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Matrix metalloproteinase 14 modulates signal transduction and angiogenesis in the cornea

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Abstract

The cornea is transparent and avascular, and retention of these characteristics is critical to maintaining vision clarity. Under normal conditions, wound healing in response to corneal injury occurs without the formation of new blood vessels. However, neovascularization (NV) may be induced during corneal wound healing when the balance between pro-angiogenic and anti-angiogenic mediators is disrupted to favor angiogenesis. Matrix metalloproteinases (MMPs), which are key factors in extracellular matrix (ECM) remodeling and angiogenesis, contribute to the maintenance of this balance, and in pathological instances, can contribute to its disruption. Here, we elaborate on the facilitative role of MMPs, specifically MMP-14, in corneal NV. MMP-14 is a transmembrane MMP that is critically involved in ECM proteolysis, exosome transport, and cellular migration and invasion, processes that are critical for angiogenesis⁵⁶. To aid in developing efficacious therapies that promote healing without NV, it is important to understand and further investigate the complex pathways related to MMP-14 signaling, which can also involve vascular endothelial growth factor, basic fibroblast growth factor, Wnt/ β -catenin, transforming growth factor, platelet-derived growth factor, hepatocyte growth factor/chemokines, epidermal growth factor, prostaglandin E₂, thrombin, integrins, Notch, Toll-like receptors, PI3k/Akt, Src, RhoA/RhoA kinase, and extracellular signal-related kinase. The involvement and potential contribution of these signaling molecules/proteins in NV is the focus of the current review.

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Disclosures

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Keywords

Corneal neovascularization; MMP-14; VEGF-A; bFGF

I. Introduction

Neovascularization (NV) is the term used to describe the local formation of new vascular structures at previously avascular sites. Several models of neovascular processes have been proposed^{10; 14; 18; 24; 25; 43; 82; 105; 118} including: (i) vasculogenesis, which is the formation of new blood vessels from bone marrow-derived angioblasts, predominantly during embryogenesis; (ii) local recruitment of endothelial progenitor cells (EPCs); and (iii) angiogenesis, which is the formation of new vessels from pre-existing vascular structures^{31; 171}. Angiogenesis is a common feature of corneal and retinal disorders as well as cancer metastasis⁴⁶ and is usually stimulated by changes in the endothelial cell microenvironment (e.g., trauma, hypoxia, oxidative stress, mechanical strain, and genetic changes). Physical changes that occur during NV include extracellular matrix (ECM) degradation, ECM remodeling, cellular migration, and cellular invasion. The pathologies associated with NV in the normally avascular cornea include herpetic stromal keratitis, diverse inflammatory disorders, systemic/autoimmune diseases, corneal graft rejection, infectious keratitis (and other corneal infection/inflammation), contact lens-related hypoxia, alkali burns, stromal ulceration, corneal epithelium weakness, recurrent erosion syndrome, diabetes mellitus-related epithelial weakening, and limbal stem cell deficiency¹⁷². Corneal NV is also seen in some congenital disorders such as aniridia, which involves complete or partial absence of the iris. In such conditions, the balance between pro-angiogenic and anti-angiogenic factors favors NV, with both upregulation of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), and downregulation of anti-angiogenic factors, such as endostatin and thrombospondin-1^{43; 47; 177}.

The cornea is avascular under normal conditions and, even when damaged, typically heals without NV²⁴. This persistent avascularity of the cornea, which is necessary for vision clarity, may be facilitated by: (i) the tightly organized packing of collagen fibrils, (ii) the angiostatic nature of corneal epithelial cells, (iii) the immune privilege of the cornea, mediated by factors such as transforming growth factor- β (TGF- β) in tears, (iv) the comparatively hypothermic nature of the cornea, (v) extensive neuronal innervation, (vi) the movement of the aqueous humor on the endothelium, (vii) low levels of pro-angiogenic matrix metalloproteinases (MMPs), (viii) active production of anti-angiogenic factors following corneal injury, and (ix) the barrier function of the limbus^{14; 43}.

To maintain corneal clarity, proper corneal wound healing is critical. Corneal stromal wound healing occurs in four phases. In the first phase, the keratocytes adjacent to the area of the epithelial defect undergo apoptosis, leaving a central zone devoid of cells. This cell death has been suggested to initiate the healing response¹¹². In the second phase, the keratocytes immediately adjacent to the area of cell death proliferate to repopulate the wound area. For example, in rat corneas, proliferation occurs 24 to 48 hours post-wound³⁴. Within this phase, the keratocytes transform into fibroblasts that migrate into the wound area, a process

which may take up to 1 week³⁴. This transformation is evident at the molecular level, with reorganization of the actin cytoskeleton in the development of stress fibers and focal adhesion structures and activation of new genes encoding ECM components, such as fibronectin, cell adhesion molecule, $\alpha 5$ integrin, ECM-degrading MMPs, and cytokines^{14–16}. The same transition can be seen *in vitro*. When keratocytes are isolated from the corneal stroma and subcultured in serum-containing medium, they acquire the fibroblast phenotype¹⁰⁵. The migratory repair fibroblasts contain filamentous-actin and are elongated, spindle shaped, and highly reflective. These fibroblasts induce the synthesis of the $\alpha 5$ integrin chain, which results in the formation of the $\alpha 5\beta 1$ integrin heterodimer, the classic fibronectin receptor. This occurs concomitant with a reduction in fibronectin content in the wound area. In addition to forming the ECM, these repair fibroblasts synthesize several MMPs, including MMP-1, -2, -3, -9, and -14¹⁰⁵.

In the third phase of stromal wound healing, transformation of fibroblasts into myofibroblasts may occur and can be observed *via* α -smooth muscle actin staining. Myofibroblasts appear as stellate cells and are highly reflective, but are limited to within the wound area. The extent of fibroblast transformation into myofibroblasts seems to be dependent on the type of wound and the integrity of the Bowman's membrane. In general, gaping wounds and wounds in which the Bowman's membrane is removed result in greater myofibroblast generation than wounds that do not penetrate the Bowman's membrane. This process, which may take up to 1 month to become histologically apparent, can lead to a decrease in corneal clarity and vision deterioration.

The fourth and final phase of stromal wound healing involves stromal remodeling and is largely dependent on the characteristics of the original wound. Within this setting, intricate, but incompletely understood, relationships among keratocytes, fibroblasts, and myofibroblasts play key roles⁴⁵. It is theorized that TGF- β and fibroblast-like synoviocytes (FLS) are key intermediaries in the process of remodeling³⁴. Wounds that have completely healed contain few, if any, myofibroblasts, presumably because these cells revert to the fibroblast phenotype or undergo apoptosis during wound healing⁷⁰. The entire process of corneal healing after an injury may take more than 1 year³⁴.

In the setting of certain inflammatory, infectious, degenerative, and traumatic states, corneal NV may be induced during wound healing^{24; 184}. When NV does occur, blood vessel invasion into the cornea is associated with significant visual impairment, which can ultimately progress to blindness. Three distinct morphologies of corneal NV are most commonly diagnosed: (i) deep NV overlying Descemet's membrane (Fig. 1A), (ii) stromal NV observed in interstitial keratitis (Fig. 1B), and (iii) superficial vascular pannus (Fig. 1C).

NV occurs when there is a disturbance in the balance between pro- and anti-angiogenic factors. Pro-angiogenic factors include VEGF, bFGF (also referred to as FGF-2), and platelet-derived growth factor (PDGF). Anti-angiogenic factors include angiostatin, endostatin, pigment epithelium-derived factor (PEDF), thrombospondin-1, and soluble VEGF receptor 1 (sVEGFR1). A striking indication of this delicate balancing act is that many of the anti-angiogenic factors are proteolytic degradation products derived from ECM fragments formed during the initial invasion of cells into the ECM during angiogenesis^{7; 14}.

MMPs have been implicated as both pro- and anti-angiogenic molecules that are, in part, responsible for orchestrating the delicate balance between corneal angiogenesis and avascularity^{14; 64}. In this review, we present evidence for the facilitative role of MMPs, specifically MMP-14, in corneal NV.

