MMP14-Containing Exosomes Cleave VEGFR1 and Promote VEGFA-Induced Migration and Proliferation of Vascular Endothelial Cells

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Purpose. Investigate the impact matrix metalloproteinase 14 (MMP14) delivered via exosomes produced by corneal fibroblasts on vascular endothelial growth factor receptor 1 (VEGFR1) cleavage on endothelial cells, and other key processes of angiogenesis.

Methods. Proteolysis of VEGFR1 and R2 by the catalytic domain of MMP14 was investigated via immunocytochemistry with anti-VEGFR1, anti-VEGFR2, and anti-MMP14 antibodies. Exosomes were isolated via precipitation and serial ultracentrifugation from wild-type (WT) and MMP14 exon4-deficient corneal fibroblasts. Transmission electron microscopy and nanotracking analysis were used to characterize the isolated exosomes. The presence of MMP14 in exosomes from WT fibroblasts was confirmed by Western blotting. VEGFR1 cleavage upon treatment with WT-derived exosomes, Δexon4-derived exosomes, or the pan-MMP inhibitor GM60001 was examined via in vitro proteolysis analysis using recombinant mouse (rm) VEGFR1/R2. Endothelial cell migration and proliferation were investigated using a Boyden chamber assay and BrdU incorporation, respectively.

Results. WT-derived exosomes specifically cleaved rmVEGFR1 in vitro, whereas Δexon4-derived exosomes did not. Treatment with the pan-MMP inhibitor GM6001 effectively inhibited VEGFR1 cleavage by WT-derived exosomes, confirming the role of MMP14 in this cleavage. WT-derived exosomes induced greater endothelial cell migration ($P < 0.01$) and proliferation ($P < 0.5$) compared to Δexon4-derived exosomes.

Conclusions. MMP14-containing exosomes may be involved in the regulation of corneal neovascularization through degradation of VEGFR1 and VEGFA-induced endothelial cell proliferation and migration.

Keywords: matrix metalloproteinase 14, exosome, vascular endothelial growth factor receptor, angiogenesis

Matrix metalloproteinase 14 (MMP14) is primarily a membrane-anchored Zn^{2+}-dependent MMP enzyme that can degrade extracellular matrix (ECM) proteins including type I, II, III, and IV collagens, fibronection, vitronection, laminin, fibrin, and proteoglycan.[1–3] As a membrane-bound protein, MMP14 has a limited function, but other MMPs that are secreted from the cell have various functions.[4,5] MMP14 expression has been demonstrated in a wide variety of human tissues including lung, kidney, ovary, spleen, intestine, prostrate, and placenta, and has been implicated in tissue-remodeling events such as invasion and migration.[6] MMP14 becomes concentrated in lamellipodia in normal cells and invadipodia in cancer cells, where it creates a path through the surrounding tissues by degrading ECM and enhancing cell migration.[7] MMP14 has been shown to activate MMP2 and bind to its natural inhibitor tissue inhibitor of metalloproteinases 2 (TIMP2). TIMP2 recruits pro-MMP2 to form a tri-molecular complex on the cell surface.[8] Interestingly, active MMP2 expression is reduced but not eliminated in MMP14-null mice,[9] which suggests that alternative mechanisms may exist including those involving MMP15 and MMP16.[10,11] Expression of MMP14 has been observed during the proliferation, invasion, and metastasis of cancer cells in numerous cancer types. Transcriptional changes during tumor formation have been associated with upregulation of MMP14, which also upregulates vascular endothelial growth factor (VEGF) production for tumor formation.[12] Previous studies indicated that with MMP14 knock-out in a mouse model, the implantation of a basic fibroblast growth factor (bFGF) pellet within the cornea no longer resulted in neovascularization in the mouse cornea.[13,14] Moreover, bFGF-induced VEGF production was reduced in MMP14 knock-out corneal fibroblasts compared with that in wild-type corneal fibroblasts.[14] These findings are consistent with studies showing that MMP14 regulates transduction signaling via modulation of receptor tyrosine kinases (RTKs), including FGF receptor 2 (FGFR2) through ADAM9, and cleavage of VEGF receptor 1 (VEGFR1).[15–18]

Nanoparticles are formed by cells via internalization of portions of the plasma membrane; such nanoparticles are referred to as "endosomes" and form multivesicular bodies (MVBs) within the cytoplasm.[19] The MVBs include an extracellular domain of transferrin receptors and cytosol at their surface.[20] Secreted nanomembrane vesicles (30–150 nm) called "exosomes" are shed into the extracellular space via

exocytosis from almost all types of cells—including immune cells, epithelial cells, tumor cells, nerve cells, embryonic cells, and corneal stromal cells—both under normal and pathological conditions.[21] Exosomes are also abundantly found in bodily fluids like saliva, urine, blood, semen, amniotic fluid, synovial fluid, ocular fluid, pleural effusion, and breast milk. In recent years, exosomes have been found to act as important messengers among cells, transferring miRNA, lipids, and proteins between cells.[22] Exosomes are quite unique in their lipid and protein content, depending upon the cell type from which they originate.[23] Thus, exosomes play roles in diverse physiological processes.[24] Such diversity makes understanding the formation, content, and functions of exosomes challenging but also suggests their therapeutic potential based on the possibility of specific targeting of tumor cells and delivering various effectors to specific cells.

VEGFR1 is a member of the RTK family, and its involvement in angiogenesis and lymphangiogenesis is well established.[25] This receptor consists of an extracellular ligand-binding domain, with seven immunoglobulin-like motifs, a single transmembrane domain, a kinase domain split by a kinase insert, and a carboxyl terminus.[26] Although VEGFR1 is an RTK, its kinase activity is relatively weak. However, its binding affinity for its ligand, VEGFA, is approximately 10 times stronger than that of VEGFR2.[25] Thus, VEGFR1 commonly acts as a decoy receptor for VEGFR2.[27] VEGFR1 activity is first observed as early as embryogenesis when it recruits VEGFA to the cell membrane and plays an inhibitory role in embryonic vasculogenesis.[28] VEGFR1 is expressed in vascular endothelial cells (VECs), monocytes and macrophages,[29] and with the loss of VEGFR2, the embryonic endothelial cells are grossly disorganized and abnormally overgrown in blood vessels.[30] VEGFR1 has been found to be a positive regulator of monocyte and macrophage migration and a positive or negative regulator of VEGFR2 signaling capacity, depending upon the pathological or normal conditions.[31] Furthermore, soluble VEGFR1 (arising via alternative splicing, or proteolysis by a catalytic enzyme) can trap VEGF and serve as a specific antagonist of VEGF function.[15,32] Thus, VEGFR1 has been found to function in signaling pathways that promote angiogenesis as well as in pathways that inhibit angiogenesis.[16,31] However, it remains unknown whether the opposing effects of VEGFR1 on VEGFR2 activity occur in the same or different types of endothelial cells and under the same or different pathological conditions.

