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Matrix Metalloproteinase Control of Capillary Morphogenesis

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Abstract

Matrix metalloproteinases (MMPs) play crucial roles in a variety of normal (e.g. blood vessel formation, bone development) and pathophysiological (e.g. wound healing, cancer) processes. This is not only due to their ability to degrade the surrounding extracellular matrix (ECM), but also because MMPs function to reveal cryptic matrix binding sites, release matrix-bound growth factors inherent to these processes, and activate a variety of cell surface molecules. The process of blood vessel formation, in particular, is regulated by what is widely classified as the angiogenic switch: a mixture of both pro- and anti-angiogenic factors that function to counteract each other unless the stimuli from one side exceeds the other to disrupt the quiescent state. While it was initially thought that MMPs were strictly pro-angiogenic, new functions for this proteolytic family such as mediating vascular regression and generating matrix fragments with antiangiogenic capacities have been discovered in the last decade. These findings cast MMPs as multi-faceted pro- and anti-angiogenic effectors. The purpose of this review is to introduce the reader to the general structure and characterization of the MMP family and to discuss the temporal and spatial regulation of their gene expression and enzymatic activity in the following crucial steps associated with angiogenesis: degradation of the vascular basement membrane; proliferation and invasion of endothelial cells within the subjacent ECM, organization into immature tubules; maturation of these nascent vessels; and the pruning and regression of the vascular network.

Introduction

The convective transport of nutrients, gases, signaling molecules, and circulating blood and immune cells is achieved in normal physiology primarily by blood vessels. The molecular mechanisms by which blood vessels form have been a major topic of scientific inquiry for the past several decades, as it is widely believed that understanding these mechanisms is vital to treat pathologies characterized by misregulated capillary morphogenesis, including cancer, diabetic retinopathy, rheumatoid arthritis, and numerous ischemic conditions. In the context of cancer, much of the explosion of research in the past three decades can be traced to the pioneering hypothesis that growing tumors recruit host vasculature to fuel their growth and metastatic spread.[1] This idea has already led to the development and clinical testing of several new compounds, which have shown great promise in their ability to retard tumor growth and spread.[2] On the other hand, the absence of capillary growth is an important hallmark of several other pathologies, including various ischemic diseases.[3] An improved molecular understanding of capillary morphogenesis has contributed to the hypothesis that reperfusion of ischemic tissues can be achieved by delivering growth factors that enhance vessel ingrowth.[4, 5] This

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hypothesis has been most widely explored in the treatment of ischemic myocardium.[6] However, the success of clinical trials in this area has been mixed, perhaps due to both the mode of growth factor delivery and the fact that delivery of a single growth factor often yields an immature, overly-permeable vasculature.[7] Furthermore, clinical applications in wound healing as well as the future success of the emerging field of tissue engineering hinge critically on an improved molecular understanding of capillary morphogenesis.[8]

Fundamental to the process of capillary morphogenesis is the ability of endothelial cells (ECs) to invade through the tissue space occupied by the extracellular matrix (ECM, Figure 1). Degradation of the ECM is a critical initiating step, and it is now widely accepted that proteases, particularly the family of matrix metalloproteinases (MMPs), play a pivotal role. Besides acting to degrade the capillary basement membrane and the surrounding ECM in the initial steps of angiogenesis, it is increasingly clear that MMPs facilitate other functions necessary for capillary morphogenesis.[9] For example, specific MMPs are involved in the release of ECM-bound angiogenic growth factors, the exposure of cryptic pro-angiogenic integrin binding sites in the ECM, and the activation of other cell surface molecules.10 Conversely, MMPs also negatively regulate capillary morphogenesis through the generation of endogenous angiogenesis inhibitors by proteolytic cleavage.10 Excellent reviews on the broader topics of capillary morphogenesis/angiogenesis11 and the role of MMPs in tissue remodeling[12] have recently been published. In this review, we will focus on the known roles of the MMPs in capillary morphogenesis, particularly emphasizing the temporal regulation of MMP expression and activity during this complex morphogenetic process.

The Angiogenic Switch

Blood vessel formation is achieved by both vasculogenesis and angiogenesis.[13] Vasculogenesis refers to the process by which a nascent vasculature, called the primary capillary plexus, is assembled *de novo* from angioblasts or stem cells during embryonic development. By contrast, angiogenesis refers to the formation of new capillaries that branch from existing vasculature, a process which accounts for new blood vessel growth at later developmental stages and in fully developed adults. In the case of angiogenesis, a complex cascade of events defines the morphogenetic process, including basement membrane degradation; EC activation, migration and proliferation; organization into immature vessels; maturation and stabilization of these vessels due in part to the association of mural cells (pericytes and smooth muscle cells) and basement membrane redeposition; followed by appropriate pruning or remodeling of the vessels in response to physiologic need.[13]

Hanahan and Folkman first presented the notion that these processes are governed by a switch. 14 Perturbing the balance of proteins regulating the switch will either turn the switch “on” (in the case of greater pro-angiogenic stimuli) or “off” (in the case of greater antiangiogenic stimuli). For instance, in humans, angiogenesis ceases to occur physiologically after development except for the female reproductive cycle. Accordingly, the switch is maintained in the “off” state. However, in pathological states such as cancer and wound healing, vascular quiescence is disrupted and angiogenesis occurs. As a result, considerable effort has been made to uncover the cancer-specific cytokine which activates the switch. However, a single “gatekeeping” molecule has not been discovered. Instead, these efforts revealed a wide array of pro- and anti-angiogenic growth factors and other molecules. Amongst the most widely studied of these molecules is vascular endothelial growth factor (VEGF), a key initiator of angiogenesis. It is widely believed that hypoxia in the stroma stimulates VEGF expression via hypoxia-inducible factors (HIF-1α), stimulating ECs in nearby capillaries to initiate branching morphogenesis. The fibroblast growth factors (FGF), hepatocyte growth factor (HGF)/scatter factor, and angiopoietin-2 (Ang-2) also play vital roles in the initial phases of angiogenesis. [13] Ang-1, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and

sphingosine-1 phosphate (S1-P) are all central to stabilizing nascent vessels.13 Meanwhile, inductive cues are counterbalanced by suppressive signals derived from angiogenesis inhibitors. These include α-interferon, platelet factor-4, and thrombospondin-1, as well as cryptic protein fragments that will be discussed in detail later in this review.13 We refer the reader to two comprehensive reviews for additional details regarding the molecular regulation of angiogenesis.11, 13

It is now accepted that MMPs also play a role in the angiogenic switch, as research continues to emerge demonstrating that MMPs are essential to each step of capillary formation. However, classifying these molecules as simply pro-angiogenic is an oversimplification, and a major goal of this review will be to confer this understanding to the reader. First, however, an overview of the structure and function of the MMP family will be presented.

MMP Classification, Transcription, Activation, and Inhibition

MMPs are a family of zinc endopeptidases of the metzincin superfamily of metalloproteinases. They were named in numerical fashion, starting with the first discovered MMP, MMP-1, originally discovered when tadpole fin explants were found to degrade a surrounding collagen gel.[15] Since then, 24 more MMPs have been discovered in vertebrates, all bearing close structural similarity to their original predecessor (summarized in Table I). Generally, the MMP family shares the following domains in common with one another: a "pre" domain, a propeptide region, and a catalytic domain.[16] The "pre" signal sequence, located at the protein's N-terminus, directs the unprocessed proteinase to the endoplasmic reticulum. The propeptide domain contains one of two motifs that maintain the protease in inactive form. Proteolytic cleavage of the motif is necessary in order to activate the MMP precursor, or zymogen. The catalytic domain binds two zinc ions, one structural and one catalytic, as well as calcium ions. MMPs cleave internal peptide bonds (hence, "endopeptidases"; exopeptidases cleave terminal amino acid bonds) and generally display ECM substrate specificity in their activities (for instance, MMP-1 cleaves a variety of collagens, fibrin and vitronectin, amongst a few other ECM proteins). The catalytic domain confers specificity (in part) and is responsible for the physical proteolysis of the substrate.

Though the identity of the ECM substrate(s) and specific peptide bonds cleaved by different MMPs varies, the basic mechanism of action remains the same: displacement of water from the active site zinc leads to nucleophilic attack of the substrate's carbonyl group. A nearby pocket within the catalytic domain called the specificity pocket cradles the side chain of the substrate residue, which becomes the new N-terminus of the proteolyzed matrix protein. The size of this specificity pocket also influences substrate specificity of the various MMPs.[17]

MMPs can crudely be characterized as either secreted or membrane-bound. In either case, they are extensively regulated at both the transcriptional and post-translational levels, allowing cells within a tissue to act in concert by regulating MMP activity spatially and temporally. This section will explore MMP classification, synthesis, regulation, activation, and inhibition in greater detail.

