1, consistent with the delayed regression response in panel A. (**c**) ECs transfected with the indicated siRNAs were suspended in 3D collagen matrices and cultured in the absence of serine proteases for 46 hours prior to fixation, staining, and photography. As illustrated, MMP-1 siRNA does not affect the ability of ECs to undergo lumenal morphogenesis and establish multicellular tubular networks. Bar = 100 μm.

regression) as well as in collagen remodeling (Wu *et al.*, 2002, 2003). Its expression pattern does not correlate with a time course involving the formation of new blood vessels in this repair response and significantly lags behind collagen type I gene synthesis (Wu *et al.*, 2003). Importantly, studies in mice, pig, and human tissues during *in vivo* wound repair (Stricklin *et al.*, 1993, 1994; Stricklin and Nanney, 1994; Wu *et al.*, 2002, 2003) are very consistent with our conclusions shown in Figure 1 that MMP-13 and MMP-1 are expressed in a temporal time course that is consistent with a role in the regression phase of granulation tissue rather than its formation. Both MMP-13 and MMP-1 are found within granulation tissue and are expressed within the wall or are present around vessels in these settings (Stricklin *et al.*, 1993, 1994; Stricklin and Nanney, 1994; Vaalamo *et al.*, 1997; Wu *et al.*, 2002, 2003). In support of such conclusions are new data from our laboratory showing that adenoviral-induced production of MMP-1 *in vivo* during vascular endothelial growth factor-induced angiogenesis in the chick chorioallantoic membrane results in vascular collapse and regression of these new vessels (Saunders *et al.*, in preparation). These previous studies, our *in vitro* models, and our new *in vivo* models are all strongly supportive of the conclusion that MMP-1, MMP-10, and MMP-13 play a role as vascular regression factors *in vivo* and *in vitro*.

To experimentally address the possibility that mouse or human MMP-13 may be capable of inducing capillary tube regression in our models, we developed recombinant adenoviral vectors that express these MMP-13 proteins as well as human MMP-1. In Figure 8a, data are presented showing that increased MMP-1 expression in ECs strongly accelerates the capillary tube regression response when serine proteases such as plasminogen are added. Plasminogen is converted by ECs through plasminogen activators to plasmin, which then directly activates pro-MMP-1 as well as other pro-MMPs such as MMP-10 and MMP-13 (Davis *et al.*, 2001; Pepper, 2001; Saunders *et al.*, 2005). The timing of this response directly correlates with the amount of plasminogen added and directly correlates with activation of pro-MMP-1 (Figure 8a). In a similar manner, murine MMP-13, when expressed in human ECs, strongly regulates capillary tube regression in a manner dependent on the concentration of plasminogen (Figure 8b). The regression response following mouse MMP-13 expression in human ECs is clearly accelerated by the presence of plasminogen (Figure 8b, left panel time course). However, expression of murine MMP-13 in human ECs is distinct from MMP-1 in that it eventually will cause a regression response even in the absence of added plasminogen (Figure 8b), suggesting that there is another mechanism for activation of pro-MMP-13 independent of