Corneal neovascularization is the process of new blood vessel growth into avascular corneal tissue in response to insults such as corneal ulcers, inflammation related to infection, chemical injury, and corneal transplantation.[33] The presence of blood vessels in the deeper or superficial stroma causes problems to corneal transparency and optimal vision.[34] It was reported that MMP14 is overexpressed in the corneal stroma corneal wound healing and corneal neovascularization.[35] Our previous studies have suggested that MMP14 may be involved in the regulation of tyrosine kinase activity, specifically that of VEGFR1 on human umbilical vein endothelial cells (HUVEC).[15,16] Once VEGFR1 is cleaved on the surface of HUVEC by MMP14, these cells can be induced to undergo VEGFA-induced cell mitogenesis, migration, and invasion through breakdown ECM. Since our discovery that exosomes from mouse corneal fibroblasts contain MMP14,[36] we have remained interested that these nanoparticles may influence corneal neovascularization. As a follow-up to our studies showing the pro-angiogenic activities of MMP14, research was needed to determine whether MMP14 delivery via corneal fibroblast-derived exosomes to endothelial cell has pro-angiogenic effects. Previous studies have revealed key information about exosome generation, secretion, fusion to target cells, and bio-molecular composition.[37,38] MMP14 was even identified as an exosome-associated protein,[39] but its function remained unclear.

In our previous study, we investigated exosomal transport of MMP14 and its target, MMP2, from corneal fibroblasts to VECs as a possible mechanism governing MMP14 activity in corneal angiogenesis.[36,40] We found that HUVEC and calf pulmonary artery endothelial cells (CPAEC) readily absorb MMP14-containing exosomes isolated from corneal fibroblasts in vitro.[36] We also found that MMP14 is enriched in exosomal fractions of corneal fibroblasts. Moreover, loss of the MMP14 enzymatic domain results in accumulation of pro-MMP2 protein in exosomes, whereas MMP2 was almost undetectable in exosomes from MMP-14 null fibroblasts.[36] The results of our previous research were important in establishing the capability of corneal fibroblast-secreted exosomes to transport proteins, including MMP14, to HUVEC. The shuttling of MMP14 from corneal fibroblasts to HUVEC via exosomes during angiogenesis has important implications for the therapeutic potential of using exosomes to target angiogenic processes in the cornea.

In the present study, we hypothesized that exosomes may be agents for the modulation of VEGFR1 activity in angiogenesis. We explored a possible mechanism through which MMP14-containing exosomes induce cleavage of VEGFR1 during corneal angiogenesis. We found that exosomes could have a pro-angiogenic effect in corneal HUVEC via MMP14-mediated cleavage of VEGFR1, which would allow increased VEGFR2 activation by VEGFA.

Materials and Methods

Cleavage of VEGFR1 by Catalytic Domain of MMP14 on HUVEC

To investigate the proteolytic activity of catalytic domain of MMP14 on VEGFR1 cleavage, levels of VEGFR1/R2 were stained with immunocytochemistry analysis after incubation with or without catalytic domain of MMP14. HUVEC (1×10^4 cells; ScienCell, Carlsbad, CA, USA) was seeded onto 1.5 mm circled cover glass (Thermo Fisher Scientific, Rochester, NY, USA) for 24 hours. Cells were washed 3 times with PBS and then in fresh media (EBM-2; Lonza, Allendale, NJ, USA) including 50 ng/mL of catalytic domain of MMP14 (Calbiochem, Nottingham, UK) was incubated for 4 hours. Cells were fixed with 4% of paraformaldehyde/PBS (Electron Microscopy Sciences, Hatfield, PA, USA) for 15 minutes. Fixed cells were washed 3 times with PBS and then incubated for 10 minutes with 0.5% Triton X-100/PBS (Sigma-Aldrich Corp., St. Louis, MO, USA). Cells were incubated with 10% goat serum/PBS for 1 hour at room temperature and then briefly washed with PBS. Anti-VEGFR1 (1:500 dilution; R&D Systems, Minneapolis, MN, USA), anti-VEGFR2 (1:500 dilution; R&D Systems), and anti-MMP14 (1:1000 dilution, Abcam, Cambridge, MA, USA) in 3% BSA/PBS were added to cover glass and then incubated for at 4°C. Each cover glass was washed with PBST for 3 times to remove primary antibodies. FITC or 647 conjugated secondary antibodies (1:1000 dilution; Thermo Fisher Scientific) in 3% BSA/PBS was then added for 1 hour at room temperature. Cells were washed 3 times with PBST and mounted on glass slides (Thermo Fisher Scientific) with DAPI containing mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). Images were analyzed using Leica LSM 710 confocal microscopy (Leica Microsystems, Wetzlar, Germany). Fluorescence density was measured by Zen 2 software (Carl Zeiss GmbH, Oberkochen, Germany) using the histo analysis option. Arithmetic mean intensity was observed from five different images: The mean and SD values were calculated using Prism 6 software (Graphpad, San Diego, CA, USA).

Investigative Ophthalmology & Visual Science

Immortalized Cell Lines and Exosome Isolation

Mouse corneal fibroblast cells were generated from WT and MMP14 KO mice as described previously.[41] Briefly, excised mouse corneal stroma under surgical microscopy was incubated with Dulbecco's modification of Eagle's medium (DMEM; HyClone Laboratories, Logan, UT, USA) containing 3.3 mg/ml collagenase type II (Sigma-Aldrich Corp.) at 37°C, shaking for 90 minutes. Isolated keratocytes were maintained in DMEM supplemented with 10% fetal calf serum (FCS; HyClone) at 37°C in a 5% CO_2 humidified atmosphere. For immortalization, stromal fibroblasts were grown with a mixture containing polybrene (4 ng/ml) and an equal volume of pZIPTEX virus (containing SV40T antigen). To isolate exosomes from conditioned-medium (CM) from mouse corneal fibroblast cultures, we used a modified method with total exosome isolation reagent (Invitrogen, Carlsbad, CA, USA) and ultracentrifugation (Beckman Coulter, Indianapolis, IN, USA). Briefly, mouse corneal fibroblasts were maintained with DMEM containing 10% fetal bovine serum (FBS; Sigma-Aldrich Corp.) and antibiotics. Upon reaching confluence, cells were washed at least 3 times with phosphate-buffered saline (PBS). To prepare exosome-depleted FBS, FBS was ultracentrifuged at 100,000*g* for 18 hours, and supernatant was stored at −20°C until use. Fresh DMEM, including 1% exosome-depleted FBS, was added and cell cultures were returned to the incubator. The next day, the CM was collected and cell debris and macro particles were removed by serial centrifugation at 750*g* for 10 minutes and 3050*g* for 30 minutes. The supernatant was filtered through a 0.22-μm filter (Millipore, Billerica, MA, USA). Filtered CM was concentrated using an ultra-filtration system with a 100 kDa molecular weight cut-off (MWCO) membrane (Millipore). An equal amount of total exosome isolation reagent was added, and then the samples were incubated overnight in a cold room to allow the exosomes to precipitate. Samples were centrifuged at 10,000*g* for 1 hour, and pellets were re-suspended in PBS. Ultracentrifugation at 100,000*g* for 1 hour was performed to remove the remaining total exosome isolation reagent and single proteins. The obtained pellet was stored at −80°C until use. Hereafter, exosomes derived from WT fibroblasts are denoted as "WT-derived exosomes," and those derived from MMP14 exon4-deficient corneal fibroblasts are denoted as "Δexon4-derived exosomes."