MMP Classification

Secreted MMPs can be classified based on either substrate specificity or domain structure. Based on the former, secreted MMPs can be described as collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), matrilysins (MMP-7 and MMP-26), enamelysins (MMP-20), epilysins (MMP-28) or others (MMP-19, -21 -22, -23, -27).16, 18 In contrast, by characterizing secreted MMPs via divergences in their domain structure, six groups emerge: 1) the minimal domain MMPs, 2) the simple hemopexin domain-containing MMPs, 3) the gelatin-binding MMPs, 4) the furin-activated secreted MMPs, 5) the vitronectin-like

insert linker-less MMPs, and 6) the cysteine/proline-rich IL-1 receptor-like domain MMPs (Figure 2).[16]

Minimal domain MMPs, as implied by their name, solely contain the 3 domains common to every MMP (described above): the "pre", propeptide, and catalytic domains.[19] MMPs included in this category are MMP-7 and -26. The simple hemopexin domain-containing MMPs add a vitronectin-like (hemopexin) domain which is connected to the core structure at the catalytic domain by a hinge region. This hinge varies in length amongst the various MMPs and contributes, along with the catalytic domain, to substrate specificity. The hemopexin domain confers further substrate specificity, and is also responsible for binding the substrate as well as a family of MMP inhibitors called the tissue inhibitors of metalloproteinases (TIMPs, described in detail below).16 The collagenases, stromelysin-1 and -2, metalloelastase, enamelysin, as well as MMPs-19, -22, and -27 are all simple hemopexin-domain containing MMPs. Gelatin-binding MMPs, gelatinase-A (MMP-2) and -B (MMP-9) have a structure similar to that of their hemopexin-domain containing brethren, but contain three head-to-tail cysteine-rich repeats within their catalytic domain similar to the gelatin-binding domains of fibronectin.19 MMP-11 (stromelysin-3) and MMP-28 (epilysin) are furin-activated MMPs. These are similar in structure to the simple hemopexin domain-containing MMPs, except, rather than a cysteine switch, they contain a furin-susceptible site in their propeptide region that, when cleaved, leads to their activation. MMP-21 and -23 each differ structurally from other MMPs in a unique fashion. MMP-21 lacks a hinge region altogether (hence, "linker-less") and also contains a vitronectin-like insert in its propeptide region.16 MMP-23 lacks the hemopexin domain, instead containing a cysteine- and proline-rich sequence followed by an immunoglobin-like domain.20

Membrane-type MMPs (MT-MMPs) can be classified one of two ways: transmembrane or GPI-anchored (Figure 2). Both types contain a pre sequence, a propeptide region with a furin-susceptible site, a catalytic domain, a hinge region, and a hemopexin domain. Upon intracellular activation by the protease furin and processing to the cell membrane, the catalytic, hinge, and hemopexin regions lie extracellularly, and are anchored to the cell surface via either a transmembrane region attached to a short amino acid tail that resides within the cytoplasm (transmembrane MMPs; MMP-14/MT1-MMP, MMP-15/MT2-MMP, MMP-16/MT3-MMP, and MMP-24/MT5-MMP) or a glycophosphatidyl inositol (GPI) domain fixed within the cell membrane (GPI-anchored MMPs; MMP-17/MT4-MMP and MMP-25/MT6-MMP).[16] Membrane-anchored MMPs provide a means to spatially confine proteolytic activity to the cell surface. Mechanisms to provide temporal control are also built into the cascade of events that result in transcription of the proenzyme and inhibition of the active protease.

Transcriptional Control of MMP Synthesis

Paralleling the diversity and complexity of the MMP family itself is the multifaceted nature of their transcriptional regulation, which contributes to the tissue-specific regulation of MMP expression and thereby activity. As one might expect based on the structural similarities and overlapping substrate specificities of the MMPs, their transcriptional regulation also shares common elements, including structural features in their promoters, which may enable multiple MMPs to be co-regulated to some extent.

MMP promoters harbor several *cis*-elements, which include AP-1, PEA3, Sp-1, β-catenin/Tcf-4, and NF-κB. Exactly which elements are involved in transcriptional regulation depends on the identity of the MMP. Collectively, these elements allow for the regulation of MMP gene expression by a diverse set of *trans*-activators, including interleukins, interferons, epidermal growth factor (EGF), keratinocyte growth factor (KGF), nerve growth factor (NGF), HGF, bFGF, VEGF, PDGF, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β. The majority of MMP promoters contain a TATA box at approximately −30 bp (relative to

the transcription start site) and an activator protein-1 (AP-1) binding site at approximately −70 bp. Most promoters also contain a PEA3-binding site (PEA3 is an ETS family transcription factor) which can cooperate with the adjacent AP-1 binding site. The presence of the AP-1 and/or PEA3 binding sites in most MMP promoters renders these genes responsive to the large number of *trans*-activating growth factors or cytokines listed above. Transcriptional regulation of the MMPs induced by these factors occurs primarily through MAPK-mediated phosphorylation, but activated p38 MAP kinases can also increase the expression of AP-1 targets indirectly by increasing transcription from *c-jun* and *c-fos* promoters. Furthermore, the AP-1 and PEA3 sites provide a downstream target for ECM proteins to induce MMP expression, likely through the FAK/MAPK signaling axis.[21]

In addition to AP-1 and PEA3 binding sites, a Tcf-4 site (for β-catenin/LEF binding) in the *MMP-7, -12, -14*, and *-26* promoters and an NF-κB binding site in the *MMP-9* promoter also regulate transcription. The NF-κB pathway may also contribute to the regulation *of MMP-1, -3*, and *-11* expression,[22] despite the absence of a functional NF-κB binding site within the promoters of these genes. The Tcf-4 site mediates MMP expression in response to Wnt signaling via β-catenin.[23] For example, Wnt-2 expression increases MMP-7 expression by stabilizing β-catenin,[24] while disrupting E-cadherin mediated cell-cell junctions augments *MMP-14* expression in lung cancer cells by permitting β-catenin to accumulate in the nucleus.[25] Interestingly, β-catenin also trans-activates the *c-jun* promoter,[23] providing a mechanism for the control of those MMP genes lacking the Tcf-4 binding site in their regulatory regions.[26, 27]

Other more restrictive regulatory mechanisms exist, as in the case of the *MMP-13* promoter regulation by a complex consisting of Runx-2 (an osteogenic transcription factor) and AP-1 in osteoblasts.[28] In the case of MMP-9, there is also evidence from human astroglioma and fibrosarcoma cell lines demonstrating that interferon (IFN)-γ and IFN-β inhibit MMP-9 enzymatic activity and protein expression induced by phorbol esters and TNF-α.29 These inhibitory effects are due in part to transcriptional suppression of the *MMP-9* gene by the transcription factor STAT-1α. Whether these or similar mechanisms play a role in regulating MMP gene expression in capillary morphogenesis remains unknown.

In the context of angiogenesis specifically, consensus binding sites in the MT1-MMP gene for transcription factors include ETS-1, Sp-1, Egr-1, AP-4, NF-κB, and Nkx-2 (mammalian homolog of tinman), but not AP-1 nor AP-2. There are also no TGF-β, hypoxia, or shear stress response elements, nor a TATA box. However, ECs undergoing angiogenesis in a 3D collagen matrix have been shown to increase production of one of these transcription factors, Egr-1.[30] Egr-1 is known to up-regulate a number of targets in response to tissue injury or mechanical stress, and is an important activator for multiple endothelial cell genes transcribed during vascular remodeling, including PDGF. The authors of this particular study speculate that tractional forces developed within collagen matrices as cells attach, elongate, and migrate may provide the trigger for enhanced Egr-1 production in 3D matrices.30 This is a particularly intriguing possibility given that ECM biophysical properties, and perhaps cell-generated tractional stresses, do correlate with capillary morphogenesis *in vitro*.[31]

There are several other studies that have investigated the transcriptional regulation of MMP genes other than MT1-MMP in animal models that indirectly possess phenotypes associated with capillary morphogenesis. Lee, et al. studied the transcriptional activation of the *MMP-2* gene in a model of hindlimb ischemia using various MMP-2-lacZ reporter mice.[32] They reported that ischemia induced the expression and binding of c-Fos, c-Jun, JunB, FosB, and Fra2 to a noncanonical AP-1 site present in the *MMP-2* promoter and decreased binding of the transcriptional repressor JunD. Ischemia also activated the expression and binding of p53 to an adjacent enhancer site (RE-1) and increased expression and binding of nuclear factor of

activated T-cells (NFAT)-c2 to consensus sequences within the first intron. Likewise, Jansen, et al. probed the transcriptional activation of the *MMP-2* gene in a mouse model of skin injury.[33] They found that MMP-2 expression was upregulated following skin injury, and that deletion of an RE-1 site at −1241/+ 423 bp eliminated *MMP-2* transcription in vivo.

Recent *in vitro* data from Licht, et al. demonstrate that EC morphogenesis and tube formation require the AP-1 subunit JunB.34 Interestingly, the expression of MMP-13, typically thought to be restricted to osteoblasts, was impaired in JunB-deficient ECs in this study. The authors implicated core-binding factor β (CBFβ), which together with the Runx proteins forms the heterodimeric core-binding transcription complex CBF, as a key target of JunB and regulator of capillary morphogenesis. Consistent with a role for Jun, Zhang, et al. reported that silencing Jun reduces neovascularization in a murine model of proliferative retinopathy in part by repressing MMP-2 expression and activity.[35] On the other hand, there is also evidence that transcriptional regulation of MMP-9 expression by the NF-κB pathway, which is known to mediate the expression of many downstream target genes including VEGF, interleukin-8 (IL-8), urokinase plasminogen activator (uPA), and MMP-9, can regulate the bioavailability of VEGF liberated from the matrix by MMP-9.36

Collectively, these transcriptional regulatory mechanisms provide a means for soluble factors as well as cell-cell and cell-ECM interactions to regulate MMP expression. Since all of these types of signals are important in the context of capillary growth, it is clear that many transcriptional regulators control MMP expression in capillary morphogenesis, and that the interactions are complex and multivariable. Considering the strong structural similarities across MMP promoters, exactly what determines distinct MMP expression patterns in specific cell types or tissues remains unclear. Certainly one possibility is that the expression of certain transcription factors may be restricted to specific cell types. However, MMP gene expression may also be regulated by polymorphisms, epigenetic regulation (such as histone modifications), and post-transcriptional mechanisms (such as mRNA stabilization), all of which are discussed in greater depth in a recent review.[37]

Activation and Cell Surface Localization of MMPs

Once translated, MMPs are tagged by the “pre” region for processing by the endoplasmic reticulum before emerging from the cell surface. At this junction, the proteases remain in their precursor/zymogen form; the cysteine switch motif must be cleaved before the protease is active. This can be accomplished extracellularly by other proteases, including MMPs. However, for a subset of MMPs, activation occurs intracellularly via the endopeptidase furin, which selectively cleaves paired base residues within a protein.[38] Thus, MMPs containing a furin-recognition sequence (for instance, RRKR) in their propeptide region are susceptible to activation by the intracellular enzyme. A number of MMPs are activated in this fashion, including all membrane-bound MMPs,[39–44] MMP-11,45 MMP-23,46 and MMP-28.[47] As a result, this subset of proteases is processed to the cell surface in an active form, ostensibly placing the onus to regulate their activities at either the transcriptional level or on soluble inhibitors.