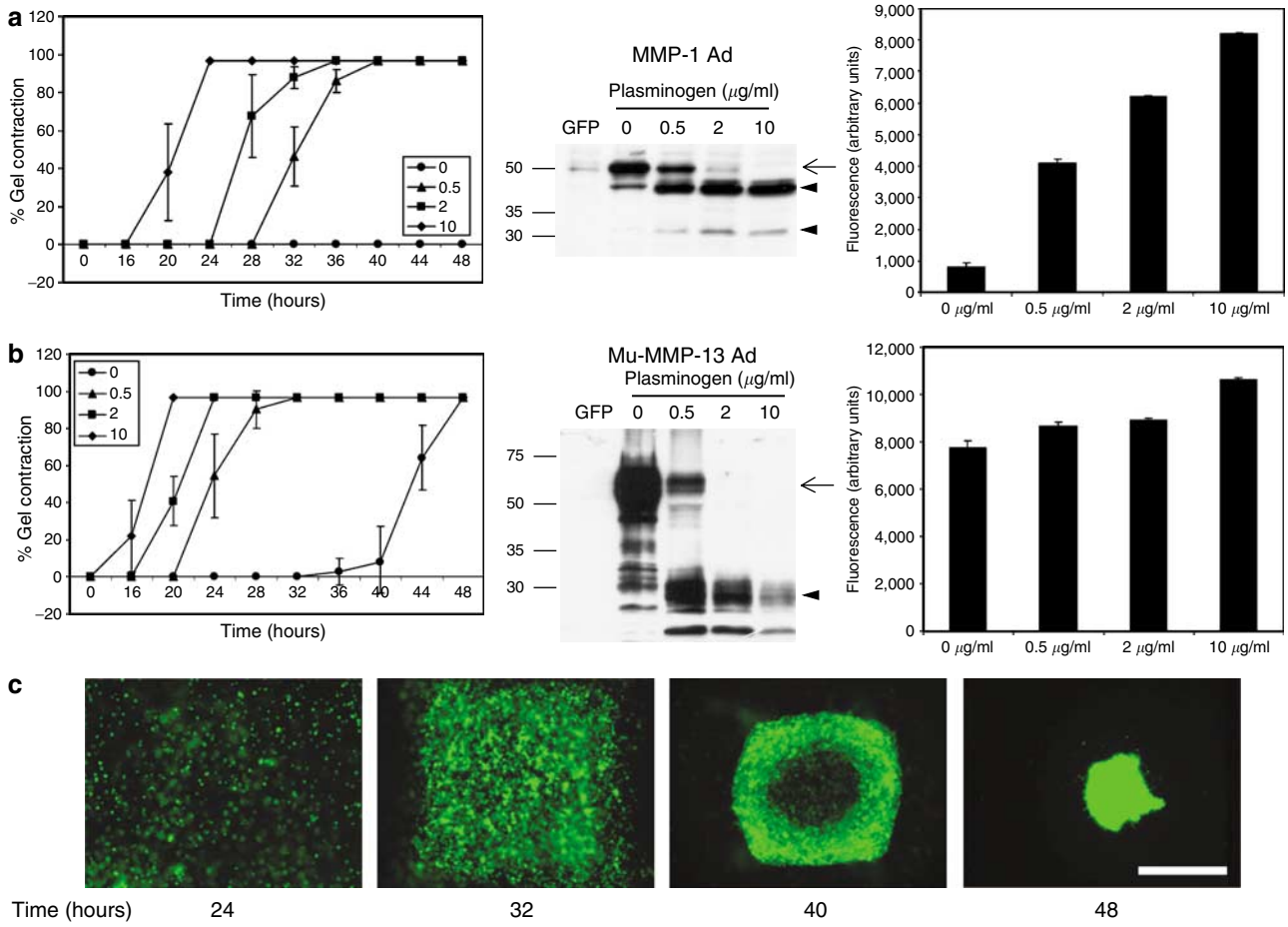

Figure 8. **Expression of human MMP-1 or murine MMP-13 in ECs via adenoviral mediated gene transfer leads to accelerated capillary tube regression and collagen gel contraction in 3D collagen matrices.** ECs were induced to express GFP, human MMP-1, or murine MMP-13 prior to suspension in the 384 micro-well regression assay as described (Saunders *et al.*, 2005). ECs were cultured in the absence or presence of varying amounts of plasminogen (μg/ml) and cultures were monitored every 4 hours for capillary tube regression and collagen gel contraction. At 48 hours, conditioned media were collected and analyzed via Western blot for MMP-1 or MMP-13 expression and activation, as well as total collagenase activity via a fluorescent DQ collagen substrate assay performed as described (Saunders *et al.*, 2005). (**a**) ECs expressing increased levels of MMP-1 underwent plasminogen-dependent regression that correlated with activation of MMP-1 on Western blot and elevated collagenase activity in conditioned media. MMP-1 did not induce regression in the absence of plasminogen. (**b**) ECs expressing murine MMP-13 underwent plasminogen dependent-regression in a manner similar to MMP-1; however, ECs expressing MMP-13 also underwent regression in the absence of plasminogen, a phenomenon unique to both murine and human MMP-13 (data not shown). (**c**) ECs suspended in the 384 micro-well regression assay (from panel A) were photographed under fluorescence at original magnification ×4 at the indicated time points to illustrate the capillary tube regression and gel contraction response which is analogous to wound contraction in the latter stages of wound healing. Bar = 1.0 mm.