II. General Features of MMPs

A. MMP Members

Among the 25 MMPs identified to date, at least 16 have been found in the cornea, including collagenases (MMP-1, -8, and -13), gelatinases A and B (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), matrilysin (MMP-7), macrophage metalloelastase (MMP-12), and the membrane type MMPs (MT-MMPs; MMP-14, -15, -16, -17, -24, -25)^{6; 9; 19; 24; 38; 39; 41; 66; 82; 99; 100; 115; 131; 135; 161; 169}. MMPs display dual functions during angiogenesis based on their ability to: (i) degrade ECM, which allows tissue invasion by MMP-bearing endothelial cells, and (ii) generate and/or release anti-angiogenic fragments from precursors (e.g., endostatin proteolytically produced from type XVIII collagen), which otherwise lack anti-angiogenic properties^{26; 90; 96–98; 126}. MMPs thus catalyze the normal turnover of ECM molecules, such as collagens, proteoglycans, and fibronectin. Furthermore, they modify the activity of signaling molecules, cleave molecules to produce smaller entities that maintain independent functions, create space for cells to migrate, and regulate ECM components with which cells interact via receptors.

B. MMP Expression

MMPs are expressed in the normal corneas of mice, rats, rabbits, and humans^{21; 51}. Experimentally identified MMP expression patterns in injury and disease vary widely due to the use of different experimental models and also due to the collections of samples at different times after corneal injury. Multiple studies have shown that MMP-1, -3, -7, and -12 are upregulated in corneal epithelial cells in rats during Wnt 7a-induced wound healing, whereas MMP-1, -2, -3, and -9 are upregulated in rabbit corneas and MMP-2 and -9 are upregulated in the rat cornea following excimer laser keratectomy^{23; 82; 95; 180; 181}.

Gordon et al. carried out a comprehensive expression analysis of corneal MMP expression in mice following corneal abrasion injury⁵¹. They reported that mRNA levels of MMP-1, -9, -10, -12, and -13 are significantly upregulated in the corneal epithelium at 18 hours post-wounding, with MMP-1 and -13 mRNA expression remaining elevated at 24 hours post-wounding. Furthermore, MMP-2, -3, -7, and -14 mRNA levels are elevated in both normal and wounded samples to a similar extent, whereas MMP-16, -17, -24, and -25 mRNAs were not detected in corneas. Together, these findings suggest a highly complex pattern of expression that may differ from one animal model to another or even between patients in the clinical setting. Although MMPs are upregulated during corneal angiogenesis, the exact roles they play in the regulation of angiogenesis remain unclear due to their dual pro- and anti-angiogenic functions depending on their unique microenvironment. However, it is known that quiescent endothelial cells produce little to no active MMPs, whereas MMPs are strongly upregulated in capillary sprouts during wound healing, inflammation, and tumor growth.

C. MMP Regulation

MMPs are secreted as zymogen proteinases (pro-MMPs) that are in a dormant state established by the interaction between a cysteine residue in the pro-domain with a zinc ion in the active site, which effectively blocks the active site. They are activated *via* the “cysteine switch” mechanism¹⁷⁶, through which the cysteine-zinc bridge is broken by a chemical disruption such as amino phenyl mercuric acid or through proteolytic cleavage of the pro-domain. This activation of an MMP leads to a proteolytic cascade that results in the activation of several different types of MMPs²². Most MMPs also contain a hemopexin domain, characterized by a four-bladed β -propeller structure that allows for proper protein–protein interactions, enzyme activation, and substrate recognition, among other functions.

MMPs are regulated at the levels of gene expression, compartmentalization, pro-enzyme activation, external molecule manipulation, and enzyme inactivation. They can be further regulated post-transcriptionally through signaling molecules that alter mRNA stability and production levels⁸⁹. Tissue inhibitors of metalloproteinases (TIMPs) play a pivotal role in modulating MMP activity. These molecules contain N-terminal cysteine residues that insert into the MMP-binding site in a way that blocks the catalytic zinc ion²¹. TIMPs therefore inhibit MMPs at a 1:1 inhibitor to enzyme ratio. Notably, TIMPs play a greater role in angiogenesis than simple inhibition of MMPs. It has been shown that TIMP-3 binds to VEGFR-2 and competes for binding with VEGF, a potent pro-angiogenic molecule¹³⁰.

D. Membrane-type MMPs

MMPs that have a transmembrane domain are known as membrane-type MMPs (MT-MMPs). The transmembrane domain is a hydrophobic region (~11 amino acids) between the pro-peptide and catalytic domains¹⁴¹. Members of the transmembrane MMP family include MMP-14, -15, -16, -17, -24, and -25^{124; 129; 141; 152; 168}. Like other metalloproteinases (Fig. 2A), these transmembrane MMPs have several conserved domains: a signal peptide, a pro-peptide, a catalytic domain, a hinge region, and a hemopexin-like (PEX) domain. In addition, MMP-14 has a short cytoplasmic tail through which it interacts with molecules intracellularly (Fig. 2B)^{122; 141; 168; 182}, and the PEX domain interacts with molecules on the cell surface, leading to activation and subsequent signaling events within the cell. The PEX domain of MMP-14 interacts with CD44 *via* heterodimerization, leading to cytoskeletal rearrangements and processing of cellular ‘machinery’ that is necessary for inducing cellular migration and invasion^{71; 182}.

III. MMP-14 Processing and Regulation

The precise nature of MMP-14 processing, shedding, and endocytosis conveys unique regulatory qualities with inherent complexity. MMP-14 is produced as an inactive zymogen and then cleaved into a 57-kDa peptide that inserts into the plasma membrane. MMP-14 can undergo autocatalysis to generate a 44-kDa peptide that is an inactive degradation product. Osenkowski et al. demonstrated that the hinge region of MMP-14 is the “hot spot” for autocatalytic activity, which produces the 44-kDa species, a peptide specifically lacking the catalytic domain present in the full 57-kDa molecule¹¹⁹. Synthetic MMP-14 inhibitors and TIMPs have been shown to prevent the autocatalytic process. Thus, the inhibition of

MMP-14 activity slows its autocatalytic processing, causing accumulation of MMP-14 on the cell surface^{59; 60; 119}. When TIMPs inhibit MMP-14, they further hinder the molecule's ability to autocatalyze its components, and therefore, the ratio of TIMP to MMP-14 dictates the amount of net active enzyme present. Because MMP-14 plays an important role in angiogenesis and tumor cell invasion, a clear understanding of the consequences of its will prove to be of high value to the fields of both ophthalmology and oncology^{11955; 17717376}.

An inhibitor of both MMP-14 and MMP-2 is RECK (reversion-inducing, cysteine-rich protein with Kazal motifs). RECK is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein located at the cellular surface previously shown to regulate cell motility and development of circulatory system¹¹⁶. RECK deficiency results in disrupted vasculature and premature death of mouse embryos, presumably because upregulated MMP activity allows cells to permeate the ECM more easily¹⁰⁷. This cell migratory behavior is reflected in studies of human cancers, which are more aggressive following RECK inactivation³⁰. In addition, RECK has been shown to inhibit MMP-14-induced migration of hematopoietic stem and progenitor cells, suggesting that this molecule is crucial for proper cell migration and differentiation⁴⁹.

Transmembrane proteins often have the ability to undergo proteolytic release of their extracellular portion *via* sheddases. This proteolytic activity by sheddases, which are membrane-bound enzymes that cleave extracellular portions of transmembrane proteins, releases soluble ectodomains into the ECM that can participate in various biological functions. Examples include TGF- α and Notch. MMP-14 has been demonstrated to be a sheddase, and the autocatalytic shedding of MMP-14 results in the release of an 18-kDa enzymatically inactive fragment^{84; 85; 119}. MMP-14 can also shed an ectodomain in a non-autocatalytic process, generating a 50-kDa peptide that possesses enzymatic activity. However, previous studies have demonstrated that a secreted recombinant transmembrane domain/cytoplasmic tail-deleted MMP-14, similar to the enzymatically active 50-kDa fragment, fails to give cells the ability to invade a collagen matrix^{61; 119}. However, naturally shed MMP-14 has a unique advantage over secreted recombinant forms in that it is shed where MMP-14 clustering and proteolysis occurs, thus achieving targeted delivery of the 50-kDa molecule to the migration front^{86; 119}.

Endocytosis is a common theme in the regulation of transmembrane proteins. Endocytosis leads to either lysosomal degradation of the protein or its internalization and reinsertion in the plasma membrane^{33; 119}. It has been demonstrated that MMP-14 fails to undergo internalization without its cytoplasmic tail component, based on experiments showing that cytoplasmic tail deletion mutations lead to an increase in pro-MMP-2 activation and ectodomain shedding¹¹⁹. After internalization, the protein is either degraded or recycled back into the plasma membrane. It has been hypothesized that this relocation serves the purpose of reinserting MMP-14 into specific areas of the cell membrane to contribute to targeted proteolysis on the invasive front of the cell^{119; 132}.