On their own, MT-MMPs are able to proteolytically process a variety of substrates, such as a variety of collagens, fibronectin, gelatin, laminin, and fibrin (Table I).[16] Additionally, MT1-MMP can be liberated from the cell surface as smaller fragments, some of which remain proteolytically active.[48] However, one key to localizing MMP activity to the pericellular compartment is the ability of MT-MMPs to either directly or indirectly activate soluble MMPs.

Five out of the six MT-MMPs (MT4-MMP being the lone exception)[49] have the demonstrated capability of activating pro-MMP-2.[39, 40, 43, 50] Out of these, MMP-2 activation by MT1-MMP is the most extensively studied, likely because the molecular complex that is formed

ironically contains a soluble inhibitor of these MMPs, TIMP-2. Active MT1-MMP is generally maintained in high amounts intracellularly near the centrosome, and is rapidly trafficked to the cell surface via microtubules,[51] where it can be secreted on its own[52] or in a complex with TIMP-2.[53] In either case, MT1-MMP clustering is necessary for the activation of MMP-2. The N-terminal domain of TIMP-2 engaged in a pro-MMP-2/TIMP-2 complex can bind unassociated MT1-MMP, at which point a neighboring, unbound MT1-MMP cleaves the cysteine switch of the TIMP-2-engaged pro-MMP-2.[52] From here, a nearby fully active MMP-2 completes the activation of its sister molecule.[54] Alternatively, pro-MMP-2 can bind a MT1-MMP/TIMP-2 complex on the cellular surface,[53] again requiring a neighboring, free MT1-MMP and an active MMP-2 to complete its activation. Conceivably, saturating amounts of TIMP-2 could occupy all of the unbound MT1-MMPs, depriving pro-MMP-2 of the active, unoccupied MT1-MMP necessary for its activation, thus providing an indirect means by which TIMP-2 can influence MMP activity. MT1-MMP has only been shown to directly activate one other MMP, MMP-13. This activation is apparently accelerated by the presence of MMP-2 and is independent of, but can be inhibited by, TIMP-2.[55]

A variety of MMP-dependent and independent means exist to activate some of the 14 remaining soluble MMPs, yet seemingly all can be traced to some form of surface localization, either indirectly by MT1-MMP (through MMP-2), or by the serine protease plasmin, which can be pericellularly activated by uPA tethered to the cell membrane via its receptor, uPAR.[56] MMP-2 activates both MMP-9 and MMP-13. MMP-13, in turn, also activates MMP-9. On the other hand, plasmin activates MMP-1, -2, -3, -7, -8, -9, -10 and -11.20 MMP-3, in turn is involved in the activation of MMP-1, -7, -8, -9 and -13.57 In fact, synergistic activation of MMP-1 by plasmin and MMP-3 is nearly ten-times more efficient than activation by plasmin alone.58 Further, MMP-10, also plasmin-activated, is capable of fully activating MMP-1, -8, and -9, and partially activating MMP-7.[59] Proteolytic activation mechanisms of the remaining MMPs (-12, -18, -20, -21, -22, -23, -26, -27) have not been sufficiently described in the literature, although other serine proteases like kallikrein and chymase play a role in activating certain MMPs. These activation mechanisms are compiled in Table I.

Aside from MT-MMPs and uPAR, other membrane receptors function to retain active MMPs at the cell surface. For instance, active MMP-2 has been shown to colocalize with integrin $\alpha_v\beta_3$ in growing blood vessels,[60] while active MMP-9 is bound by the transmembrane hyaluronan receptor CD44 in invasive lamellipodia.[61] CD44 also plays a role in recruiting MT1-MMP to the membrane in lamellipodia,[62] ostensibly promoting the activation of MMP-2 and subsequently MMP-9 to maximize the proteolytic capability of the ECs at a location where ECM degradation is necessary to facilitate migration. Collagenase-I can also be localized to the cell surface: α_2 integrin can bind pro- and active MMP-1,[63] which coincides with the ability of $\alpha_2\beta_1$ to bind collagen-I and induce the MMP-1 expression.[64]

MMP Inhibitors

TIMPs specifically inhibit MMPs and limit their activation *in situ*. While this subsection is meant merely to introduce the reader to this small family of proteins and briefly describe their anti-MMP activities, more comprehensive reviews are available.[20, 65]

Four TIMPs have been identified. All are secreted proteins with molecular weights between 20 and 29 kDa which inhibit their respective substrates in a 1:1 stoichiometric ratio,[65] with K_i values falling in the 10–100 nM range. TIMPs contain only two domains, and though both have the demonstrated ability to bind MMPs, the N-terminal region is the domain primarily responsible for MMP inhibition. TIMPs exert their function by binding the active site cleft in the catalytic domain of an MMP as an ECM substrate would.[20] Experiments conducted thus far indicate that each TIMP can inhibit the activity of all tested MMPs, the one exception being that TIMP-1 poorly inhibits MT1- and MT3-MMP.[66] In contrast, TIMPs exhibit differences

in their substrate binding affinities; one such difference is the ability of TIMP-1 to preferentially complex with pro-MMP-9 while TIMP-2 has a much higher affinity for binding pro-MMP-2.[67, 68]

Although TIMP-1, -3, and -4 expressions are induced by a multitude of cytokines, expression of TIMP-2 is largely constitutive.[65] One of the primary biological functions of TIMP-2 is the mediation of MMP-2 activation by MT1-MMP, which was earlier discussed in greater detail. Another unique aspect of TIMP-2 is that while the N-terminal region is responsible for its MMP inhibitory activities, the C-terminal region can also suppress angiogenesis by reducing EC proliferation.[69]

TIMP-3 is the most "promiscuous" of this family because, in addition to its ability to inhibit MMPs, it also acts on another class of metalloproteinases, the a disintegrin and metalloproteinase domain (ADAM) family.20 TIMP-3 possesses two other attributes that distinguish it from the rest of the TIMP family. First, TIMP-3 blocks the binding of VEGF to its receptor VEGFR-2, which is likely responsible for some of the antiangiogenic capabilities observed with the use of this inhibitor.70 Second, TIMP-3 is the only TIMP that is not freely diffusible. Instead, it is bound to sulfated glycosaminoglycans within the ECM (e.g. heparin sulfate).71 This implies a role for TIMP-3 that is distinct from the others; while freely diffusible TIMPs are likely responsible for maintaining vascular quiescence, TIMP-3 presumably functions to regulate or suppress angiogenesis once the switch has already been flipped on; that is, MMP degradation of the ECM liberates matrix-bound TIMP-3 which then acts to inhibit further MMP activation at the EC-ECM interface.

Unlike other family members, TIMP-4 expression is mostly confined to the human heart. In contrast to TIMP-2, TIMP-4 does not reduce EC proliferation, but instead restricts EC motility when exogenously added to *in vitro* assays. However, this does not translate into suppression of angiogenesis in an *in vivo* model, while addition of exogenous TIMP-2 does.[72]

In addition to the TIMPs, the GPI-anchored glycoprotein RECK has the demonstrated capacity to inhibit MMP activity. RECK inhibits the release of pro-MMP-9 from the cell surface, and also inhibits the enzymatic activity of MT1-MMP and MMP-2, thus preventing MMP-2 activation.[73, 74]

In summary, it is clear that substantial means to extensively check the production and activation of MMPs exist and involve control at the transcriptional level, via multiple *cis*- and *trans*-regulatory elements, and the post-translational level, where intracellular activation, transport to the cell membrane, and activation (as well as inhibition) in the pericellular compartment are all regulated in a spatiotemporal fashion.

The Role of MMPs in the Angiogenic Switch

Given their ability to degrade the ECM and facilitate crucial steps in the angiogenic cascade, it is tempting to classify MMPs as proangiogenic factors. However, as mentioned early, doing so is inaccurate. MMPs also dampen proangiogenic momentum, as MMP-generated cleavage products of a number of substrates have demonstrated antiangiogenic effects. Nonetheless, numerous studies have shown that known angiogenic factors induce or upregulate MMP activity. Thus, classifying MMPs, at least in part, as angiogenic *effectors* seems most appropriate.