Transmission Electron Microscopy (TEM)

For analysis of the morphology of exosomes, 15 μL of isolated exosomes in PBS were dropped onto 300 mesh Formvar/carbon coated copper grids. The mesh that absorbed the solution was stained with 2% aqueous phosphotungstic acid. Air-dried exosomes samples were observed using a JEOL JEM-1220 transmission electron microscope, operating at an accelerating voltage of 80 kV at 120,000× magnification.

Protein Concentration

The micro BCA protein assay (Thermo Fisher, Rochester, NY, USA) was performed to measure the exosome-associated protein concentration. Exosomes were suspended in 150 μL PBS (1:10 ratio), and this solution was pipetted into a microplate well before serial dilution of BSA (range, 0–200 μg/mL) for the preparation of a standard curve. An equal volume of BCA working solution was thoroughly mixed with samples and standards. Covered plates were incubated at 37°C for 2 hours. The mean and SD values were calculated from three samples for the absorbance at 562 nm on a plate reader (BioTek, Winooski, VT, USA).

In Vitro Proteolysis of VEGFR1 by Exosomes

One microgram of rmVEGFR1 or rmVEGFR2 (R&D Systems, Minneapolis, MN, USA) was incubated alone or with 1 μg of exosomes. The rmVEGFR1 and exosomes were mixed together in a total of 30 μL MMP developing buffer, including 0.02% Brij 35 (w/v) (Invitrogen) and then incubated overnight at 37°C. Pan-MMP inhibitor GM6001 (Calbiochem) was used for inhibition of MMP14 enzymatic activity. Samples were incubated at 37°C for 4 hours and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) for separation for Western blotting. Anti-His (Abcam) was used to determine rmVEGFR expression via Western blotting analysis.

Western Blotting

Samples were mixed with SDS loading dye and then boiled for 10 minutes. Samples were subjected into 4% to 20% SDS-PAGE (Bio-Rad, Hercules, CA, USA). Separated proteins in gel were transferred to a nitrocellulose membrane (Bio-Rad) and blocked with 3% to 5% skim milk in Tris-buffered saline (TBS) for 1 hour. The membranes were first incubated with anti-TSG101 and anti-MMP14 (Abcam) diluted in blocking solution (1:1000) for 1 hour at room temperature and then with fluorescence-conjugated secondary antibody (1:5000; Li-Cor, Lincoln, NE, USA). Blots were developed using the Li-Cor Odyssey system (Li-Cor).

Chemotaxis Assays

Modified Boyden chamber assays were used to investigate VEC migration after treatment with WT-derived exosomes or Δexon4-derived exosomes. Briefly, 2×10^4 HUVEC (ScienCell) were seeded into the lower well of chambers containing polycarbonate membranes with 12-mm pores. The chambers were inverted and then incubated at 37°C for 2 hours. Medium containing WT-derived exosomes or Δexon4-derived exosomes was added to the upper well for incubation at 37°C for 4 hours. Cells that had migrated onto the membrane were fixed with methanol after nonmigrating HUVEC had been washed away. After staining with hematoxylin and eosin (H&E), the mean numbers of migrated HUVECs were counted in four wells per group on a bright field microscope (DMi1; Leica Microsystems), and SD values were calculated.

Cell Proliferation Assay

Endothelial cell growth medium including supplements (EBM-2; Lonza) was used to maintain HUVEC (ScienCell). Mouse corneal fibroblast cells were maintained with DMEM including 10% FBS (Sigma-Aldrich Corp.) under 37°C, 5% CO_2, and 95% of humidity. Three thousand cells were seeded in wells of 96-well clear-bottom plates (Millipore) with 100 μL complete medium. The next day, the culture medium was replaced with fresh media, including various concentrations (12.5–200 μg/mL) of WT-derived or Δexon4-derived exosomes. To evaluate the degree of VEGFA-induced HUVEC proliferation following exosome treatment, 100 μL fresh serum-free medium containing 5 ng/mL VEGFA or lacking VEGFA was added to each well. Bromodeoxyuridine (BrdU, 10 M; Roche, Mannheim, Germany) was added for measurement of HUVEC proliferation following the manufacturer's instructions. Incorporated BrdU was detected with anti-BrdU-peroxidase-conjugated antibody, and chemiluminescence was measured using in a microplate luminometer (BioTek).

Immobilization of Exosomes Onto Latex Beads

To investigate the spatio location of MMP14 on exosomes, we used aldehyde/sulfate latex beads (Invitrogen) to capture

exosomes. Briefly, 4×10^8 exosome particles were mixed with 10 μL of 4% of aldehyde/sulfate latex beads and incubated for 10 minutes at room temperature. Two hundred microliters of 1% BSA/PBS was added for blocking of the aldehyde/sulfate latex beads for 1 hour. Samples were washed with 1 mL PBS using centrifugation at 3050*g* for 5 minutes. Residual aldehyde/sulfate was inactivated by 50 mM glycine solution. The exosomes/latex complexes were resuspended in 10 μL PBS and stored at 4°C until use.

Zymography Analysis

Equal amounts of exosomes/latex complex were resuspended in 20 μL detergent free-MMP activation buffer (50 mM Tris-HCl, 50 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnSO_4$, pH 7.5) with a total of 50 ng of pro-MMP2 (Calbiochem). Samples were incubated at 37°C for 4 hours and then mixed with 5 μL of reducing agent free-4X SDS loading dye (Invitrogen). Samples were loaded and separated on a 10% zymogram gelatin gel (Invitrogen). The zymogram gel was rinsed with deionized water and then incubated with denaturing buffer (Invitrogen) for 2 hours to remove the SDS from the gel. Each gel was incubated overnight with developing buffer (Invitrogen). Coomassie brilliant blue R-250 (Bio-Rad) was used to stain the gel, and images were taken using the ChemiDOC image system (Bio-Rad). Pro-MMP2 with only aldehyde/sulfate latex was used as a control.