IV. MMP-14 Mechanisms of Action

MMP-14 is the most prevalent MMP involved in angiogenesis and ECM remodeling¹²¹⁷⁶. It leads to the disruption of endothelial tight junctions, reorganization of the actin cytoskeleton, and proteolysis of the basement membrane and interstitial matrix. MMP-14 exerts its effects *via* four key mechanisms: 1) cleavage of ECM molecules, such as type I collagen^{73; 157}, 2) upregulation of angiogenic factors, such as VEGF¹⁵⁷, 3) interactions with the cellular adhesion molecules expressed on the cell surface, such as CD44^{108; 157}, and 4) degradation of anti-angiogenic factors, such as decorin in the cornea¹⁰⁵. Cleavage of type I collagen not only degrades the ECM, but also stimulates migration, organization, and guidance of endothelial cells to sprout new vessels¹⁵⁷. MMP-14 is also involved in the processing other important ECM substances such as gelatin, fibronectin, tenascin, aggrecan, and nidogen²³.

In addition to exerting direct effects in angiogenesis and ECM remodeling, MMP-14 also activates a number of downstream MMPs, such as MMP-2, which degrades type IV collagen and shares an expression pattern similar to that of MMP-14³. The activation of MMP-2 by cleavage from the inactive zymogen, pro-MMP-2, has been directly linked to the formation of a ternary complex at the cell surface. MMP-14 has been shown to homodimerize and bind to TIMP-2 at the cell surface. This complex acts as a receptor for pro-MMP-2, which is then cleaved by another free MMP-14, to form active MMP-2³. Overall, the formation of this complex leads to the activation of MMPs and collagen degradation^{3; 32}. In fact, MMP-14 is generally considered to be the most prominent factor in pericellular proteolytic activity and migration, likely because MMP-14 has a broad spectrum of proteolytic activities towards a variety of ECM components (Fig. 3)¹⁷⁵.

MMP-2 has been shown to interact with MMP-14 to degrade ECM molecules. Researchers have investigated the effect of inactivating mutations on different MMPs. Although an individual inactivating mutation in either MMP-2 or MMP-14 is nonlethal for the first few weeks of life, inactivating mutations in both MMP-2 and MMP-14 are lethal in mice immediately after birth due to abnormal blood vessel formation and immature muscle fibers, which contribute to subsequent respiratory failure. Notably, MMP-14 and MMP-2 were found to be necessary for the formation of blood vessels with wide lumens, but are not required for vessel extension and sprouting. These findings indicate functional overlap between MMP-2 and MMP-14. Although TIMP-2 may complex with MMP-14 to facilitate the activation of MMP-2, TIMPs also are effective inhibitors of MMPs as mentioned above. This is demonstrated by the decrease in pro-MMP-2 and pro-MMP-13 activation in the presence of either TIMP-2 or TIMP-3 in a concentration-dependent manner. Although TIMP-2 is an MMP-2 inhibitor, its presence is required for MMP-2 activation, as previously explained, through the formation of the ternary membrane complex (Fig. 4)^{32; 94}. Furthermore, MMP-14 recruits TIMP-2 to the cell surface, and TIMP-2 is endocytosed for lysosomal degradation in an MMP-14-dependent process¹⁴⁸. This presents a paradox in the effects of TIMPs on the activity of MMPs^{32; 94}, which may at least be partly explained by the differences in the affinity of the TIMP N- (inhibitory) and C- (stimulatory) terminal domains to the PEX domain of MMPs and MT-MMPs^{133; 163}. Additionally, it has been shown that the interactions between MMP-14, MMP-2, and TIMP-2 may be regulated at the

transcriptional level through certain epigenetic mechanisms, such as DNA hypermethylation and histone modification ²⁹.

V. Pathways of MMP-14 Signaling

MMP-14 expression is regulated through a multitude of signaling pathways, and its expression leads to the production of multiple factors that support or inhibit angiogenesis and ECM remodeling. Furthermore, MMP-14 expression is associated with various disease processes, such as NV and cancer metastasis, through its ability to degrade ECM components and participate in multiple signaling pathways. Such interactions illustrate the complexity of MMP-14 signaling and demonstrate the delicate balance between pro- and anti-angiogenic factors.

A. VEGF Pathway

VEGF-A was initially identified as a stimulator of vascular permeability (then called vascular permeability factor or VPF) ⁹³ and was subsequently shown to be an endothelial cell-specific mitogen and angiogenic factor ⁷⁴. It is upregulated in inflamed and vascularized corneas in both humans and animal models ¹²⁵. VEGF-A expression has been correlated with embryonic, physiological, and pathological blood vessel growth *in vivo* ^{20; 43; 44}. In several systems, the spatial and temporal expression patterns of VEGF and its tyrosine kinase receptors, VEGFR-1 (or *flt-1*) and VEGFR-2 (or *flk-1/KDR*), suggest that VEGF-A is a key mediator of vasculogenic and angiogenic events associated with a wide range of biological events such as tissue repair and tumorigenesis ^{7; 102; 113}. Local and systemic signals, including cyclic AMP, steroid hormones, protein kinase C agonists, polypeptide growth factors, oxygen, free radicals, glucose, cobalt, and iron, are responsible for orchestrating the growth and regression of new blood vessels and for regulating VEGF gene expression. The potential mechanisms by which these agents modulate gene expression vary and include transcriptional regulation through the activator proteins 1 and 2, p53, and nuclear factor (NF)- κ B ^{123; 149}. The expression of VEGF-A and its receptors (VEGFR-1 and VEGFR-2) in the cornea is implicated in vascular endothelial cell proliferation and NV. Moreover, VEGF-A expression is tightly controlled, in part because elevated VEGF-A levels contribute to corneal NV and tumorigenesis ^{106; 162; 178}.

Cleavage of VEGFR-1 has been shown to occur during corneal NV. Our research has demonstrated that MMP-14 binds and cleaves VEGFR-1 but not VEGFR-2 or -3, to produce 59.8-kDa (N-terminal fragment, Ig domain 1–5), 35-kDa (C-terminal fragments containing, IgG and His-tag), and 20-kDa (Ig domain 6–7) fragments. The 59.8-kDa fragment can bind to VEGF and inhibit VEGF-induced endothelial cell mitogenesis ⁵⁴. VEGFR-1 cleavage by MMP-14 may decrease angiogenic potential by increasing VEGF-trap, thereby reducing free VEGF in the extracellular space (Fig. 5). This is consistent

Additional VEGF members include VEGF-B, VEGF-C, and VEGF-D, and these factors differentially bind to the various VEGF receptors and regulate angiogenesis and lymphangiogenesis ^{4; 7; 53; 87; 117}. VEGF-B binds to VEGFR-1, and alone, it is an inefficient endothelial cell mitogen. VEGF-C and -D are mitogenic for endothelial cells and thus stimulate cell division. They activate VEGFR-2 and VEGFR-3 and, therefore, are involved

in the regulation of the growth and/or differentiation of the lymphatic and blood vessel endothelium.

VEGF binding at the cell surface activates an intracellular signaling cascade that ultimately mediates aspects of angiogenesis, cell migration, and endothelial gene expression. VEGF is a pro-angiogenic molecule and the factor most frequently suppressed by anti-angiogenic medical therapy^{11; 25}. In human breast cancer cell lines, MMP-14 has been shown to regulate the expression of VEGF-A and its receptor, VEGFR-2, at the cell membrane. Localization of VEGFR-2 to the cell surface has been shown to increase as MMP-14 expression increases. Furthermore, VEGFR-2 associates with MMP-14 to form a complex that then interacts with Src to induce the phosphorylation of Akt and mammalian target of rapamycin (mTOR), ultimately leading to increased transcription of VEGF-A⁴². This is thought to be facilitated through the catalytic activity of MMP-14 by three mechanisms: (i) the extracellular catalytic domain, (ii) the intracellular amino acid C574, and (iii) complexing with Src kinase^{156; 158}.