Soluble Proangiogenic Growth Factors Promote MMP Expression and Activity in EC

The list of proangiogenic factors, cytokines that enhance EC basement membrane degradation, proliferation, motility, and differentiation, is extensive. Not surprisingly, several of the

molecules implicated also have roles in promoting EC MMP production. Two of the most potent angiogenic chemokines, VEGF and bFGF, greatly increase the amount of pro-MMP-2 and -9 contained in vesicles shed by endothelial cells,[75] thus enhancing the proteolytic capacity of ECs in their pericellular compartment. In addition, these two growth factors are also known to upregulate the ETS-1 transcription factor, which results in a marked increase in MMP-1, -3, and -9 levels.[76] VEGF on its own has also been shown to upregulate mRNA expression of MT2-MMP,[77] and partially increases the proteolytic capacity of MT1-MMP. Another potent proangiogenic factor, HGF, stimulates increased MT1-MMP mRNA expression as well as increased latent and active MMP-2.[78] Platelet-derived lipids S1-P and lysophosphatidic acid may cooperate with MT1-MMP to enhance EC motility through the activation of EGF receptors.[79, 80] Ang-2, but not Ang-1, enhances the expression of MMP-1 and -9 in the presence of VEGF.[81] Interleukins also upregulate MMP expression in endothelial cells: IL-1, IL-6 and IL-8 all enhance MMP-9 expression,[82–84] while IL-8 also upregulates MMP-2 levels.[84] Another growth factor with a known pro-angiogenic capacity, TNFα, stimulates the production of MMP-3 and -8.[84, 85] TGF-β1, which possesses confounding pro- and anti-angiogenic functions, upregulates MMP-2 and -9 production by ECs and, in concert with phorbol myristate acetate, decreases the production of TIMP-1.[86]

It is not coincidental that these pro-angiogenic factors upregulate an array of MMPs with known proteolytic activities towards the major components of basement membrane and the primary components of the interstitial (type I collagen) and provisional (fibrin) matrices. How these MMPs then function in concert to facilitate the formation of new blood vessels will be detailed below.

MMPs as angiogenic effectors

The process of angiogenesis can be divided into five distinct steps: (1) degradation of the EC-lining basement membrane, (2) proliferation and invasion of the ECs through the surrounding interstitial matrix, (3) differentiation of ECs into a tube-like structure, (4) stabilization of the vessels, which involves laying down a new basement membrane as well as pericyte incorporation into the vessel structure, and (5) vascular pruning or regression. The great majority of MMP literature explores changes in MMP expression during steps (2) and (3) jointly. As such, for the purposes of this review, these steps will be combined. Thus, we will elucidate roles for the relevant MMPs involved in the angiogenic program by characterizing their expressions according to these steps (see Figure 3 for illustration).

Basement membrane degradation—Certain ECs within the vasculature are essentially selected to initiate sprouting.[11] In contrast to their quiescent counterparts, these so-called tip cells express high levels MT1-MMP.[87] The first barrier that these cells encounter and must proteolyze is the capillary basement membrane, primarily composed of type IV collagen, laminin, heparin-sulfated proteoglycans, and entactin.[88] The MMPs able to degrade at least one of these components include MMP-2, -3, -7, -9, -10, -12 and MT1-MMP.[16] Thus, it comes as no surprise that VEGF and bFGF, the two growth factors most heavily implicated in commencing the angiogenic cascade, elicit rapid shedding of MMP-2, -9, and MT1-MMP containing vesicles at the EC surface.[75] The upregulation of this group of MMPs is associated with the increased ability of ECs to invade reconstituted basement membrane.[75, 89]

Aside from purely facilitating degradation of the basement membrane, collagen IV proteolysis by both MMP-2 and MMP-9 exposes cryptic binding sites within the triple-helical structure of the molecule which are able to further stimulate angiogenesis. MMP-2 exposes a hidden $\alpha_v\beta_3$ binding site on collagen IV that facilitates EC adhesion and migration, and appears to stimulate pathologic angiogenesis.[90] In a similar fashion, MMP-9 reveals a cryptic binding site

within the vascular basement membrane in the retina that is critical to EC migration and neovascularization independent of MMP-2 *in vivo*.[91]

Proliferation, Migration, and Capillary Formation—Upon initial breakdown of the basement membrane, which ostensibly also functions as a barrier to diffusive transport, it is quite possible that ECs induce MMP expression from interstitial cells through secretion of extracellular matrix metalloproteinase inducer (EMMPRIN).[92] The resulting production of soluble MMPs coupled with interstitial flows present within the tissue may actually create chemotactic gradients that foster EC invasion by providing a greater number of proteolytic substrates to be activated at the EC surface.93

How these MMPs function in concert to promote capillary formation despite potentially radically different microenvironments becomes an important question. In a physiologic setting, the interstitial matrix is likely to be dominated by type I collagen. However, in a pathologic setting, extravasation of the plasma protein fibrinogen and subsequent invasion of ECM-secreting fibroblasts results in a provisional matrix rich in fibrin, fibronectin, and vitronectin.[94] Yet, despite significant changes in the environment, sweeping alterations in MMP expression patterns are not observed. ECs undergoing angiogenesis in fibrin express more MMP-2 (latent and active), but less MT1-MMP and MMP-11 than they do while undergoing angiogenesis in a collagen matrix.[95] The majority of the literature linking MMPs and angiogenesis has focused on how crucial these proteinases are to facilitate EC invasion, proliferation, and capillary formation in a variety of *in vitro* and *in vivo* models. Here, we briefly summarize the roles that particular MMPs play in this step of the angiogenic cascade.

MT1-MMP: One of the first studies to explicitly link MT1-MMP and angiogenesis demonstrated that transfecting ECs with a MT1-MMP expression vector, resulting in an approximately 3-fold enhancement in protein expression, led to an increase in basement membrane invasion and EC migration.[89] Since then, it has been shown rather convincingly that the cell-bound protease is critical to the formation of capillary structures in a collagen matrix. Aortic rings from mice with inactivating mutations in MT1-MMP cultured within collagen gels were not able to initiate sprouting into the surrounding matrix, let alone undergo capillary morphogenesis, apparently due to their failure to degrade the subjacent collagen; two-dimensional migration of these mutant cells was unaffected. Interestingly, deletion mutants for plasminogen, MMP-2, integrin β_3, MMP-9, or CD44 all formed extensive vessel-like structures within the matrix after 7 days.[96] The importance of MT1-MMP in capillary formation within collagen has been substantiated in other studies using a variety of assays and inhibitors. Microvascular EC vessel formation in a collagen invasion assay proceeds independent of MMP-2 but requires MT1-MMP.[97] Similarly, siRNA inhibition of MT1-MMP blocks both EC invasion and EC organization within a type I collagen matrix.[98]

Inhibition studies have also suggested a role for MT1-MMP in vessel formation atop collagen-fibrin composites.99 However, an unexpected role for MT1-MMP facilitating vessel formation in pure fibrin, where the PA/plasmin system was thought to play the critical role, has also been uncovered. Hiraoka et al. demonstrated that a soluble MT1-MMP transmembrane deletion mutant is far more fibrinolytic than other soluble MMPs (e.g. nearly 4-fold more than MMP-3, and nearly 11-fold more than MMP-2). Further, cell-bound MT1-MMP, and not the PA/plasmin system, was required for MDCK cells to undergo a tubulogenesis-like program into fibrin hydrogels under VEGF-stimulated conditions.100 Somewhat contradictory is the finding that ECs with deleted MT1-MMP still invaded the subjacent fibrin matrix and formed tubes in aortic ring assays, suggesting that MT1-MMP may not be totally critical to tube formation within fibrin matrices. This effect may be due to redundant fibrinolytic functions of other MMPs since TIMP-2 inhibition still had an inhibitory effect on EC fibrin invasion.96 Studies with other cell types have confirmed that invasion in fibrin matrices can still occur when MT1-

MMP is absent or inhibited, though the extent of invasion is significantly increased when MT1-MMP is expressed.101 Thus, while the role of MT1-MMP in vessel formation within a collagen matrix (critical) is relatively well established, its role in fibrin is not completely understood, may involve other MMPs, and may be a supplemental, enhancing role rather than a central one.

Other MT-MMPs: In time course studies of capillary morphogenesis within a fibrin matrix, mRNA expression levels of MT1-, MT-2, and MT3-MMP are all increased as tubulogenesis proceeds.[102] Given the particularly drastic multi-fold increases of both MT2- and MT3-MMP, a role for each of these membrane-bound proteases would be expected. Indeed, studies with fibroblasts have demonstrated that in the absence of MT1-MMP, transient transfection of MT2- or MT3-MMP (but not MT4-MMP) results in similar amounts and depths of invasion.[101] Thus, it is plausible that ECs are able to engage a similar mechanism when expression levels of MT1-MMP are inhibited and possibly even partially shift the fibrinolytic burden to the PA/plasmin system in order to help digest surrounding fibrin. This supposition is reinforced by the results that inhibition of microvascular EC MT3-MMP significantly reduces, but does not eliminate, tube formation atop of a fibrin matrix.103

While MT2-MMP facilitates invasion into a collagen matrix, MT3-MMP does not.[104] Moreover, RNA interference of MT3-MMP does not affect capillary morphogenesis within collagen gels.[98] The remaining MT-MMPs, MT5-MMP and MT6-MMP, are not believed to significantly contribute to capillary formation. MT5-MMP is brain-specific,[43] while MT6-MMP is mainly expressed by peripheral blood leukocytes.[44]

Soluble MMPs: The major source of soluble MMPs *in vivo* is not believed to be ECs, but rather surrounding interstitial and inflammatory cells. Thus, although prominent roles have been established for EC MT-MMPs, particularly MT1-MMP, *in vitro*, this conclusion must be considered in the context that secondary and tertiary cell types were excluded. Furthermore, the *in vivo* ECM is never comprised by solely one type of ECM molecule, and cellular crosstalk likely results in complex temporal expression patterns of soluble factors that are poorly approximated by simply replenishing growth factor-laden culture medium every other day. For these reasons, we must ask the question: despite *in vitro* evidence to the contrary, do soluble MMPs play an essential role in the intermediate phases of capillary morphogenesis?