FACS Analysis

Equal amounts of exosomes/latex complexes were resuspended with 100 μL of 10% normal goat serum/PBS for blocking. The exosomes/latex complexes were washed with 1 mL of 1% BSA/PBS and then incubated with FITC-conjugated MMP14 antibody in 1% BSA/PBS (1:100, Abcam) for 1 hour at room temperature. Unbound antibody was washed with 1 mL of 1% BSA/PBS three times. Exosomes/latex complexes were resuspended with 500 μL of 2.5 mM EDTA/1% BSA/PBS for FACS analysis using the Sony SH800S cell sorter (Sony Biotechnology, San Jose, CA, USA). Rabbit IgG was used as a control. Exosomes/latex complexes were separated based on forward and side scatter, and FITC parameter histograms were used to compare staining of fluorophore-conjugated MMP14 on the exosomes/latex complexes with WT versus Δexon4-derived exosomes.

In Vitro Proteolysis of Exosomes by Catalytic Domain of MMP14

One hundred micrograms of exosomes of each type were incubated with or without 0.2 μg of the catalytic domain of MMP14 enzyme (Calbiochem) in 40 μL of MMP reaction buffer for 1 hour at 37°C. The MMP14 enzyme reaction was terminated by adding 4X LDS sample buffer, including β-mercaptoethanol. Exosomal proteins were separated by electrophoresis using 4% to 20% polyacrylamide gel (Invitrogen) and stained by Coomassie stain solution (Bio-Rad). For mass spectrometry, proteins bands were cut off from stained SDS-PAGE gel with Coomassie blue (Bio-Rad). Samples were analyzed by the UCSD Proteomics Facility (http://bpmsf.ucsd.edu). Counts for the total spectrum from Scaffold 4 viewer software, and molecular weight and accession numbers were compared.

Statistical Analysis

Statistical analyses of immunocytochemistry, migration, proliferation, and protein concentration results were performed using Prism 6 software (GraphPad). A paired *t*-test was used to compare results between different groups.

Results

MMP14 Cleaved Membrane-Bound VEGFR1 on HUVEC

We previously determined that VEGFR1 cleavage by MMP14 affects downstream VEGFA signaling in HUVEC.[15,16] To determine that only membrane-bound VEGFR1 was cleaved by the exogenous catalytic domain of MMP14 and not VEGFR2 on HUVEC, we stained VEGFR1 and R2 after incubation with the catalytic domain of MMP14. Depletion of VEGFR1 was observed in the samples treated with the catalytic domain of MMP14 but not in the untreated control group (Fig. 1A). VEGFR2 staining was observed not to be affected by treatment with the catalytic domain of MMP14 (Fig. 1B). Further, the amount of surface VEGFR1 eliminated upon treatment with the catalytic domain of MMP14 was determined to be approximately 54% (Fig. 1C).

rmVEGFR1 Was Diminished by WT-Derived Exosomes

Exosomes were isolated from WT and MMP14 Δexon4-deficient corneal fibroblasts. The morphology of purified WT-derived exosomes was first evaluated by TEM, and a round shape was observed (Fig. 2A, white arrows). In our proteolysis analysis, we found that the amount of rmVEGFR1 was reduced after incubation with WT-derived exosomes compared with that in control medium (Fig. 2B). To determine whether this cleavage of rmVEGFR1 was caused by MMP14 within the WT-derived exosomes, pan-MMP inhibitor GM6001, a general inhibitor of MMPs was added during the incubation with WT-derived exosomes. The results showed that pan-MMP inhibitor GM6001 effectively inhibited the protease activity of WT-derived exosomes with the level of rmVEGFR1 remaining comparable to that in control conditions (Fig. 2C). Consistent with our findings for rmVEGFR2 incubated with the catalytic domain of MMP14, the amount of rmVEGFR2 was not affected by treatment with WT-derived exosomes (Fig. 2D), indicating that exposure to these exosomes did not result in VEGFR2 cleavage. Overall, the results of this experiment indicate that MMP14 in the WT-derived exosomes retain proteolytic activity for cleavage of VEGFR1.

Lack of VEGFR1 Cleavage Upon Exposure to Δexon4-Derived Exosomes

To further investigate whether VEGFR1 cleavage upon exposure to WT-derived exosomes is due to MMP14 contained within those exosomes, we purified exosomes from MMP14 Δexon4-deficent corneal fibroblasts. The morphology of the Δexon4-derived exosomes was also observed by TEM, and the general shape of Δexon4-derived exosomes was similar to that of WT-derived exosomes (Fig. 3A). Using the same proteolysis experimental design, we observed that the level of rmVEGFR1 after incubation with Δexon4-derived exosomes remained similar to that in control medium (Fig. 3B). To confirm the absence of MMP14 in Δexon4-derived exosomes, we performed Western blotting for both WT-derived and Δexon4-derived exosomes. MMP14 was found only in WT-derived exosomes and not in Δexon4-derived exosomes, and the exosomal marker protein TSG101, as a control, was present in both exosome samples (Fig. 3C). Taken together, our results indicate that VEGFR1 can be cleaved by MMP14 contained within corneal fibroblast-derived exosomes.