VEGF supports angiogenesis through its interactions with various factors. VEGF is known to modulate gene expression in endothelial cells *via* a mechanism in which histone deacetylase 7 (HDAC7) is phosphorylated by protein kinase D1 (PKD-1), thereby mediating the intracellular accumulation of HDAC7. Induction of PKD-1 phosphorylation of HDAC7 by VEGF is a critical mechanism by which VEGF induces expression of MMP-10 and MMP-14, which ultimately leads to microvessel sprouting. Increased endothelial cell vascular permeability is an early step in angiogenesis and is followed by proliferation and migration of cells for the formation of new blood vessels in the direction of the angiogenic stimuli¹³. One method by which vascular permeability is increased involves VEGF-dependent phosphorylation of vascular endothelial cadherin molecules and β -catenin, which serve to weaken adherens junctions, the connections between endothelial cells. In addition, VEGF stimulates nuclear accumulation of β -catenin, which functions as a transcriptional co-activator of both MMP-14 and MMP-2 in endothelial cells⁴⁰.

Although all the above factors govern VEGF-mediated angiogenesis in a variety of cells, the control of corneal angiogenesis is our topic of interest. PEDF has been shown to be a potent inhibitor of angiogenesis in mammalian eyes, because it inhibits retinal endothelial cell growth and migration and further diminishes ischemia-induced retinal NV. Notably, PEDF levels are reduced in patients with diabetes who demonstrate proliferative diabetic retinopathy. These findings suggest that decreased PEDF levels may contribute to the development and progression of corneal angiogenesis. Recently, Matsui et al. demonstrated the mechanism underlying the unique ability of PEDF to potently inhibit angiogenesis¹⁰³. After corneal NV induction with chemical cauterization and topical application of PEDF-derived synthetic peptides, the investigators quantified corneal NV in these two groups. Rat corneas treated with the PEDF-derived peptide had 31% less corneal NV at day 7 compared to controls, and treatment with PEDF-derived peptide was associated with reduced expression of VEGF and an oxidative stress marker. The authors suggested that a PEDF-derived synthetic peptide inhibits corneal NV by suppressing VEGF expression *via* its anti-oxidative properties¹⁰³.

Our previously obtained data indicate that VEGF expression is greatly decreased in MMP-14 knockout (KO) stromal fibroblasts and can be recovered to normal levels with MMP-14 knock-in (KI) ⁵⁷. These results strongly imply that MMP-14 plays an important role in the upregulation of VEGF and are consistent with published experimental models demonstrating that: (i) crosstalk occurs between bFGF and VEGF-A during corneal NV ⁵⁷; (ii) the pro-angiogenic effect of MMP-14 is mediated at least in part by upregulation of VEGF-A at both the mRNA and protein levels ^{155,36}; (iii) MMP-14 and VEGF-A are functionally linked in tumor vasculogenesis ¹⁵⁶; and (iv) hypoxia-induced upregulation of MMP-14 by hypoxia inducible factor (HIF)-1 α in bone marrow-derived stromal cells is correlated with VEGF-A stimulation ^{9; 128}.

B. bFGF Pathway

Another potent pro-angiogenic factor extensively used in models of corneal angiogenesis is bFGF. This factor is a member of the FGF family, which includes 23 structurally related peptides widely expressed in both developing and mature tissues during cellular differentiation, angiogenesis, mitogenesis, and wound repair. bFGF is upregulated after tissue injury in stromal fibroblast/vascular endothelial cell co-cultures. Furthermore, bFGF was shown to induce corneal NV by activating the VEGF/VEGFR system ¹². Crosstalk is thought to occur between bFGF and VEGF during corneal NV. Our previous results suggest that MMP-14 potentiates corneal NV in part through mechanisms intertwining bFGF signaling pathways and VEGF-A expression pathways ¹¹⁸. In addition, enhanced MMP-14 expression is correlated with increased bFGF-induced VEGF-A upregulation and corneal NV in mice ¹¹⁸. Thus, published studies suggest that bFGF and MMP-14 complicity in the regulation of VEGF-A expression precedes corneal vascularization. Additionally, VEGF signaling via VEGFR-2 induces the expression of MMP-14 ⁴⁸ (Fig. 6).

The functions of the FGFs are mediated through interactions with the FGF receptors (FGFRs)-1, -2, -3, and -4, which perform unique biological roles. Tissue-specific FGFR expression reflects the diversity of its biological response, which is regulated by differences in ligand specificity and function ⁶⁹. For example, FGF-1 (aFGF) is expressed in the normal corneal epithelium, and FGF-2 (bFGF) is upregulated after injury and during keratocyte/vascular endothelial cell co-culture. Interestingly, bFGF binds to Bowman's and Descemet's membranes in normal corneas and the vascular basement membrane in neovascularized corneas ¹. It has been suggested that the basement membrane actually acts as a reservoir for bFGF by sequestering it and thus acts as an anti-angiogenic control mechanism.

MMP-14 has multiple associations with FGFs. Notably, a single nucleotide polymorphism for FGFR-4 has been shown to be an activity switch, regulating the ability of FGFR-4 to interact with MMP-14 via a membrane-bound complex and modulating its activity and degradation ¹⁶⁴. FGFR-4 also was shown to promote MMP-14-mediated collagen invasion. Furthermore, MMP-14 was shown to regulate the expression of FGFs and their receptors ¹⁶⁵. Decorin, an anti-angiogenic structural protein expressed in the cornea as well as other tissues, inhibits FGF- and VEGF-induced angiogenesis by inhibiting endothelial cell migration and vascular tube formation. MMP-14 degrades decorin, either directly or indirectly, to allow FGF and VEGF to exert their pro-angiogenic effects ¹⁰⁵.

MMP-14 has a potentiating effect on bFGF-induced corneal NV. In a previous study, we implanted bFGF pellets and injected MMP-14 DNA into the mouse cornea. Using immunostaining and western blot analysis, we showed that at 7 and 10 days after implantation/injection, the areas of NV in corneas that had been injected with both MMP-14 DNA and implanted with bFGF pellets were significantly greater than those in either the group that received MMP-14 DNA injection or bFGF pellet implantation alone. Treatment with bFGF has been shown to cause upregulation of MMP-14 in the cornea, and this signaling may be mediated through activation of MAP kinases (ERK, JNK, and p38)¹¹⁸. Furthermore, activation of these MAP kinases has been shown to increase VEGF mRNA stability and enhance VEGF production. Thus, the relationships between MMP-14, bFGF, and VEGF are intertwined to promote corneal NV.

We have studied various models of corneal NV, including the intrastromal implantation of bFGF and VEGF pellets in wild-type mice, severe combined immunodeficiency (SCID) mice, a SCID mouse model of corneal transplantation, and mice with stem cell deficiency, laser-induced corneal injury, and hemilimbal injury. In these models, the onset of corneal NV was found to occur during the second stage of stromal wound healing. Although MMP production was induced in these wound healing models, corneal NV was not seen after laser keratectomy or simple corneal epithelial debridement¹⁷. This finding suggests that the enhanced MMP expression in corneal wounds is not sufficient to induce corneal NV.

We designed a study to elucidate the interactions between MMP-14 and three biological factors that may be involved in the bFGF-induced VEGF-A expression pathways in corneal fibroblasts. These biological factors are: (i) FGFR-1, an endogenous bFGF receptor; (ii) Ras, an upstream regulator of certain ERK regulatory pathways governing cell proliferation, differentiation, and survival; and (iii) ERK, a MAP kinase homolog important for MMP-14-dependent cell migration^{35;159}. Toward this end, we generated MMP-14 catalytic domain KO corneal fibroblasts and transfected them with MMP-14 DNA to generate KI corneal fibroblasts overexpressing MMP-14. We then evaluated interactions between the KI platform and FGFR-1, Ras, and ERK. Furthermore, to more clearly link such interactions within the VEGF-A expression pathways, we investigated the effects of Ras and ERK inhibitors on VEGF-A mRNA and/or protein expression levels. We observed that: (i) MMP-14 upregulated FGFR-1 expression levels and modulated Ras-GTP binding and ERK phosphorylation in KI corneal fibroblasts; and (ii) the application of Ras or an ERK inhibitor to corneal fibroblast models downregulated VEGF-A mRNA and/or protein expression levels. These results suggest an interposition of Ras and ERK activity within the MMP-14-mediated VEGF-A expression pathway of corneal fibroblasts.