This question has been partially addressed by monitoring capillary formation in explant cultures containing ECs from mice with deletions of MMP-2, MMP-9, or both. Wild-type ECs displayed extensive tube formation within collagen gels when the aortic ring assay was utilized, as did the respective single- and double-deletion mutants. However, when tumor angiogenesis was studied *in vivo* utilizing these same strains of mice, vascularization of the tumor was unhindered in single deletion mutants, but was greatly reduced when the expression of both gelatinases was knocked out simultaneously.[105] This study highlights the drawback in making broad conclusions from experiments in highly controlled *in vitro* environments, and points to the need for more experiments in which the expression of multiple MMPs are knocked out or silenced simultaneously. It also suggests a more significant function for soluble MMPs than *in vitro* studies predict. The gelatinases, in particular, apparently comprise critical but divergent functions. Though their activities would appear redundant, expression of MMP-2 is not complementarily increased as a result of deleting MMP-9, suggesting distinct regulation and activities for each proteinase.[106]

More specifically, current data signifies that MMP-2 is critical to angiogenesis within pathologic microenvironments. There are several examples to support this statement, but perhaps none more convincing than the following: 1) MMP-2 deficient mice develop normally and are fertile, yet display reduced tumor- and retinopathy-induced angiogenesis, and 2)

MMP-2 localizes to integrin $\alpha_v\beta_3$, an integrin that is generally only appreciably expressed in pathologic angiogenesis.[107, 108] In addition, MMP-2 expression and activity are significantly upregulated in ECs undergoing angiogenesis in the provisional matrix generally present during pathogenesis.[95] This observation is supported by *in vivo* studies of wound healing fluid in which levels of active MMP-2 are observed to peak during the formation of granulation tissue, when fibroblasts migrate into the tissue and revascularization of the wound occurs.[109] MMP-2 is also upregulated between days 3 and 14 after femoral artery ligation in models of mouse hindlimb ischemia, the period during which the majority of revascularization takes place.[110] Further evidence to support the involvement of MMP-2 in pathologic angiogenesis is available. The use of a proteolytically non-functional MMP-2 fragment to competitively bind MT1-MMP and prevent its cell surface activation and localization reduces morphogenesis *in vitro* on Matrigel and *in vivo* within a chick chorioallantoic membrane (CAM) model, both models that are representative of pathologic morphogenesis.[111]

Aside from simply proteolyzing the surrounding matrix, a particularly striking role for MMP-2 has been demonstrated in both *in vitro* and *in vivo* models of chondrosarcoma. Specifically, the use of antisense oligonucleotides to inhibit MMP-2 expression in tumor nodules inhibited the recruitment of microvascular ECs *in vitro* (within collagen) and drastically reduced the vasculature feeding pretreated nodules *in vivo*.[112] This study hinted that MMP-2 could function as a chemoattractant, potentially docking on EC MT1-MMP to facilitate ECM proteolysis and encourage migration towards the tumor module.

Defining the extent of MMP-9 involvement in the angiogenic cascade is somewhat more complicated. On one hand, while macrophage-derived MMP-9 is prominently expressed in its latent forms during wound healing, an active fraction is not detected.[109] Further, MMP-9 does not possess type I collagenolytic activity on its own, and thus cannot proteolyze the interstitial substrate to pave a path for a growing vessel.[113] Further complicating the interpretation of this enzyme's role is its upregulation in response to high doses of a known anti-angiogenic molecule, thrombospondin-1.[114] On the other hand, MMP-9 deletion results in fewer vessel number, perfusion capacity, and branching in a mouse hindlimb ischemia model.[115] And though mice with a targeted knockout of the MMP-9 gene survive and are fertile, they display abnormal skeletal growth plate ossification due to delayed vascularization.[116] What accounts for these different responses?

One possible explanation is that MMP-9 also conducts a variety of secondary functions, aside from cleaving certain collagens and gelatins. Stromal cells such as fibroblasts are known to secrete large quantities of VEGF which is then sequestered within the ECM, yet adult neovascularization is only observed in pathologic tissues.[14] Thus, an inherent mechanism must exist that makes this VEGF available for use by needy cells. One of the known *in vivo* functions for MMP-9 is to liberate this matrix-bound VEGF. This was demonstrated in an elegant study by Bergers et al. utilizing a mouse model of pancreatic islet carcinoma. Latent and active MMP-2 and -9 were drastically increased in angiogenic islets. Treating non-angiogenic islets with active MMP-2 did not stimulate angiogenesis in explants, but MMP-9 treatment elicited tube formation and coincided with a doubling of VEGF levels secreted by the treated islet.[117] In addition to liberating sequestered VEGF from the ECM, MMP-9 also cleaves VEGF, further processing the growth factor to create a 16 kDa VEGF fragement. This VEGF isoform promotes decreased vascular density and larger diameter vessels typical of blood vessels that grow in a pathological tissue.[118] MMP-9, when bound to CD44, is also capable of activating latent TGF-β1 in order to promote tissue remodeling.[119] These varied and divergent functions of MMP-2 and -9 define a very important role for these proteinases in the preliminary steps of angiogenesis *in vivo*, despite *in vitro* evidence that MMP-2 or MMP-9 independent angiogenesis proceeds uninterrupted.

Despite its collagenolytic activity, RNA interference of MMP-1 does not inhibit tube formation within a collagen matrix. Silencing MMP-10 yields similar results. Still, the involvement of soluble MMPs in the intermediate steps of the angiogenic cascade is not confined to the gelatinases. For example, MMP-7 also liberates matrix-bound, fibroblast-derived VEGF *in vivo*,[120] while MMP-3 and -13 are responsible for liberating heparin-tethered bFGF from the ECM.[121] Additionally, MMP-3, -7, and -19 are all capable of cleaving VEGF to generate the aforementioned 16 kDa isoform.[118] Exogenous MMP-7 also increases EC proliferation and secretion of MMP-2 and -9 *in vitro*.[122] MMP-13 stimulates blood vessel invasion in the presence or absence of VEGF and bFGF within CAM models.[123] MMP-19 and MMP-26 may function in angiogenesis given their expression patterns and substrate cleavage specificities,[54, 124] though additional studies are necessary to demonstrate whether these and other soluble MMPs play causal, auxiliary, or insignificant roles.

Vessel stabilization and maturation—After invading the subjacent matrix and forming tube-like structures, stabilization of these capillaries involves reproduction of the scaffolding surrounding the tube and the incorporation of pericytes within the scaffolding. The mechanism by which mural cells are recruited to the growing vasculature canonically involves crosstalk between ECs and pericytes: PDGF-B secreted by ECs binds pericyte PDGF receptor-β and causes a cascade of reactions apparently regulated by MT1-MMP[125] that control pericyte migration and proliferation.[126] Similarly, pericyte-derived Ang-1 binds the TIE2 receptor on ECs and further mediates the interaction between these two cell types, though the exact function of this cytokine is unknown in this respect.[127]

Evidence continues to mount for an MMP-related form of EC and mesenchymal cell crosstalk that directs the capillary stalk to a more mature state. Specifically, in the absence of vascular smooth muscle cells, growing capillaries express MT1-MMP throughout their structure and very little basement membrane is present. In contrast, growing capillaries in the presence of vascular smooth muscle cells (SMC) do not express MT1-MMP *in vitro* in their stalks. Instead, stalk ECs express basement membrane proteins, while MT1-MMP expression is instead confined to the tip cells of the growing vessels. Similar expression patterns are observed *in vivo*.[87]

On the other hand, the TIE2(TEK) receptor and collagen IV are expressed throughout the stalk and absent at the tip cells. These expression patterns appear to be mediated by SMC-derived Ang-1 acting through the EC TIE2 receptor.[87] While Ang-1 provides an indirect mechanism by which perivascular cells can regulate EC MMP activity, TIMP secretion provides a direct means. Specifically, TIMP-1, in addition to inhibiting soluble MMPs, actually induces basement membrane production by ECs to facilitate vessel stabilization128 TIMP-3, secreted by perivascular cells,[98, 129] is likely then presented to ECs through heparin sulfate proteoglycans contained in the basement membrane to suppress EC MT1-MMP activity.[98] Accordingly, EC interaction with mural cells via Ang-1 or PDGF function to essentially shut-off MT1-MMP once the basement membrane has been reestablished in order to prevent further tubulogenesis and maintain vascular quiescence.

Interestingly, despite similar origins, an analogous function for bone marrow-derived cells has yet to be described in the literature. In fact, these cells seem more likely to play a role in facilitating angiogenesis in response to imposed stresses such as fibrosis and hypoxia by functioning as a repository of soluble MMPs. The presence of MMP-9 expressing cells derived from bone marrow is greatly increased in response to hypoxia in skeletal muscle,[130] while mesenchymal stem cells have been shown to drastically increase capillary network formation in dense matrices *in vitro* while concomitantly increasing MMP-2 and -9 activities along with MT1-MMP expression levels of these cultures.[31]

MMPs as antiangiogenic effectors

As noted earlier, MMPs are not solely pro-angiogenic. While it is not surprising that MMP inhibition by their soluble inhibitors, the TIMPs, impedes blood vessel formation, MMP *expression* also correlates with impaired angiogenesis. This generally occurs through one of two mechanisms: 1) MMP-induction of vascular regression by compromising ECM stability or 2) generation of proteolyzed matrix fragments with antiangiogenic properties.