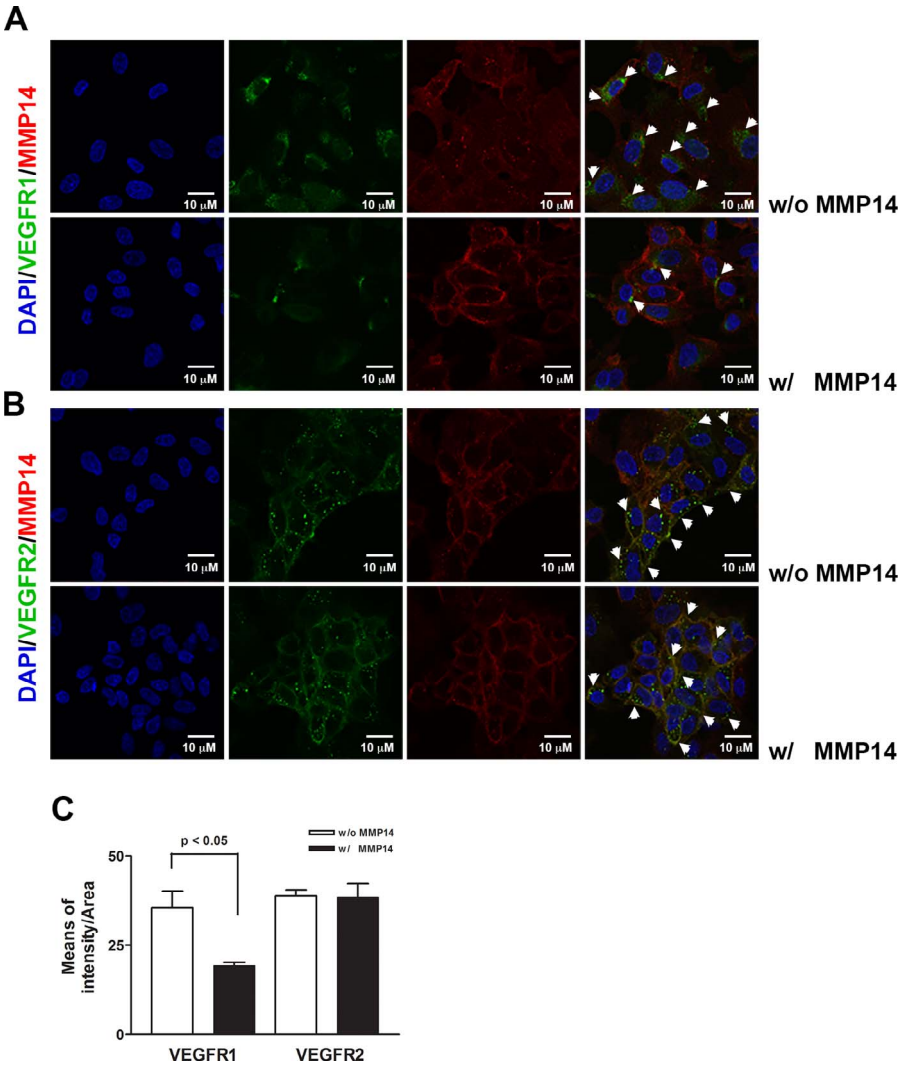

Figure 1. Cleavage of surface VEGFR1 on HUVEC by exogenously delivered catalytic domain of MMP14. (**A**) Immunostained-VEGFR1 (*green fluorescence*) was observed on HUVEC after incubation with (*bottom panel*) or without (*top panel*) the exogenous catalytic domain of MMP14 (50 ng/mL). MMP14 (*red fluorescence*) and nuclei (*blue fluorescence*) were also stained. (**B**) Immunostained-VEGFR2 (*green fluorescence*) was observed on HUVEC after incubation with (*bottom panel*) or without (*top panel*) the exogenous catalytic domain of MMP14. *Arrows* indicates positive staining of VEGFR1 and R2. *Scale bar*: 10 μm. (**C**) Quantitated intensity of green fluorescence representing the levels of VEGFR1 and VEGFR2 expression on HUVECs after incubation with or without the exogenous catalytic domain of MMP14.

Effects of Fibroblast-Derived Exosomes on Endothelial Cell Migration and Proliferation

Boyden chamber analysis was performed to compare the chemotactic effects of WT-derived exosomes and Δexon4-derived exosomes on HUVEC. We found that HUVEC migrated toward WT-derived exosomes in a dose-dependent manner. However, Δexon4-derived exosomes did not attract HUVEC, even at a high concentration (Fig. 4A). We then questioned whether a difference in protein concentration within the exosomes might affect the induced HUVEC migration. We first compared the size of the WT-derived exosomes and Δexon4-derived exosomes by NTA. Equal numbers of WT-derived exosomes and Δexon4-derived exosomes were used for determination of their protein content using the Micro-BCA method. The results showed that Δexon4-derived exosomes contained approximately twice as much protein as WT-derived exosomes (Fig. 4B). We then repeated the Boyden chamber analysis with exposure of the HUVEC to different amounts of the exosomes corresponding to equal protein concentrations and observed the same induction of migration by the WT-derived exosomes and lack of migration toward the Δexon4-derived exosomes (Fig. 4C).

Next, to investigate the potential role of VEGFR1 cleavage by exosomally delivered MMP14 in VEGFA-induced angiogenesis, HUVEC proliferation was observed upon stimulation with or without 100 ng/mL VEGFA following incubation with same protein concentration of WT-derived exosomes and Δexon4-derived exosomes. We discovered that HUVEC showed greater sensitivity (and thus greater proliferation) to this low concentration of VEGFA (100 pg/mL) after incubation with WT-derived exosomes than after incubation with Δexon4-derived exosomes (Fig. 4D). Furthermore, it was observed that exposure to Δexon4-derived exosomes delayed the proliferation of HUVEC in the absence of VEGFA stimulation (Fig. 4D). Together, these results suggest that MMP14-containing exosomes could contribute to the induction of angiogenesis by promoting cell migration and VEGFA-induced proliferation via the cleavage of VEGFR1 on endothelial cells.

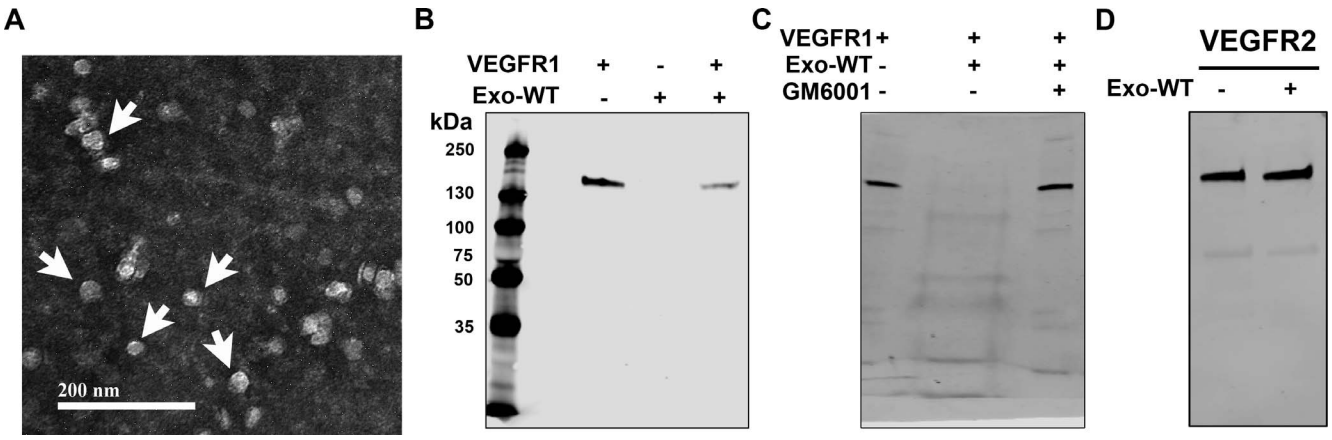

Figure 2. Cleavage of VEGFR1 by WT-derived exosomes. (**A**) Representative TEM image showing the round and uniform morphology of WT-derived exosomes. (**B**) One microgram of VEGFR1 was incubated overnight with 1 μg of WT-derived exosomes in 30 μL of reaction buffer or in control conditions. (**C**) The same concentration of VEGFR1 was incubated overnight with WT-derived exosomes and 50 nM of pan-MMP inhibitor GM6001 or in control conditions. (**D**) One microgram of VEGFR2 was incubated overnight with 1 μg of WT-derived exosomes or in control conditions. VEGFR1 and R2 were detected using anti-His antibody via Western blot analysis.