C. PDGF Pathway

Another mechanism by which c-Src is involved in MMP-14 regulation is through the PDGF receptor. PDGF binding to PDGF receptor leads to c-Src intracellular activation, which then binds to furin and facilitates its activity. Furin is the convertase that cleaves pro-MMP-14 by removing the R¹⁰⁸RKR¹¹¹ ↓ Y¹¹² motif of the enzyme's pro-domain. The released intact pro-domain maintains its potency as an MMP-14 inhibitor through a non-covalent association with the main molecule. Therefore, furin must cleave the pro-domain fragment

further at the PGD ↓ L⁵⁰ intradomain site to render it nonfunctional as an MMP-14 inhibitor. This two-step process is necessary to form the fully active MMP-14 that is expressed on the cell membrane ^{50; 145}.

MMP-14 expression upregulates the expression of PDGF receptor-β, a process that was shown to be regulated by Notch signaling. MMP-14-induced upregulation of PDGF receptor-β increases cellular invasiveness, and MMP-14 downregulates gene expression of PDGFRβ ²⁸. In developing vasculature, mural cell recruitment (vascular smooth muscle cells and pericytes) is dependent on MMP-14 expression. Furthermore, the appropriate mural cell-endothelial cell interactions are dependent on PDGF and PDGF receptor-β. MMP-14 has been shown to be a proteolytic modifier and necessary cofactor of PDGF/PDGF receptor-β signal transduction ⁸⁴.

D. HGF and Chemokine Pathway

Hepatocyte growth factor (HGF) is secreted by fibroblasts and mediates the progression of cancer. One method by which HGF exerts this effect is through upregulation of MMP-14. HGF also induces increased expression of CXCR-4, the chemokine receptor, which leads to the activation of intracellular protein kinases, such as protein kinase C ζ (PKC ζ). MMP-14 was shown to coordinate with CXCR-4 to promote the invasion of MDA-MB 436 cells in a PKC ζ-dependent manner. In addition, inhibiting Rac-1 and PI3K in these cells attenuates MMP-14 expression in HGF-stimulated cells ⁶². Therefore, HGF may activate the PI3K/Akt pathway and PKC ζ through CXCR-4, leading to activation of Rac-1 (in the Rho/Rac/ROCK pathway) and upregulation of MMP-14 ⁶². HGF also has been shown to be a strong chemo attractant for multiple malignancies, specifically human mesothelioma cells. Furthermore, HGF upregulates multiple MMPs, including MMP-14, while also upregulating TIMP-1 expression, thus promoting mesothelioma cell invasion and motility ⁵⁸.

Like other factors discussed previously, the chemokine granulocyte colony-stimulating factor (G-CSF) has been shown to affect MMP-14 production. G-CSF increases MMP-14 transcription and synthesis in hematopoietic cells. MMP-14 then incorporates itself into lipid rafts within the hematopoietic plasma membranes, in a PI3K-dependent manner. Following pro-MMP-2 activation, the bone marrow ECM and basement membrane are degraded and hematopoietic stem/progenitor cells are released into the circulation (Fig. 7) ¹⁵¹. G-CSF-induced mobilization of hematopoietic stem/progenitor cells increases serum levels of HGF. G-CSF also induces the expression of the HGF receptor (c-Met) in hematopoietic stem/progenitor cells. Both G-CSF and HGF increase the secretion of MMPs into bone marrow ECM in addition to MMP-14 at the cell surface ⁶⁸.

MMP-14 has consistently been associated with tumor invasion and growth due to its role in degrading ECM components. The chemokine stromal cell-derived factor (SDF)-1α plays a critical role in the metastasis of several cancers. SDF1α signals through CXCR-4, which activates Abl kinases downstream. Abl kinases proceed to form complexes with MMP-14 to assist in forming invadopodia complexes, specialized protrusive structures that form on the surface of invading cells ¹⁵³. It has been shown that invadopodia are actin-rich membrane extensions that promote the invasive capacity of tumor cells by recruiting MMP-14 from

secretory vesicles⁶⁵. Additionally, inhibition of Abl kinases results in the perinuclear accumulation of MMP-14 and the internalization of MMP-14¹⁵³.

E. Wnt/ β -catenin Pathway

The Wnt/ β -catenin signaling pathway is critical in the regulation of corneal epithelial stem cell proliferation in response to wound healing. Corneal stem cells remain relatively inactive until stimulated to rapidly proliferate. Wnt/ β -catenin signaling is highly complex and typically remains inactive until activated in response to wound healing. Activation of Wnt signaling is known to promote the proliferation of corneal epithelial stem cells, which otherwise remain undifferentiated in cell culture. Wnt7a is specifically upregulated in the human cornea during wound healing, and β -catenin maintains nuclear localization in corneal epithelial cells near wound edges. The Wnt/ β -catenin signaling pathway not only provides a better understanding of the regulation of corneal wound healing but also offers a potential clinical target for increasing the efficacy of corneal epithelial stem cells for transplantation¹¹⁰.

Comprehension of the Wnt pathway is pivotal for gaining a complete understanding of corneal wound healing and angiogenesis. The canonical Wnt signaling pathway is associated with both stem cell and tumor cell development, with continual pathway activation potentially contributing to the progression of cancer⁹¹. In this signal transduction pathway, Wnt binds to the Frizzled receptor, and β -catenin is uncoupled from its complex to allow translocation into the nucleus. There, β -catenin binds to the T-cell factor/lymphoid enhancing factor family transcription factors and activates transcription of the Wnt target gene, which encompasses several proteins involved in cancer cell invasion, including MMP-14, MMP-7, and CD44. Additionally, TGF- β binding causes phosphorylation of Smad proteins, which mediates rapid translocation of β -catenin to the cell nucleus¹¹¹. β -catenin can be found in different locations within a cell depending on Wnt-3a levels. In cancerous cells, Wnt-3a is known to be expressed at higher levels, allowing greater amounts of cytoplasmic β -catenin to enter the nucleus. In non-cancerous cells, where Wnt-3a levels are low, and β -catenin is located mainly in the cytoplasm. It has been demonstrated that cytoplasmic β -catenin directly interacts with MMP-14 by binding the cytoplasmic domain of MMP-14, and this interaction results in an accumulation of MMP-14 in the cytoplasm with a concomitant decrease in the expression of MMP-14 on the cell surface. Thus, a dynamic relationship exists between the location of β -catenin and its regulation of MMP-14, depending on the presence of Wnt-3a⁹².

F. TGF Pathway

One route by which MMPs coordinate vascular remodeling and angiogenesis is by activating growth factors, such as TGF- β . MMP-14 regulates the availability of active TGF- β by inducing the release of this factor from decorin, which functions to sequester TGF- β in the ECM. This is important in preventing vascular leakage and maintaining vascular homeostasis. This signaling axis is regulated by type I collagen, which induces MMP expression and, in turn, TGF signaling¹⁵⁷. Sounni et al. found that *in vivo* treatment with broad-spectrum MMP inhibitors or neutralizing antibodies to TGF- β enhances vessel leakage, whereas in tissues with chronically elevated TGF- β and MMP-14 activity,

cutaneous vessels are resistant to leakage¹⁵⁴. Together, the steady-state levels of MMP-14 and TGF- β regulate the extraversion of plasma proteins into the interstitial tissue and are critical in maintaining vessel stability¹⁵⁴.

TGF- β regulation is integral to both the commencement and resolution of angiogenesis. Its regulation is dependent upon targeting of TGF- β to the ECM by latent TGF- β binding proteins (LTBPs) during endothelial cell activation, migration, and apoptosis. In the ECM, LTBPs are proteolytically cleaved from TGF- β by MMP-14, which thereby regulates the concentration of TGF- β ¹⁷⁰. TGF- β also induces MMP expression, thus accelerating the angiogenic process¹⁴³. It has been shown in pancreatic cancer cells that the expression of MMP-14 is increased through TGF- β signaling when SMAD proteins (transcription factors) are activated intracellularly after exposure to type I collagen¹⁴⁴.

The normal homeostasis of the corneal epithelium requires a constant turnover of cells: cells are shed from the apical surface and then replaced by basal cells. It has been shown that TGF- α acts *via* an autocrine mechanism to control this turnover. Khaw et al. proposed that growth factors present in tears, such as TGF- α , may provide an immediate reservoir for signaling epithelium migration and mitosis after injury⁷⁷. TGF- α can transmit a diffusible mitogenic signal or can bind and activate an epidermal growth factor (EGF) receptor in adjacent cells to support cell–cell adhesion. It is synthesized as a precursor molecule, pro-TGF- α , which is targeted to the cell surface.