MMP-induced vascular regression—The final phase of angiogenesis is the pruning of formed vasculature. Though a step in the angiogenic cascade, this phase coincides with the angiogenic switch turning "off", and as such, is characterized as anti-angiogenic. The soluble and insoluble signals that mediate this event are unknown, and determining the relevant cues is further complicated by the fact that concurrent vessel formation occurs elsewhere in the vascular bed. Nonetheless, a causal role for proteolytic breakdown of the collagen-rich ECM, thus compromising the mechanical integrity of the scaffold necessary to counter EC-generated forces during the process of tube formation, has been elucidated. Levels of active MMP-1 directly correlate to collagen matrix collapse/contraction and capillary regression due to proteolytic digestion of interstitial collagen.[131, 132] Contraction of the extracellular scaffold causes collapse of the tubular network, but is still indicative of capillary regression and not simply the destruction of ECs since 75% of the ECs remain viable.[131] In this *in vitro* model of capillary regression, MMP-1 activation was demonstrated to be induced by plasmin (as well as a variety of other serine proteases), MMP-10 and MMP-13 (both also activated by plasmin). Additionally, both MMP-10 and MMP-13 on their own are sufficient to induce regression, although neither appears to be as efficient in doing so as MMP-1.[132, 133] Further, MMP-13 is able to induce vascular regression independent of added plasmin,133 suggesting another mode of activation that may instead depend on MT1-MMP. MMP-2 and -9 may function in this regression cascade as well.131, [134] It may be that this subset of MMPs acts cooperatively due to their substrate specificities: MMP-1 and -13 proteolyze type I collagen to produce a denatured byproduct; MMP-2, -9, and -10 are highly efficient in further breaking down the denatured collagen.

MMP-mediated generation of antiangiogenic fragments—Several proteolytic degradation products with antiangiogenic capabilities derived from either ECM or non-ECM molecules have been identified. Only those generated specifically by MMPs will be discussed in detail. A more comprehensive review of this class of endogenous angiogenesis inhibitors is provided by Nyberg et al.[135]

Interestingly, several degradation products are derived from one of the primary components of the vascular basement membrane, collagen IV, and a minor component of the interstitial matrix surrounding the vasculature, collagen XVIII. Collagen IV-derived fragments include arresten, canstatin, tumstatin, and metastatin.[18] While MMP-9 is able to liberate arresten and canstatin, it is significantly more efficient in generating tumstatin. Tumstatin can also be produced by MMP-2, -3, and -13, though not as efficiently.[136] While developmental angiogenesis proceeds normally in tumstatin-null mice, pathological angiogenesis is accelerated. This is likely due to levels of tumstatin's target, the $\alpha_v\beta_3$ integrin, in these respective processes. During physiologic vessel formation, $\alpha_v\beta_3$ is not expressed at discernable levels on ECs. However, approximately 40% of blood vessels associated with tumors express the integrin.[136] Thus, deprivation of tumstatin, which binds $\alpha_v\beta_3$ in a RGD peptide-independent manner,[137] would be expected to reduce pathologic angiogenesis. Additional data support this hypothesis: proliferation of ECs expressing $\alpha_v\beta_3$ is reduced by tumstatin, while proliferation of ECs not expressing $\alpha_v\beta_3$ is not.[136]

The other collagen IV-derived fragments generated by MMP-9 cleavage inhibit EC proliferation, migration, and tube formation *in vitro*. Putatively, arresten does so by binding the $\alpha_1\beta_1$ integrin receptor for type I collagen,[138] while the means by which canstatin performs these functions has not yet been specified.[139]

Collagen XVIII-derived angiogenesis inhibitors include endostatin and endostatin-spanning fragments of various molecular weights termed neostatins. These fragments can be generated by a variety of MMPs, including MMP-3, -7, -9, -13, and MT1-MMP, but not MMP-1, -2, -8, and -12.[140, 141] Endostatin was initially shown to inhibit EC proliferation,[142] and has since been shown to effect a variety of other EC functions, including migration (through $\alpha_5\beta_1$),[143] VEGF signaling,[91] and MT1-MMP mediated activation of MMP-2 as well as the proteolytic activities of both MT1-MMP and MMP-2.[144]

Fragments of other ECM molecules including vastatin (collagen VIII),145 restin (collagen XV),146 anastellin (fibronectin),147 and endorepellin (perlecan)148 all possess antiangiogenic functions; however, definitive mechanisms for MMP generation of these fragments have not been described.

Many non-ECM-associated protein fragments with antiangiogenic activity are derived from proteins involved in the blood clotting cascade. Of these, angiostatin, a 38 kDa fragment of the plasmin precursor plasminogen, is the most famous. Angiostatin inhibits EC proliferation[149] and reduces plasmin-induced EC migration through its interaction with integrin $\alpha_v\beta_3$,[150] resulting in significantly reduced blood vessel formation within CAM and corneal assays and greatly reduced tumor neovascularization *in vivo*.[149] MMP-2, -3, -7, -9, and -12 are all capable of hydrolyzing plasminogen to generate angiostatin.[151–154] Fragments derived from fibrinogen also display antiangiogenic activities,[155] although further characterization is necessary to determine which MMPs play a role in their production.

In summary, the generation of antiangiogenic fragments through the degradation of extracellular and thrombolytic proteins provides yet another level of spatiotemporal control over MMP activity. Though these fragments generally do not act directly to inhibit MMP activity, they do inhibit processes such as EC migration and proliferation that MMPs are trying to effect, attenuating the pro-angiogenic effects of these proteases and resulting functionally in impaired vascularization.

The Link Between Cytoskeletal Tension and MMP Production in EC

The intact ECM also provides several insoluble cues to an attached cell, including substrate-specific binding motifs, which influence which integrins are activated on the cell surface; ligand-binding concentration, which affects the clustering of integrins; and mechanical rigidity, which affects cytoskeletal tension. It is now taken for granted that a cell attached to a two-dimensional substrate needs to anchor to that substrate on its leading edge in order to pull itself forward, but once in a three-dimensional environment, as present *in vivo*, different modes of migration are possible and simultaneous degradation of the matrix is necessary in most cases to facilitate movement. Are there cues transmitted by the added dimensionality of the ECM, and, if so, how do they alter MMP production in order to achieve the desired result?

Placing ECs in a 3-D microenvironment does in fact induce MMP expression. ECs cultured within collagen or fibrin gels produce more latent and active MMP-2 (and MT1-MMP for collagen) than cells cultured on these respective matrices.[102, 156] The signaling mechanism responsible for communicating this change in the microenvironment and inducing coordinate upregulation of MT1-MMP and MMP-2 reportedly involves proteins in the MAPK family.[157] Furthermore, it is also clear that ECs are able to alter MMP expression based not only on their dimensional context, but also in response to matrix identity. For example, ECs placed in

a collagen matrix differ, albeit slightly, in their expression patterns compared to ECs in a fibrin tissue.95

Given that MAPK signaling is a major factor in transducing cues from the extracellular environment to the cell, this pathway would be expected to play a major role in determining EC behavior in response to such cues as increased matrix density. Increased matrix density is demonstrated to have an adverse affect on capillary morphogenesis in a wealth of studies,[31, 158–161] possibly through a reduction in MT1-MMP.[31] Whether increased stiffness, ligand density, imposed diffusion limitations,[162] or all three are responsible for modulating a cell's MMP activity is unknown. A role for MAPK is logical given that changes in matrix stiffness and ligand concentration are transduced through integrins and affect actin polymerization, which in turn alters phosphorylation of MAPK family proteins, and perhaps ultimately MMP expression.[163, 164] At this juncture, the evidence for such an association is not concrete, but provides an impetus for greater exploration in the near future.

Conclusions and Future Perspectives

Ever since Folkman's theory that tumors need a vascular supply to grow beyond a critical size became widely accepted, researchers have gone to great lengths to uncover the factors and associated mechanisms that tilt the angiogenic switch towards an active state. As a result, increased attention has been paid of late to the MMP family, and roles have been defined for many members of this family with regards to initiating the formation of immature vasculature, stabilization and maturation of nascent vessels, and regression/pruning of the vascular network. The discoveries that the first identified membrane bound MMP, MT1-MMP, is absolutely critical to capillary morphogenesis in an interstitial matrix (collagen) *in vitro* while also playing an unexpected yet prominent role in provisional matrix components (fibrin) has taken some attention away from other members of this family. Indeed, important roles exist for multiple soluble MMPs, not only in degrading the basement membrane/ECM, but also by revealing cryptic pro-angiogenic binding sites, liberating and activating matrix-bound pro-angiogenic growth factors, enabling vascular regression by compromising the extracellular scaffolding, and generating antiangiogenic protein fragments. These various functions cast a new light on this family as multi-faceted effectors of both pro- and anti-angiogenic processes, and may help explain why broad-spectrum MMP inhibitors have generally failed to effectively treat metastatic cancers in a clinical setting.