Distribution of MMP14 on Exosomes

To characterize the spatiotemporal location of MMP14 on exosomes, we used an aldehyde/sulfate latex system to immobilize isolated exosomes. Conversion of active-MMP2 form (62 kDa) was observed after incubation of a pro-MMP2 (72 kDa) with WT-derived exosomes/latex complexes. ΔExon4-derived exosomes/latex complexes or latex beads, as a control, did not show transition of pro-MMP2 to active MMP2 (Fig. 5A). To minimize rupture of exosomes during incubation, detergent-free MMP buffer was used. To determine the external localization of MMP14 in the exosomal membrane, exosomes/latex complexes were analyzed with anti-MMP14 antibody

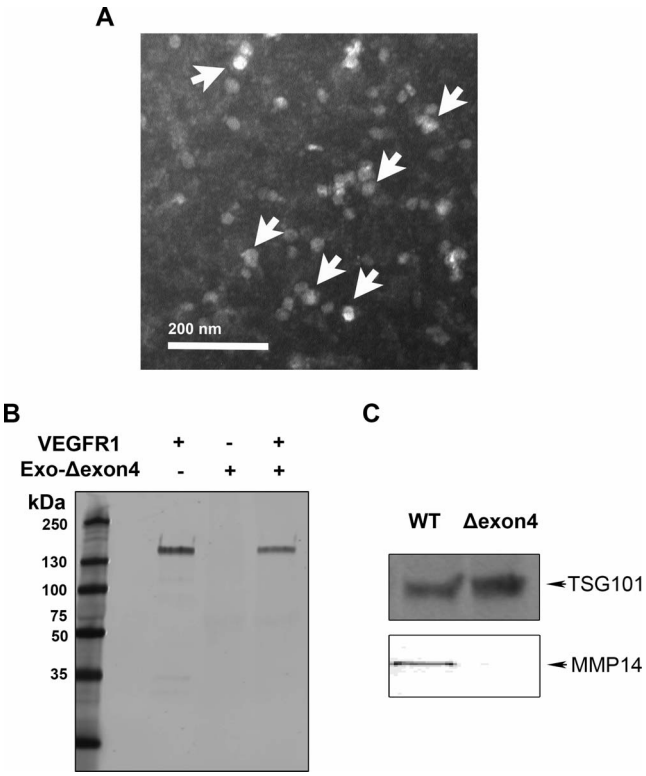

Figure 3. VEGFR1 cleavage upon exposure to Δexon4-derived exosomes. (**A**) Representative TEM image showing the round and uniform morphology of Δexon4-derived exosomes. (**B**) One microgram of VEGFR1 was incubated with 1 μg of Δexon4-derived exosomes or in control conditions. VEGFR1 was detected using anti-His antibody via Western blot analysis. (**C**) Western blot detection of MMP14 and TSG101, an exosomal marker protein, in 10 μg of WT-derived exosomes and Δexon4-derived exosomes.

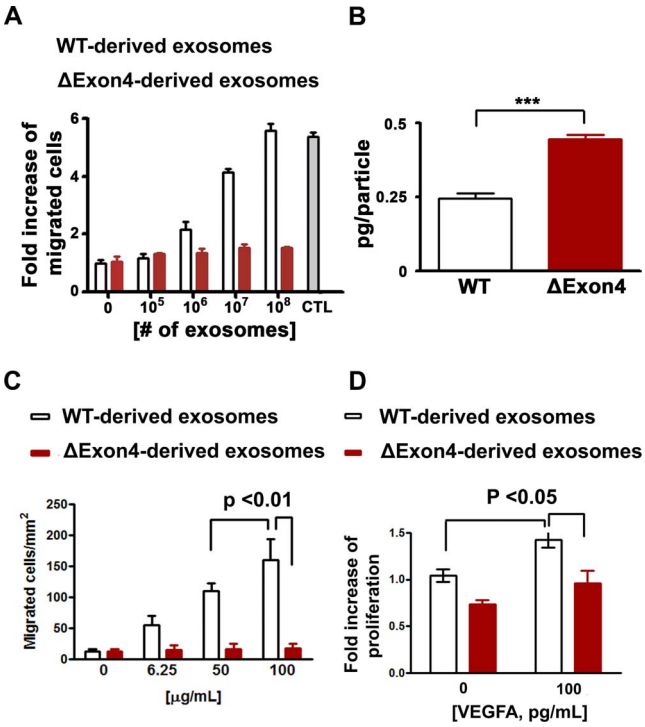

Figure 4. Migration and VEGFA-induced proliferation of HUVECs with exposure to WT-derived exosomes and Δexon4-derived exosomes. (**A**) HUVEC migration toward medium containing different amounts of WT-derived exosomes or Δexon4-derived exosomes. Medium only was used as a negative control, and complete medium including supplements was used as a positive control. (**B**) Protein content of WT-derived exosomes vs. Δexon4-derived exosomes. (**C**) HUVEC migration toward medium containing WT-derived exosomes or Δexon4-derived exosomes in amounts corresponding to equal protein concentrations. (**D**) VEGFA-induced HUVEC proliferation rate after treatment with WT-derived exosomes or Δexon4-derived exosomes.