G. EGF Pathway

EGF is known to induce MMP-14 expression⁶³, whereas MMP-14 is known to induce EGFR activation through crosstalk with CD44, a cell surface cellular adhesion molecule. Specifically, the PEX domain of MMP-14 associates with CD44 to induce EGFR phosphorylation, leading to MAPK and PI3K pathway activation. Furthermore, silencing CD44 interferes with the ability of MMP-14 to mediate cell migration without affecting ECM proteolysis by MMP-14. To this end, MMP-14 plays a significant role in cancer metastasis, and high levels of MMP-14 expression are inversely correlated with cancer patient survival rates. Therefore, MMP-14 may be useful as prognostic marker in some types of cancer¹⁸². Zhang et al. demonstrate that EGF induces MMP-14 expression and inhibits MMP-2 expression in the cervical cancer cell line SiHa¹⁸³. EGFR activation by ligand binding activates multiple signaling pathways in the cell cytoplasm. One pathway, the MAPK/ERK signaling pathway, leads to increased synthesis of MMP-14 and decreased synthesis of MMP-2. Simultaneously, the PI3K/AKT pathway transmits a mild positive regulatory signal for MMP-2 synthesis. Notably, MMP-2 activity is not increased upon EGF treatment in comparison to no EGF treatment, despite the downregulation of the proenzyme form of MMP-2¹⁸³.

MMP-14 has been shown to cleave the laminin5 γ 2 chain and to shed the ectodomain of the heparin-binding EGF-like growth factor to generate EGF-like fragments that bind to EGFR and activate the EGF signaling pathway^{80; 81}. Heparin-binding EGF has been shown to play a critical role in promoting the growth of ovarian carcinomas. The co-expression of MMP-14 with heparin-binding EGF potentiates the activity of heparin-binding EGF to promote invasive tumor growth. This interaction is dependent upon MMP-14 cleaving an N-terminal

fragment from the heparin-binding EGF, which allows EGF to function without the presence of heparin in a hyperactive capacity^{80; 81}. In addition, in another form of interaction, EGF stimulation results in the phosphorylation of the MMP-14 cytoplasmic tail, which causes defective internalization of MMP-14. Also, the cytoplasmic domain of MMP-14 mediates the association of factor inhibiting HIF-1 (FIH-1) with Mint3 and thereby abrogates FIH-1 activity. This inactivation of FIH-1 induces substantial activation of the transcription factor activity of HIF^{136–139}. This may be the major mechanism by which MMP-14 induces VEGF expression. Interestingly, this new regulatory mechanism of HIF is also mediated by mTOR activity¹⁴⁰. Internalization of MMP-14 is a mechanism by which surface expression is regulated, and as a result of EGFR phosphorylation, MMP-14 activity is not suppressed¹⁰⁹.

H. PGE2 Pathway

In the eye, prostaglandin E2 (PGE2) triggers multiple physiologic events including miosis, vasodilation, and changes in intraocular pressure⁸⁸. Liclican et al. investigated the role of PGE2 in corneal angiogenesis after injury and found that endogenous levels of PGE2 do not change significantly after an acute epithelial abrasion injury, but do increase strikingly after more severe and chronic corneal injury. This increase in PGE2 is correlated with the development of pathologic angiogenesis. After injury in the corneal suture injury model, NV is amplified with a 54% increase in total blood vessels upon treatment with PGE2 compared to saline-treated control mice. This finding is valuable as PGE2 appears to mediate significant corneal inflammatory and angiogenic responses and is a major target of current ocular disease treatments⁸⁸.

PGE2 is a major product of cyclooxygenase-2 (COX-2) signaling and mediates the pro-angiogenic effects of COX-2. COX-2 signaling is stimulated by several pro-angiogenic factors and is itself implicated in angiogenesis. Researchers, while investigating the relationship between PGE2 and MMP-14 in lipopolysaccharide-stimulated monocytes, found that PGE2 inhibition by indomethacin suppresses the levels of MMP-14 mRNA and the addition of PGE2 reverses this suppression of MMP-14 mRNA expression. To further establish the pathway by which PGE2 upregulates MMP-14, an inhibitor of adenylyl cyclase and protein kinase A, SQ 22536, was used, and this factor inhibited MMP-14 in a dose-dependent manner. These results demonstrate that PGE2 stimulates MMP-14 mRNA and protein production via the adenylyl cyclase and protein kinase A pathway¹⁴². PGE-2-mediated angiogenesis also requires TGF- β . It has been shown that MMP-14 activates TGF- β in endothelial cells, and once activated, TGF- β binds to its tyrosine kinase receptor Alk5, which, in turn, activates the pro-angiogenic SMAD3 pathway. Active MMP-14 and TGF- β are required for PGE2-induced angiogenesis, based on the observation that Alk5 inhibition impairs PGE2-induced angiogenesis⁵.

I. Thrombin pathway

Thrombin play an integral role in the vascular response, impacting coagulation, platelet aggregation, and thrombus formation. It is associated with angiogenesis and has been linked to various factors involved in angiogenesis, including MMPs. In endothelial cells, MMP-14 mediates the activity of thrombin in the vascular response by activating the small GTPases,

RhoA and Rac1, resulting in Ca^{2+} signaling, NADPH oxidase activity, reactive oxygen species generation, and ultimately tissue factor and plasminogen activator inhibitor-1 expression. In addition, thrombin increases MMP-14 expression at the cell surface. This signaling pathway has been implicated in cardiovascular disease, endothelial dysfunction, and oxidative stress. Studying this pathway may provide a unique opportunity to intervene in its progression^{8; 52; 160}. To advance our understanding of the relationship between thrombin and MMP-14, Shivaikar et al. used cord blood hematopoietic stem cells to demonstrate that thrombin increases MMP-14 expression in a PI3K- and Rac1-dependent manner¹⁵⁰.

Gelatinase A, or MMP-2, is another important factor involved in blood vessel formation and has a unique relationship with thrombin. MMP-2 participates in degrading collagen-rich ECM, thus allowing endothelial cell invasion, a critical step in angiogenesis¹¹⁴. Previously, thrombin was considered the only physiologic agent known to induce an increase in MMP-2 activation, and unlike collagen, thrombin can induce MMP-2 activation independent of MMP-14. Moreover, activation of MMP-2 by collagen matrix is slower than MMP-2 activation by thrombin; however, collagen sustains MMP-2 activation for several days longer than thrombin¹¹⁴. Lafleur et al. found that thrombin alone inefficiently activates pro-MMP-2 but can efficiently cleave the MMP-14-processed form of MMP-2 in human umbilical vein endothelial cells. Therefore, although thrombin can activate a small proportion of pro-MMP-2, its effects are greatly enhanced on pro-MMP-2 previously processed by MMP-14^{5; 83}.

J. Notch Pathway

It has been demonstrated that Notch receptors and ligands are present in the human corneal epithelium and that Notch signaling regulates cell differentiation in the cornea. Furthermore, decreased expression of Notch receptors achieved *via* γ -secretase inhibition results in decreased corneal cell proliferation and increased differentiation. Moreover, Notch signaling has been shown to be essential for vasculature formation during embryogenesis, a process dependent upon VEGF. In cultured endothelial cells, VEGF can induce the expression of Notch1 and the Notch ligand Dll4. Furthermore, Notch functions downstream of VEGF to upregulate MMP-9 and MMP-14, critical angiogenic regulators⁴⁸. Cheng et al. showed that Kaposi sarcoma herpes virus (KSHV) induces the reprogramming of lymphatic endothelial cells to mesenchymal cells, and this reprogramming is accomplished by a substantial upregulation of MMP-14 in lymphatic endothelial cells. Two KSHV proteins, viral G protein-coupled receptor and viral Fas-associated death domain-like interleukin-1 β -converting enzyme-inhibitory protein, activate Notch signaling, which in turn induces MMP-14 upregulation²⁸.