An enhanced understanding of the mechanisms by which MMPs function is necessary to aid not only antiangiogenic therapies, but also pro-angiogenic therapies related to tissue engineering and the treatment of ischmeic diseases. Though certainly not all-encompassing, some significant areas where progress can be made are: (1) identifying additional transcription factors (and their mechanisms of action) that link the presence of various cytokines with the induction of MMP production; (2) silencing the expression of MMPs combinatorially to determine the integrative functions of the MMPs in key phases of angiogenesis either *in vivo* or in complex *in vitro* models; and (3) distinguishing how biophysical cues present in the ECM (e.g., stiffness, ligand density, topography, and interstitial transport) ultimately affect the array of MMPs expressed, their respective expression levels, and their ultimate activities in the pericellular compartment. Progress in these multi-disciplinary areas should have a broad effect given the criticality of MMPs to each phase of angiogenesis in both healthy and pathological settings.

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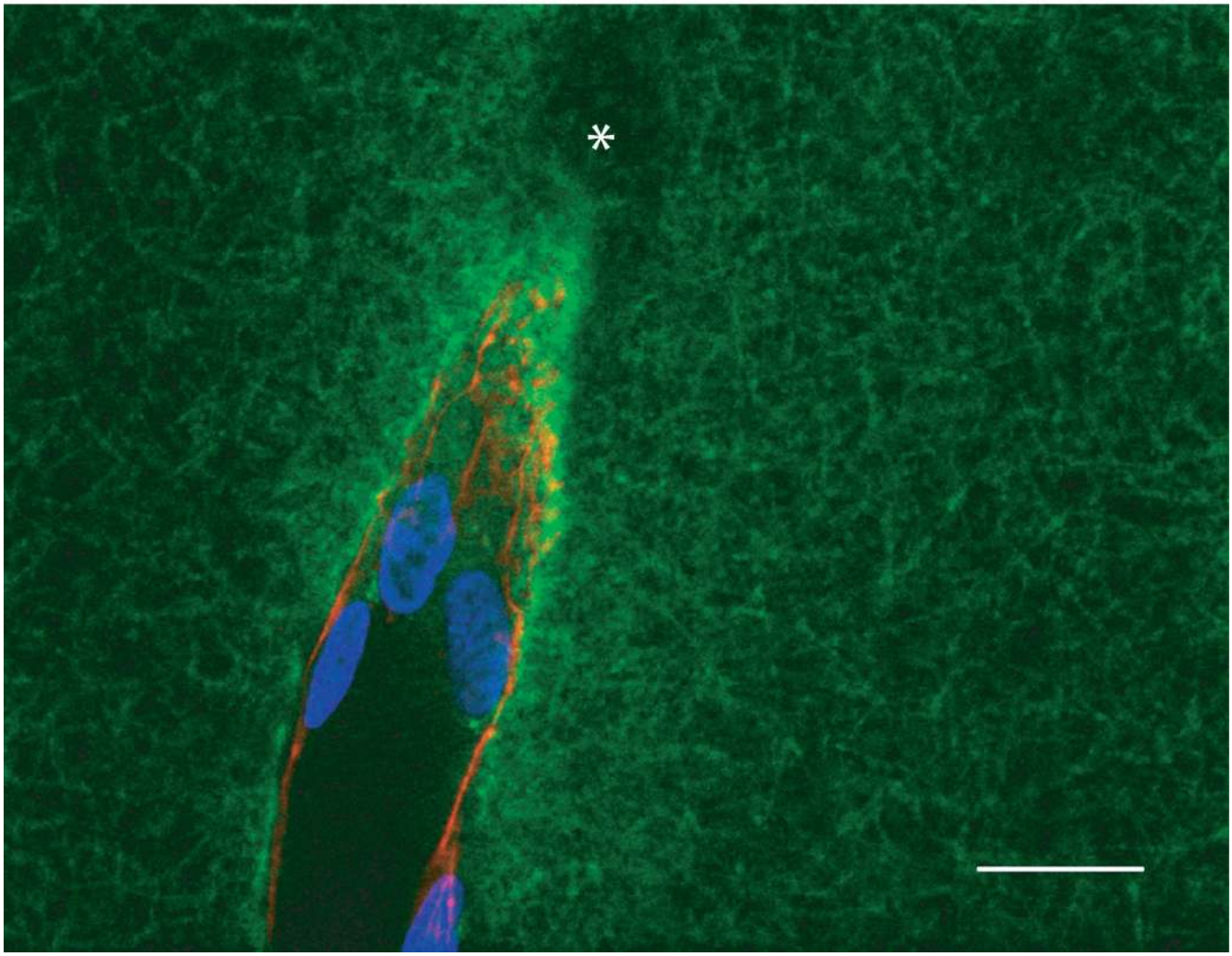

Figure 1. Endothelial cells within a growing capillary displace the ECM in order to form a hollow, tube-like structure

Shown here is a high magnification confocal image from an *in vitro* culture model of angiogenesis. Here, human umbilical vein ECs (stained with TRITC-conjugated phalloidin and counterstained with DAPI) undergo capillary morphogenesis within a 3-D fibrin matrix (labeled with Oregon Green 488-conjugated fibrinogen). The fibrin matrix is noticeably absent in the growing capillary-like structure, indicative of a hollow lumen. On the other hand, the prominent cell-ECM interface at the capillary tip (indicated by the *) underscores the need for localized proteolytic degradation of the matrix in order to permit capillary invasion into the matrix. Scale bar = 20 μm.

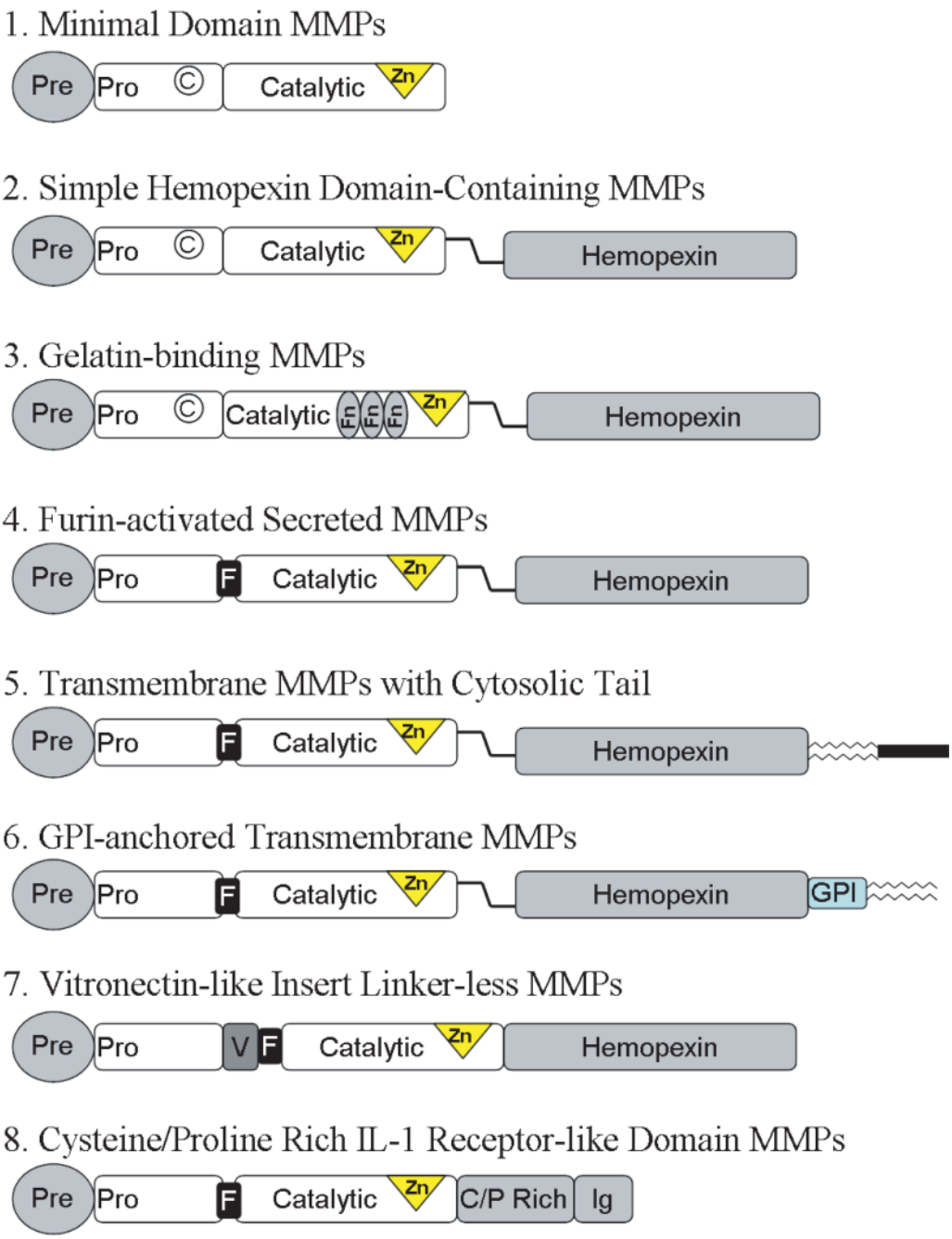

Figure 2. Schematic representation of known MMP structures

In all, 8 structural categories exist for the MMP family. These all contain a "pre" or signal sequence, a propeptide domain (pro) with either a cysteine switch (©) or a furin-susceptible site (F), and a catalytic, Zn-binding domain. Additional sequences include a hinge region (H), hemopexin domain, cytosolic tail, GPI-anchor (GPI), vitronectin-like insert (V), cysteine and proline (C/P)-rich sequences, and/or immunoglobin-like domains (Ig). Additionally, the gelatinases contain 3 fibronectin-like (Fn) inserts within their catalytic domains.