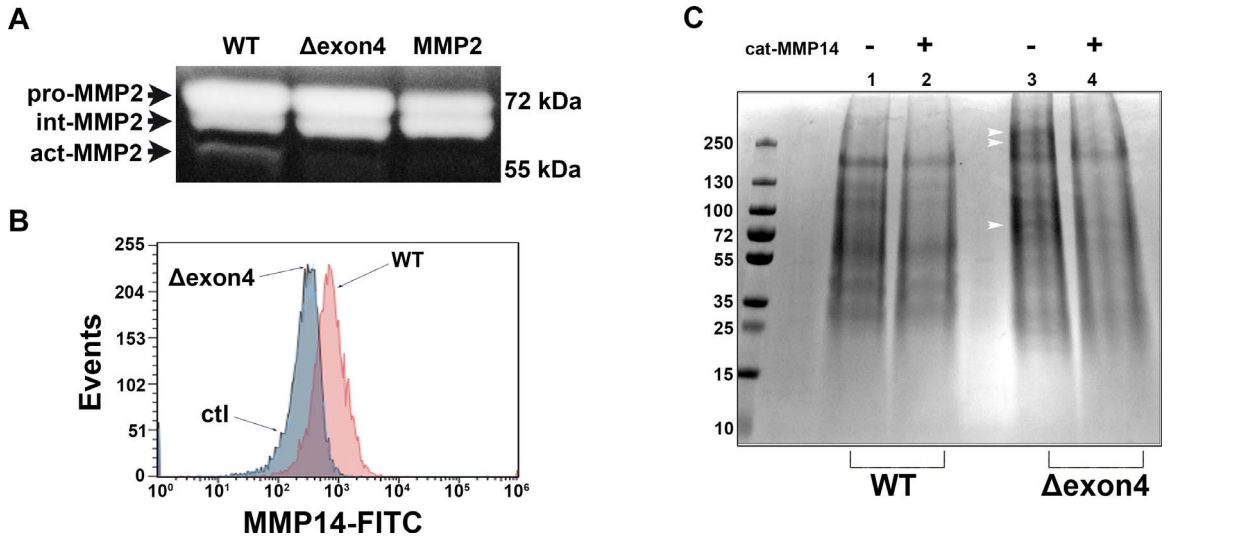

Figure 5. Distribution of MMP14 on exosomes and in vitro proteolysis of exosomes by catalytic domain of MMP14. (**A**) WT-derived exosomes or Δexon4-derived exosomes (4×10^8 particles) were immobilized on aldehyde/sulfate latex beads and incubated with 50 ng (w/v) pro-MMP2 in 20 μL reaction buffer without detergent for zymography analysis to measure active MMP2. Cleaved pro-MMP2 (around 62 kDa) as act-MMP2 was found in WT-derived exosomes/latex complexes but not in Δexon4-derived exosomes/latex complexes. Pro-MMP2 with aldehyde/sulfate latex beads was used for control. (**B**) FACS analysis was performed to identify the presence/orientation of MMP14 on exosomal surface membrane WT-derived exosomes or Δexon4-derived exosomes (4×10^8 particles) were immobilized on aldehyde/sulfate latex beads and incubated with FITC-conjugated MMP14 antibody. The positive FITC signal was observed only for WT-derived exosomes/latex complexes. The signal from Δexon4-derived exosomes/latex complexes was similar to that from aldehyde/sulfate latex beads as a control. (**C**) Cleavage of exosomal proteins by catalytic domain of MMP14. One hundred micrograms of WT-derived exosomes (*lane 1*) and Δexon4-derived exosomes (*lane 3*) without the catalytic domain of MMP14 were separated by SDS-PAGE and detected using Coomassie staining. Bands were cut out for mass spectrometry to identify proteins.

recognizing the enzymatic domain of MMP14 via FACS. The results in Figure 5B show a shift in the FITC-histogram between WT-derived exosomes/latex complexes and Δexon4-derived exosomes/latex complexes or latex beads, as a control. Together, these results indicated that activated MMP14 was located on the exosomal surface membrane and could activate pro-MMP2 to become active MMP2. Additionally, to investigate whether the composition of proteins in corneal fibroblast-derived exosomes was affected by MMP14 enzyme activity, WT-derived exosomes and Δexon4-derived exosomes were incubated with catalytic MMP14 enzyme for in vitro proteolysis analysis. We determined that some proteins bands were diminished after incubation with catalytic MMP14 enzyme according to Coomassie staining of SDS-PAGE gels (Fig. 5C, indicated with white arrows on lane 3). However, no proteins bands disappeared when WT-derived exosomes were treated with MM14 enzyme (Fig. 5C, lanes 1 and 2). The displayed image for Δexon4 derived-exosomes after proteolysis by MMP14 enzyme (lane 4) was similar with that for WT derived-exosomes (lane 1) or even MMP14 enzyme-treated WT derived-exosomes (lane 2). The three locations (marked as white arrows in lane 3) on the gel were cut out for protein identification by mass spectrometry, which identified fibronectin (253 kDa), complement C3 (186 kDa), moesin (68 kDa), and MMP2 (74 kDa) as the proteins eliminated by MMP14 enzyme.

Discussion

In the present study, we investigated whether MMP14-containing exosomes secreted by corneal fibroblasts convey enzymatic activity for the cleavage of VEGFR1. We found that the catalytic domain of MMP14 cleaves VEGFR1 and the heat-inactivated catalytic domain of MMP14 lost the ability to cleave VEGFR1 in our previous studies.[15,16] Our experiments demonstrated that WT corneal fibroblast-derived exosomes had active MMP14 enzyme that could cleave VEGFR1 in a manner similar to the purified catalytic protein. Further experiments with exosomes isolated from MMP14-exon4 deficient corneal fibroblasts (Δexon4-derived exosomes) as well as pan-MMP inhibitor GM6001 confirming that MMP14 within the WT-derived exosomes was responsible for the observed cleavage of VEGFR1.

VEGFR1 is a decoy receptor for VEGFR2 and has a weaker kinase activity but higher affinity for VEGFA than does VEGFR2 on HUVEC. Our findings that MMP14-containing exosomes cleaved VEGR1 but not VEGFR2 on HUVEC suggest that the WT-derived exosomes may promote angiogenesis by cleaving VEGFR1 to facilitated greater VEGFR2 activation upon binding of VEGFA. The physiological serum concentration of VEGF ranges from 192.8 to 242.5 pg/mL for healthy individuals.[42] However, geometric mean VEGF levels are elevated to 325.5–484.3 pg/mL in patients with conditions such as chondrosarcoma and Ewing sarcoma.[42] All VEGF receptors are structurally similar, with the seven Ig-type receptors and VEGFR1 probe having high (>95%) homology among humans, mice, and rats.[43] The homology of MMP14 also was found to be more than 92% among humans, mice, and rats.[44] Cross-reaction between WT-derived exosomes containing MMP14 could cleave VEGFR1 of human endothelial cells because of the high homology between these proteins in humans and mice. Mouse fibroblast-derived exosomes may have undesired consequences in human endothelial cells, due to the presence of mouse-derived biomaterials on the exosomes. However, human endothelial cells are widely used in experiments studying VEGF-induced migration and proliferation in which complexes are formed between VEGF and the full-length VEGF receptors.[45,46] In our previous research, we observed similar results for fusion of exosomes and exosome-induced proliferation among human, mouse, and calf endothelial cells. Notably, cultured endothelial cells did not exhibit ERK activation in response to stimulation with 100 pg/mL VEGFA, but after