MMP-14 expression has been shown to negatively regulate Notch signaling, indicating the existence of a negative feedback loop between MMP-14 and Notch⁷². MMP-14 cleaves Notch ligand Dll1, leading to the inhibition of Notch1 signaling, whereas loss of MMP-14 enhances Notch signaling⁷². Notch signaling is known to act laterally, causing Notch signaling activation in neighboring cells. Cleavage of Dll1 by MMP-14 on the cell surface also downregulates lateral (paracrine) Notch1 signaling. For instance, Notch signaling controls the differentiation of hematopoietic progenitor cells (HPCs) in an MMP-14

dependent manner. MMP-14 loss increases Notch signaling, thus impairing B-lymphocyte differentiation from HPCs. However, the increased Notch signaling in MMP-14-deficient mice is still insufficient for inducing T-cell differentiation. MMP-14 acts to negatively regulate Notch signaling in the bone marrow, allowing for precise control of HPC differentiation into B-lymphocytes ⁷².

K. Integrin Pathway

Integrins mediate MMP binding to ECM molecules, such as collagen, and thereby regulate proteolysis and degradation of the ECM ¹⁴⁸. Integrins are essential cell adhesion molecules that are involved in vascular morphogenesis and ECM remodeling. Inhibition or disruption of either cell adhesion or matrix degrading factors has been shown to inhibit angiogenesis. Integrins facilitate connections between the ECM and cell cytoskeletal elements as well as other molecules that are involved in cell migration, invasion, and ECM degradation ⁷⁹. In female rat corneas, the angiogenic response pattern following alkaline-burn induction was examined using RT-PCR and the expression of integrins and MMPs was analyzed. The results demonstrated that expression of CD31, integrins α_1 and β_3 , and MMP-14 correlates with the angiogenic response and more specifically, that the $\alpha_v\beta_5$ integrin appears to be the principal α_v integrin associated within the corneal alkaline burn model of inflammation-mediated angiogenesis. These results lend support to therapeutically targeting β_5 integrin inhibition to prevent alkaline-burn induced angiogenesis in the cornea.

Endothelial cells interact with the ECM via integrins, and these interactions are essential for endothelial cell migration, invasion, and assembly into tube networks to form new vasculature. Integrins regulate endothelial cell tube formation and sprouting by inducing Rho GTPase activation, which is critical for tube and lumen formation of endothelial cells during vasculogenesis ¹³⁴. These Rho-induced processes involve cytoskeletal changes that are known to involve integrin-based adhesion complexes ¹⁰⁴. β_1 -integrin also mediates cell motility and scattering, as shown in collagen matrix assays using AsPC1 cells (a pancreatic cancer cell line). This process is regulated by Snail, a transcription factor that upregulates MMP-14 and β_1 -integrin expression and also induces cell motility and invasion as pancreatic cancer progresses. β_1 -integrin blocking antibodies attenuate Snail-induced motility, indicating a dependency on β_1 -integrin within this process ^{140; 148; 166}. MMP-14 is critical for extracellular fibronectin cleavage and the subsequent endocytosis of fibronectin fragments for degradation, a process that is important in ECM remodeling and degradation. MMP-14 also regulates integrin endocytosis, and this process is enhanced by fibronectin endocytosis ¹⁴⁶.

Prakash et al. reported a molecular signaling pathway by which integrin $\alpha_v\beta_3$ leads to increased MMP-14 expression. Integrin $\alpha_v\beta_3$ is the most specific invasion marker in melanoma ¹²⁷. Hyaluronan-binding protein 1 (HABP1), an ECM mucopolysaccharide, has been implicated in tumor migration and metastasis and is involved in cellular adhesion and invasion. HABP1 activity is mediated through binding to integrin $\alpha_v\beta_3$, which then leads to Nck-interacting kinase (NIK) phosphorylation and the activation of $I\kappa B\alpha$. $I\kappa B\alpha$ is an upstream regulator of $\text{NF}\kappa\text{B}$, a well-known transcription factor involved in multiple integrin-mediated signaling pathways. $\text{NF}\kappa\text{B}$ translocates to the nucleus and activates MMP-14

transcription. MMP-14 is then expressed at the cell surface, where it activates pro-MMP-2 and leads to ECM degradation and cell migration ¹²⁷. MMP-14 expression is also regulated by syndecans, co-receptors for integrins that modulate integrin-mediated adhesion to the ECM ¹²⁷.

L. TLR Pathway

Toll-like receptors (TLRs) are cell surface pattern recognition receptors that are involved in sensing pathogens for the innate immune system and have many other functions. TLRs are diversely expressed in the healthy cornea and may play a role in the pathogenesis of certain corneal infections, including infection with herpes simplex virus. TLR signaling in glioma cells has been shown to upregulate MMP-14 expression. Tumor expansion via cell proliferation and invasion was also shown to be mediated by TLR-induced MMP-14 expression. TLR signaling occurs via MyD88, the TLR adaptor, and activation of the p38 MAPK pathway. In addition, MMP-14 expression facilitates further TLR signaling through degradation of ECM, which promotes TLR signaling, leading to a vicious cycle. Gliomas are exceptionally aggressive tumors, and this may be because these tumor cells exhibit high levels of MMP-14 expression unlike tumor-free brain samples, which display virtually no MMP-14 expression ¹⁰¹.

As mentioned above, TLRs are most recognized for their role in the innate immune response, yet they have diverse pathological functions as well. TLR-2 expression has been demonstrated in healthy odontoblasts, and infection of a tooth with gram-negative bacteria, as in the case of dental caries, causes TLR-4 expression. Lipopolysaccharide produced by gram negative bacteria triggers the TLR-4 signaling axis with concomitant upregulation of MMP-2, TIMP-2, and MMP-14 ²⁷. In the antigen-processing dendritic cell, the TLR signaling axis causes a reorganization of the cytoskeleton such that dendritic cells have enhanced macropinocytosis with loss of migratory capacity. Specifically, podosomes, areas with concentrated MMP-14 ECM degradation activity, are lost following TLR activation, whereas endocytic activity is increased ¹⁷⁹. Finally, TLR activation may play a role in plaque progression. In this process, accumulation of cholesterol in the plasma membrane or within endosomes of macrophages stimulates TLR signaling, which sustains phosphorylation of p38 MAP kinase and causes a downstream induction of MMP-8, MMP-14, and cathepsins. MMPs and cathepsins have been implicated in aneurysm formation and plaque instability ¹⁶⁷.

M. PI3k/Akt Pathway

Activation of the PI3k/Akt pathway is associated with increased cell surface expression and reduced turnover of MMP-14. Upregulation of the PI3k/Akt pathway can be achieved through the loss of phosphatase and tensin homolog (PTEN), a tumor suppressor that downregulates the PI3k/Akt pathway. PTEN is a phosphatase that dephosphorylates PIP3 to decrease the level of activated Akt, which promotes cell survival, proliferation, and migration. Increased expression of MMP-14 and MMP-2 is associated with the activation of this pathway, which explains the enhanced proliferation and migration of PTEN knockout cells ⁷⁸.

N. Src Pathway

Corneal epithelial cells rapidly respond to injury by proliferating and migrating to cover the defect and re-establish the epithelial barrier. Cellular migration and proliferation are driven by growth factors and cytokines. Injury can be an inciting event leading to corneal angiogenesis, and thus, a thorough understanding of the events occurring directly after injury is necessary. As explained previously, MMP-14 regulates both the localization of VEGFR-2 to the cell membrane and the expression of VEGF, and these activities are regulated by Src signaling^{42; 157}. Src is a non-receptor tyrosine kinase that is involved in the process of cell invasion and metastasis, and Src activation is critical to wound closure; blocking Src activation with a Src kinase inhibitor inhibits wound closure in mouse corneal epithelial cells. MMP-14 creates a complex with VEGFR-2 and Src at the cell surface via its cytoplasmic domain⁴². The signaling events that follow the formation of this complex lead to the activation of Akt, a known pro-survival, anti-apoptosis intracellular factor, and mTOR, which regulates cell growth, proliferation, motility, and survival⁴². Src activation by MMP-14 has also been linked to the activation of several other factors involved in cell migration, such as RANKL, the NF- κ B ligand, and ERK1/2. Inhibiting Src leads to the inhibition of MMP-14 activation and signaling events associated with activated MMP-14¹⁶⁵.

O. RhoA/ROCK Pathway

Cellular migration and invasion, processes linked to angiogenesis, are also regulated by signaling through the RhoA/ROCK pathway. This pathway has been shown to have both stimulatory and inhibitory effects on these cellular processes. Rho and Rac are small guanosine triphosphatases (GTPases) that are downstream effectors of Rho-associated kinase 1 and 2 (ROCK1/2) as well as the PI3K/Akt pathway¹⁰⁴. Members of the Rho family of GTPases are key regulators of the contractility of the cytoskeletal elements actin, microtubules, and actomyosin¹⁴⁸. On one hand, suppressing the RhoA/ROCK pathway through exposure to a ROCK inhibitor enhances MMP-14 expression and promotes cellular migration. On the other hand, overexpression of Rho has been shown to induce cellular invasion *in vivo*¹⁰⁴.