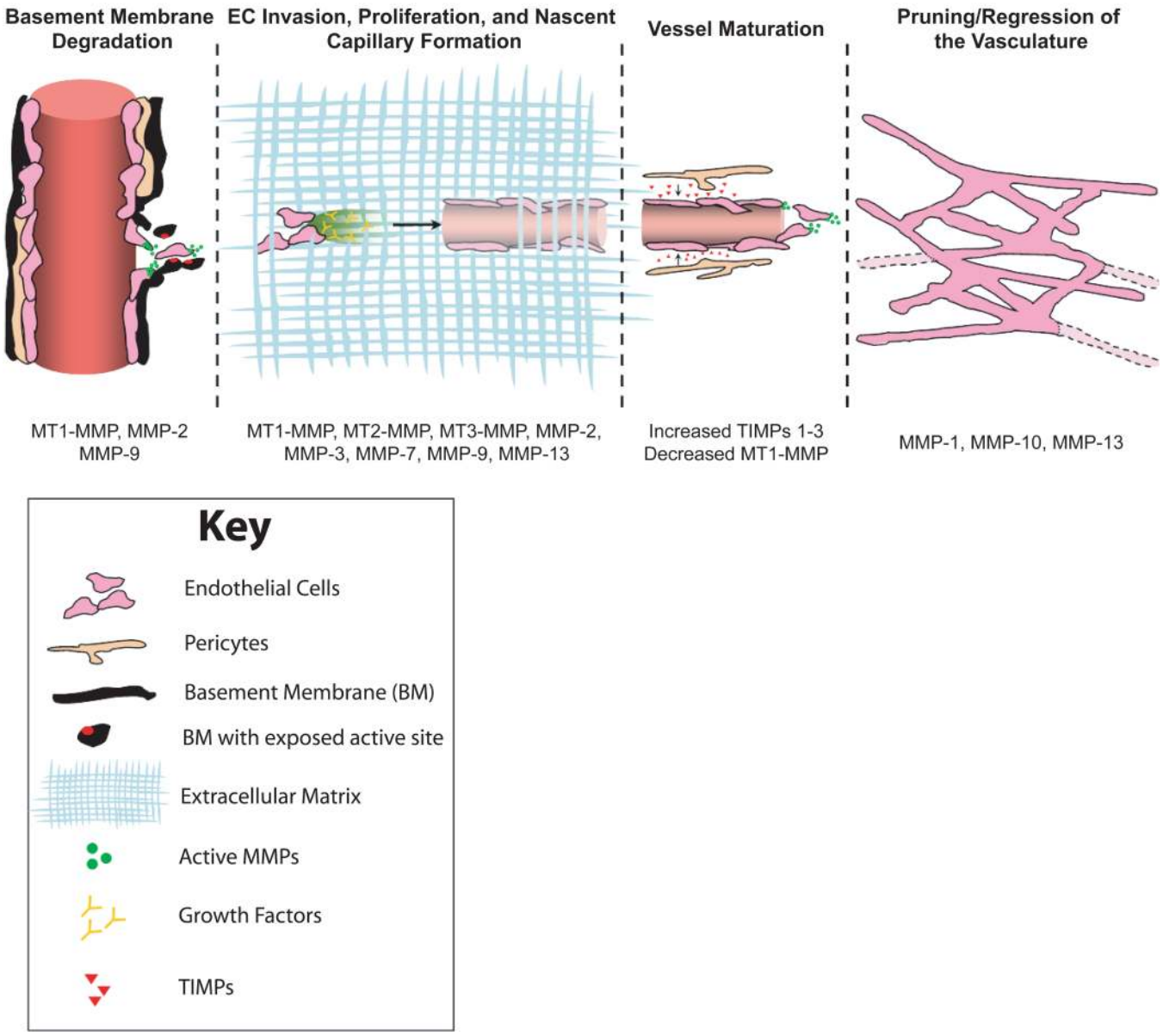

Figure 3. MMPs are critical to every step of the angiogenic cascade

MMPs serve a variety of functions that affect the process of capillary morphogenesis. MT1-MMP, MMP-2, and MMP-9 on activated endothelial tip cells facilitate basement membrane degradation. In particular, MMP-2 and -9 cleavage of basement membrane proteins produce pro-angiogenic cryptic binding sites. As these ECs invade the subjacent ECM, they proliferate and eventually reform tubular structures. MT1-MMP, MMP-2 and MMP-9 continue to be the dominant MMPs in this process; however, other MMPs (MMP-3, -7 -13, MT2-MMP, and MT3-MMP) play auxiliary roles, particularly in the release and activation of pro-angiogenic cytokines sequestered in the ECM. Crosstalk between EC and smooth muscle-like pericytes is a key factor in initiating vessel stabilization, as TIMP-1 expression induces basement membrane redeposition while EC-derived TIMP-2 and pericyte-derived TIMP-3 drive down MT1-MMP expression (and thus ostensibly the activity of other MMPs) in the stalk region. Finally, as the capillary bed matures, pruning of the vasculature occurs. Roles in vascular regression for MMP-1, -10, and -13 have been described, likely by compromising the

mechanical integrity of the underlying ECM such that it no longer supports the tractional forces necessary to maintain the tube-like structure.

Table I

MMPs organized in ascending order with common name, structure category, mode of activation, and ECM substrates for which the respective MMP has the demonstrated capacity to degrade. Adapted from references [16] and [57] with additional data from references [59, 101, 104, 132], and 166.

MMP	Common Name	Structure Category (refer to Fig. 2)	Mode of Activation	Known ECM Substrates
MMP-1	Collagenase-I	2	MMP-3, -10 plasmin, kallikrein, chymase	Collagens I, II, III, VII, VIII, X; fibrin, gelatin, proteoglycan link protein, aggrecan, ceriscan, tenacin, entactin, vitronectin
MMP-2	Gelatinase A	3	MMP-1 -7. -13 -14 -15. -16, 24, -25 plasmin	Collagens I, IV, V, VII, X, XI, XIV; fibrin, gelatin, aggrecan, proteoglycan link protein, elastin, fibronectin, vitronectin, laminin-1 & -5. galectin-3, decorin, osteonectin, hyaluronidase-treated versican
MMP-3	Stromelysin-1	2	plasmin, kallikrein, chymase, tryptase	Collagens III, IV, V, IX; fibrin, gelatin, aggrecan, fibronectin, laminin, proteoglycan link protein, versican, perlecan, vitronectin, decorin, entactin, osteonectin, large tenascin-C
MMP-7	Matrilysin-1	1	MMP-3, -10, plasmin	Collagen I, IV, X; aggrecan, gelatin, casein, fibronectin, laminin, vitronectin, entactin, elastin, decorin, transferrin, osteonectin, small and large tenascin-C
MMP-8	Collagenase-2	2	MMP-3, -10, plasmin	Collagens I, II, III, V, VII, VIII, X; gelatin aggrecan
MMP-9	Gelatinase B	3	MMP-2, -3, -10, -13, plasmin	Collagens IV, V, VII, X, XIV; fibrin, gelatin, aggrecan, fibronectin, elastin, vitronectin, galectin- 3, proteoglycan link protein, entactin, osteonectin, hyaluronidase-treated versican
MMP-10	Stromelysin-2	2	plasmin, kallikrein, chymase, tryptase	Collagens III, IV, V; gelatin, casein, aggrecan, fibronectin, laminin, proteoglycan link protein, versican, perlecan decorin, entactin, osteonectin, large tenascin-C
MMP-11	Stromelysin-3	4	Furin, plasmin	Collagen IV, gelatin, casein, fibronectin, laminin, transferrin
MMP-12	Metalloelastase	2	*not yet determined*	Collagen I, IV, gelatin, casein, fibronectin, laminin, elastin, entactin, fibrillin, vitronectin
MMP-13	Collagenase-3	2	MMP-2, MMP-14, plasmin, kallikrein, chymase, tryptase	Collagens I, II, III, IV, IX, X, XIV; gelatin, aggrecan, perlecan, fibronectin, osteonectin, large tenascin-C
MMP-14	MT1-MMP	5	Furin	Collagens I, II, III, fibrin, gelatin, casein, aggrecan, fibronectin, laminin, elastin, vitronectin, proteoglycans, entactin, large tenascin-C
MMP-15	MT2-MMP	5	Furin	Collagen I, fibrin, aggrecan, fibronectin, laminin, perlecan, entactin, large tenascin-C
MMP-16	MT3-MMP	5	Furin	Fibrin, collagen III, gelatin, casein, fibronectin
MMP-17	MT4-MMP	6	Furin	*not yet determined*
MMP-18	Collagenase-4 (*xenopus*)	2	*not yet detenmined*	*not yet determined*

MMP	Common Name	Structure Category (refer to Fig. 2)	Mode of Activation	Known ECM Substrates
MMP-19		2	Trypsin	*not yet determined*
MMP-20	Enamelysin	2	*not yet determined*	ameloqenin
MMP-21	XMMP (xenopus)	7	*not yet determined*	*not yet determined*
MMP-22	CMMP (*gallus*)	2	*not yet determined*	*not yet determined*
MMP-23		8	*not yet determined*	*not yet determined*
MMP-24	MT5-MMP	5	Furin	*not yet determined*
MMP-25	MT6-MMP	6	Furin	*not yet determined*
MMP-26	Endometase, Matrilysin-2	1	*not yet determined*	Collagen IV, gelatin, fibronectin
MMP-27		2	*not yet determined*	*not yet determined*
MMP-28	Epilysin	4	Furin	Casein