added plasminogen. Also, human MMP-13 (Freije *et al.*, 1994) acts just like murine MMP-13 in our system and will induce capillary tube regression following its expression (Saunders and Davis, unpublished observations). Interestingly, MMP-13 is strongly expressed in human granulation tissue (Vaalamo *et al.*, 1997) and thus could participate in the regression response that we describe. One possibility is that MT-MMPs may regulate pro-MMP-13 activation during EC morphogenesis since they have been reported to perform such a function for MMP-13 but not MMP-1 (Murphy *et al.*, 1999). In Figure 8a and b, panels are shown illustrating the influence of EC conditioned media on the level of collagenase activity using a microplate fluorescence collagenase activity assay (Saunders *et al.*, 2005). EC conditioned media alone has minimal collagenase activity (Saunders *et al.*, 2005) and increased expression of either human MMP-1 or murine MMP-13 strongly induces collagenase activity in conditioned medium. This detectable collagenase activity directly correlates with the capillary tube regression response shown in Figure 8a and b. Our adenoviral vectors also induce the co-expression of green fluorescent protein in ECs and a time-lapse regression experiment with increased expression of MMP-1 is shown following the addition of plasminogen illustrating collagen gel contraction of a population of green fluorescent protein expressing ECs (Figure 8c). This work shows that murine MMP-13 can substitute for human MMP-1 in our regression assay system and suggests that it may be capable of inducing capillary tube regression responses during rodent wound repair events (Wu *et al.*, 2002, 2003).

PERICYTES CAN BLOCK MMP-1 AND MMP-10-DEPENDENT CAPILLARY TUBE REGRESSION RESPONSES

EC–pericyte interactions are well known to stabilize newly formed capillary tube networks during development and angiogenic responses (Armulik *et al.*, 2005; von Tell *et al.*, 2006). To address whether pericytes might influence MMP-1 and MMP-10-dependent capillary tube regression responses, we developed a co-culture model whereby bovine retinal pericytes are co-cultured with human ECs during the capillary tube regression response (Saunders *et al.*, in preparation). Under these conditions, it is clear that the addition of pericytes strongly inhibits EC MMP-1 and -10 dependent regression. In this system, pericyte addition to 30% of the total EC concentration results in complete blockade of MMP-1 and MMP-10 activation in response to plasmin or plasma kallikrein. Using siRNA technology approaches, we have identified that EC-derived TIMP-2 and pericyte-derived TIMP-3 are together responsible for the EC–pericyte co-culture inhibition of tube regression in our model (Saunders *et al.*, in preparation). Pericyte recruitment markedly blocks this EC-derived proteolytic activity by delivering TIMP-3 (which is strongly induced by EC–pericyte interactions) (Saunders *et al.*, in preparation). These new data provide additional support for the MMP-1 and MMP-10 model of tube regression and may provide mechanistic insight into how pericytes stabilize newly formed EC-lined tubes. Importantly, this same combination of inhibitors, TIMP-2 (Stetler-Stevenson and Seo, 2005) and TIMP-3 (Vranka *et al.*, 1997; Lafleur *et al.*, 2001), also strongly blocks EC tube formation (see Figures 2 and 3) through inhibition of MT1-MMP (Figure 4), which is also necessary to regulate capillary tube stabilization. Thus, our data suggest that specific EC or pericyte-derived MMPs or inhibitors regulate a proteolytic molecular balance, which either drives EC tube formation (MT1-MMP), regression (MMP-1 and MMP-10 and serine proteases), or eventual tube stabilization (delivery of TIMP-2 and TIMP-3 following pericyte recruitment) by inhibition of both the morphogenic and regression pathways (Figure 9). Key EC molecular changes that characterize the development of capillary tube stabilization include cessation of tube morphogenesis, assembly of basement membrane matrices, development of EC apical–basal polarity, suppression of EC proliferation, blockade of capillary tube regression events, and establishment of EC quiescence (Davis *et al.*, 2002; Davis and Senger, 2005). Considerable further study is necessary here to investigate how factors such as transforming growth factor (TGF)-β, angiopoietins and HB-EGF affect these stabilizing events since the levels of these factors are modulated and regulated by EC–pericyte interactions and play an important role in vascular stabilization (Hirschi *et al.*, 1998, 1999; Jain, 2003; Thurston, 2003; Bjarnegard *et al.*, 2004; Leask and Abraham, 2004; Armulik *et al.*, 2005; Davis and Senger, 2005; von Tell *et al.*, 2006). Preliminary experiments suggest that TGF-β enhances vascular stabilization in our systems by partially inhibiting MMP-1 and MMP-10-dependent capillary tube regression when ECs are cultured alone and enhancing the ability of pericytes to stabilize tubes undergoing MMP-dependent regression in EC–pericyte cocultures (Davis *et al.*, unpublished observations).