Src signaling activates RhoA, which co-localizes with other pro-migratory molecules and thus promotes migration of cells at the leading edge of their locomotion¹²⁰. Members of the Snail family of transcription factors (Snail and Slug) as well as ERK1/2 also are activated through Rho/ROCK signaling. These molecules are known to upregulate the expression of MMP-14 (Fig. 8). In addition, Snail and Slug were found to reduce the levels of TIMP-2, the endogenous MMP-14 inhibitor¹⁴⁸.

P. ERK Pathway

Phosphorylation of ERK is important for endothelial cell activation, which promotes angiogenesis. In addition, suppression of ERK activation inhibits angiogenesis both *in vitro* and *in vivo*. When MMP-14 binds TIMP-2 on the cell surface, TIMP-2 mediates its binding to lipoprotein-related receptor protein-1 (LRP1). LRP1 signals through its cytoplasmic tail to stimulate the ERK/MEK pathway¹⁶³. Finally, it has been shown that MMP-14 activates ERK, which is important for MMP-14-dependent cell migration.

VI. Conclusions

Corneal NV is a pathological condition characterized by angiogenesis and ECM remodeling within the cornea, a physiologically avascular tissue. Pro-angiogenic factors such as MMP-14 play important roles in inducing corneal NV. The signaling pathways by which MMP-14 induces angiogenic processes and ECM degradation in conjunction with other pro-angiogenic factors such as VEGF, FGF, HGF, and Src have been summarized here.

Currently, anti-MMP14 (9EB and DX2400) antibodies have been successfully used to treat experimental breast carcinoma, lymphatic vessel sprouting, tumor growth, invasion and angiogenesis^{26,437}. These antibodies can be administered topically as eye drops or via subconjunctival injection before and after experimental injury-induced corneal angiogenesis. Their efficiency and efficacy can serve as the basis for clinical trials of their use in the treatment of corneal angiogenesis-related diseases, such as corneal transplant rejection. Angiogenesis is an incredibly complex process that is either inhibited or initiated by a milieu of factors in the local environment. The interactions of MMP-14 with multiple other molecules clearly play critical roles in the delicate balance of pro- vs. anti-angiogenic signaling. The current data indicate that MMP-14 can be a molecular target for treating corneal NV. The ultimate goal of elucidating the factors and signaling pathways involved in MMP-14 production and function is to facilitate the possibility of targeting these pathways to inhibit MMP-14-mediated corneal angiogenesis in order to maintain clarity of vision after an inciting injury or inflammation.

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Abbreviations

bFGF	Basic fibroblast growth factor
BM	Basement membrane
COX	Cyclooxygenase
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FBS	Fibroblast synoviocyte
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FIH-1	Factor inhibiting HIF-1

G-CSF	Granulocyte colony-stimulating factor
GTPase	Guanosine triphosphatase
HABP1	Hyaluronan-binding protein-1
HDAC7	Histone deacetylase 7
HIF-1	Hypoxia inducible factor-1
HPC	Hematopoietic progenitor cell
IL-1β	Interleukin-1 β
KI	Knock-in
KO	Knockout
KSHV	Kaposi sarcoma herpes virus
LRP1	Lipoprotein-related receptor protein-1
LTBP	Latent TGF- β binding protein
MMP	Matrix metalloproteinase
MT-MMP	Membrane-type matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor κ B
NIK	Nck-interacting kinase
NV	Neovascularization
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PEDF	Pigment epithelium-derived factor
PEX	Hemopexin-like
PGE2	Prostaglandin E2
PKC	Protein kinase C
PKD-1	Protein kinase D1
PTEN	Phosphatase and tensin homolog
RECK	Reversion-inducing cysteine-rich protein with Kazal motifs
ROCK	Rho-associated kinase
SCID	Severe combined immunodeficiency

SDF-1α	Stromal cell-derived factor 1 α
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TGF	Transforming growth factor
TRK	Tyrosine kinase receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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Figure 1.

Three common corneal neovascularization (NV) morphologies are (A) deep NV overlying Descemet's membrane, (B) stromal NV and (C) superficial vascular pannus.

Figure 2.

Conserved domains of a typical MMP and MMP-14. (A) Domains of a typical released MMP family member (e.g., MMP-1,-3,-8,-10,-12,-13,-18,-19,-20, and -27). (B) Domains of membrane-bound MMP-14. [Adapted from Pahwa et al. ¹²²]

Figure 3. Schematic presentation of MMP-14 activation, activity, and disappearance at the surface of the invading endothelial sprout. Adapted from a previously published overview of MMP-14 at the cell surface ^{67; 175}.

Figure 4.

Schematic model depicting the balance between MMP-2/-14 and TIMP-2 expression and its effects on MMP-2 activation in glioblastoma. (A) Tumor cells such as U87MG cells that overexpress MMP-2 and MMP-14 but secrete relatively low levels of TIMP-2 are able to activate MMP-2 at a basal level. TIMP-2 binds to MMP-14 on the cell surface and acts as a receptor for proMMP-2. A second TIMP-free MMP-14 molecule in close proximity then cleaves the MMP-2 propeptide domain to generate active MMP-2, which is then released. (B) Intermediate upregulation of TIMP-2 expression, such as in U87-C1 cells, allows more MMP-14/TIMP-2/proMMP-2 complexes to assemble on the cell surface and results in increased MMP-2 activation. (C) Very high levels of TIMP-2 expression are inhibitory to both MMP activity and MMP-2 activation due to excessive TIMP-2 binding of MMP-14 as well as direct binding to MMP-2. (D) Relationship between MMP expression, local TIMP-2 levels, and MMP-2 activation. Solid black arrowhead lines represent MMP-2 activation in tumor cells with high MMP-2 and MMP-14 expression as a function of the TIMP-2 level. MMP-2 activation initially increases as TIMP-2 increases until TIMP-2 levels reach the optimum for maximal MMP-2 activation. Thereafter, increases in TIMP-2 are inhibitory to activation of and, at higher levels, inhibitory to MMP activity. Points A, B, and C are representative positions along this plot for the scenarios depicted in (A)–(C). In nontumor tissues that presumably express low levels of MMP-2 and MMP-14, even low amounts of TIMP-2 are sufficient to inhibit MMP activation and activity. [Adapted from Lu et al.⁹⁴ with permission from Nature Publishing Group.]

Figure 5.

Cleavage of VEGFR-1 by MMP-14 into soluble (s)VEGFR-1. with the findings of previous studies, which showed that soluble VEGFR-1, generated by alternative splicing, can bind free VEGF to inhibit VEGF-induced proliferation in endothelial cells ⁷⁵ and trap free VEGF to ultimately preserve avascularity in the cornea ⁷.

Figure 6.

As a result of corneal epithelial and stromal injury, bFGF mediates fibroblast activation, whereas stromal fibroblast MMP-14 initiates enzymatic activity. bFGF-mediated fibroblasts and stromal fibroblasts show upregulation of VEGF, and MMP-14 also mediates the degradation of ECM. Both upregulation of VEGF and ECM degradation enhance vascular endothelial cell proliferation, migration, and tube formation.

Figure 7.

In the absence of mobilizing stimuli, such as G-CSF, proteolytic activities of MMP-14, -2, and -9 are relatively low due to inhibition by RECK. Functional membranous CD44 contributes to progenitor cell adhesion to the basement membrane components (retention). G-CSF signaling induces PI3k-mediated Akt phosphorylation, increasing MMP-14 and decreasing RECK expression. The opposed changes in MMP-14 and RECK levels result in MMP-14-mediated CD44 proteolysis as well as MMP-2 and MMP-9 secretion and activation. Collectively, these changes reduce progenitor cell retention and facilitate their egress and mobilization. [Adapted from Vagima et al. ¹⁷⁴.]

Figure 8.

Collagen upregulates MMP-14 through activation of the TGF- β /T β RI/Smad3/Snail pathway. MMP-14 can also be induced in the collagen microenvironment through the β 1-integrin/Src/Egr1 signaling pathway. [Adapted from Shields et al.¹⁴⁷ with permission from Portland Press Limited.]