Other EC–mesenchymal cell interactions which are relevant during wound repair responses include EC interactions with fibroblasts. Recent studies have indicated that fibroblasts deliver factors to ECs, which strongly affect the ability of ECs to form tubes in a key provisional wound repair ECM such as fibrin (Nakatsu *et al.*, 2003). Since fibroblasts preceed ECs invasion into fibrin matrices in a wound environment (Greiling and Clark, 1997), it is possible that ECM modifications delivered by fibroblasts or growth factors

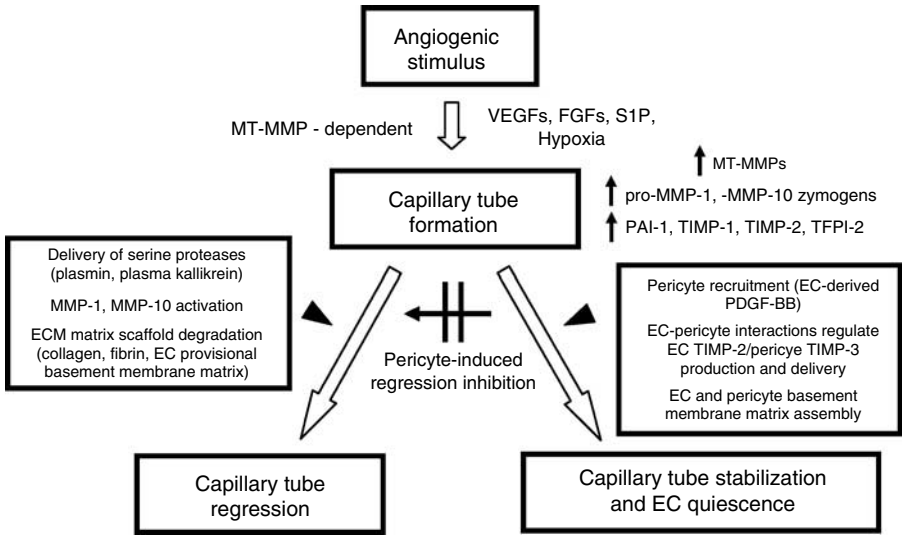

Figure 9. **Schematic diagram illustrating the role of MMPs and TIMPs in capillary tube formation versus regression in wound repair.** S1P indicates sphingosine-1-phosphate, a regulator of EC tube morphogenesis (Bayless and Davis, 2003); PAI-1 indicates plasminogen activator inhibitor-1, an important inhibitor of EC plasminogen activators (Pepper, 2001) and inhibitor of MMP-1-dependent tube regression dependent on plasminogen (Davis *et al.*, 2001); TFPI-2 indicates tissue factor pathway inhibitor-2, an induced serine protease inhibitor during tube formation (Bell *et al.*, 2001).

may be necessary to stimulate EC tube morphogenesis under these conditions (Bayless *et al.*, 2000; Lafleur *et al.*, 2002; Nakatsu *et al.*, 2003). Further work will be necessary to identify the relevant fibroblast-derived factors that are responsible for the facilitation of EC tube morphogenesis during early wound healing events (Nakatsu *et al.*, 2003).

CONCLUSION

In this review, we have discussed the critical role of specific MMPs in either capillary tube formation or regression. We have developed *in vitro* models that mimic granulation tissue formation versus regression by using human ECs suspended in 3D collagen matrices. In these models, we have shown that MT1-MMP is required for tube formation while the soluble MMPs, MMP-1, and MMP-10, are required for tube regression. siRNA suppression of MT1-MMP blocks tube formation, while siRNA suppression of MMP-1 and MMP-10 selectively blocks tube regression without affecting tube formation. Interestingly, TIMP-1 has no ability to block tube formation while it strongly inhibits tube regression consistent with its known ability to block soluble MMPs such as MMP-1 and MMP-10 while being unable to block membrane MMPs such as MT1-MMP. In contrast, TIMPs-2 and -3 can block both tube formation as well as regression in our model systems. These latter two TIMPs may have important roles in tube stabilization events and in fact EC–pericyte interactions appear to regulate the delivery or presentation of TIMP-2 and TIMP-3 to facilitate tube stabilization in our model. This occurs due to suppression of further EC tube morphogenesis by blockade of MT1-MMP and inhibition of tube regression by blockade of MMP-1 and MMP-10-dependent tube regression. Thus, MT1-MMP may be required for formation of angiogenic vessels during the initial phases of wound repair, while soluble MMPs such as MMP-1, -10, or murine MMP-13 may be induced in the wound environment for initiating regression of granulation tissue during later stages of wound healing. The recruitment of pericytes to newly formed vessels facilitates their stabilization and may prevent their regression through proteinase inhibition during this process.

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