treatment of these cells with MMP14 for VEGFR1 cleavage, ERK was dramatically activated by same concentration of VEGFA.[16] In zymography analysis to determine the activity of MMP14-containing exosomes, we found that active MMP14 was anchored on exosomes. In our study, immobilized WT-derived exosomes on latex beads showed cleavage of the propeptide of MMP2 in the condition of MMP activation buffer without detergent. Furthermore, WT-derived exosomes/latex complexes were stained by anti-MMP14-FITC on FACS analysis. These results indicate that active MMP14 protein faced externally on the exosomal surface membrane. Coomassie staining revealed that Δexon4-derived exosomes had additional proteins compared with WT-derived exosomes. We found that substrates of MMP14 including fibronectin, complement C3, moesin, and MMP2 were present in diminished quantities in WT-derived exosomes. We propose that Δexon4-derived exosomes contained more proteins due to MMP14 inactivation.

Considering that MMP14 is a membrane-anchored protein, exosomes may be the perfect natural delivery vehicle for transferring active MMP14 enzyme to HUVECs for the regulation of VEGFA-induced angiogenesis via modulation of VEGFR1 levels. It is well established that corneal stroma, keratocytes are already differentiated, quiescent cells that transform into fibroblasts during corneal wound healing and neovascularization.[40,47] In this process, MMP14-containing exosomes produced by corneal fibroblasts have pro-angiogenic activity and may provide a mechanism of cell-to-cell communication with endothelial cells. Together, the results of the present study suggest that inhibition of MMP14 and regulation of MMP14-containing exosomes are potential mechanisms by which corneal fibroblast-derived exosomes modulate VEGFR1 activity, particularly in VEGFA-induced corneal neovascularization.

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References

1. Massova I, Kotra LP, Fridman R, Mobashery S. Matrix metalloproteinases: structures, evolution, and diversification. *FASEB J*. 1998;12:1075–1095.
2. Lindsey ML. Assigning matrix metalloproteinase roles in ischaemic cardiac remodelling. *Nat Rev Cardiol*. 2018;15:471–479.
3. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science*. 2002;295:2387–2392.
4. Jablonska-Trypuc A, Matejczyk M, Rosochacki S. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. *J Enzyme Inhib Med Chem*. 2016;31:177–183.
5. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. *Nat Rev Mol Cell Biol*. 2014;15:786–801.
6. Rabelink TJ, van den Berg BM, Garsen M, Wang G, Elkin M, van der Vlag J. Heparanase: roles in cell survival, extracellular matrix remodelling and the development of kidney disease. *Nat Rev Nephrol*. 2017;13:201–212.
7. Pahwa S, Stawikowski MJ, Fields GB. Monitoring and inhibiting MT1-MMP during cancer initiation and progression. *Cancers (Basel)*. 2014;6:416–435.
8. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. *Biochim Biophys Acta*. 2010;1803:55–71.
9. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol*. 2007;8:221–233.
10. Mattei MG, Roeckel N, Olsen BR, Apte SS. Genes of the membrane-type matrix metalloproteinase (MT-MMP) gene family, MMP14, MMP15, and MMP16, localize to human chromosomes 14, 16, and 8, respectively. *Genomics*. 1997;40:168–169.
11. Apte SS, Fukai N, Beier DR, Olsen BR. The matrix metalloproteinase-14 (MMP-14) gene is structurally distinct from other MMP genes and is co-expressed with the TIMP-2 gene during mouse embryogenesis. *J Biol Chem*. 1997;272:25511–25517.
12. Deryugina EI, Quigley JP. Tumor angiogenesis: MMP-mediated induction of intravasation- and metastasis-sustaining neovasculature. *Matrix Biol*. 2015;44-46:94–112.
13. Kato T, Kure T, Chang JH, et al. Diminished corneal angiogenesis in gelatinase A-deficient mice. *FEBS Lett*. 2001;508:187–190.
14. Onguchi T, Han KY, Chang JH, Azar DT. Membrane type-1 matrix metalloproteinase potentiates basic fibroblast growth factor-induced corneal neovascularization. *Am J Pathol*. 2009;174:1564–1571.
15. Han KY, Dugas-Ford J, Lee H, Chang JH, Azar DT. MMP14 cleavage of VEGFR1 in the cornea leads to a VEGF-trap antiangiogenic effect. *Invest Ophthalmol Vis Sci*. 2015;56:5450–5456.
16. Han KY, Chang JH, Lee H, Azar DT. Proangiogenic interactions of vascular endothelial MMP14 with VEGF receptor 1 in VEGFA-mediated corneal angiogenesis. *Invest Ophthalmol Vis Sci*. 2016;57:3313–3322.
17. Han KY, Chang JH, Azar DT. MMP14 regulates VEGFR3 expression on corneal epithelial cells. *Protein Pept Lett*. 2016;23:1095–1102.
18. Chan KM, Wong HL, Jin G, et al. MT1-MMP inactivates ADAM9 to regulate FGFR2 signaling and calvarial osteogenesis. *Dev Cell*. 2012;22:1176–1190.
19. Piper RC, Katzmann DJ. Biogenesis and function of multivesicular bodies. *Annu Rev Cell Dev Biol*. 2007;23:519–547.
20. Tachiyama R, Ishikawa D, Matsumoto M, et al. Proteome of ubiquitin/MVB pathway: possible involvement of iron-induced ubiquitylation of transferrin receptor in lysosomal degradation. *Genes Cells*. 2011;16:448–466.
21. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 2014;30:255–289.
22. Witwer KW, Buzas EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles*. 2013;2:10.3402/jev.v2i0.20360.
23. Willms E, Johansson HJ, Mager I, et al. Cells release subpopulations of exosomes with distinct molecular and biological properties. *Sci Rep*. 2016;6:22519.
24. Rashed MG, Bayraktar E, Helal GK, et al. Exosomes: from garbage bins to promising therapeutic targets. *Int J Mol Sci*. 2017;18:538.
25. Shibuya M. Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) signaling in angiogenesis: a crucial target for anti- and pro-angiogenic therapies. *Genes Cancer*. 2011;2:1097–1105.
26. Tao Q, Backer MV, Backer JM, Terman BI. Kinase insert domain receptor (KDR) extracellular immunoglobulin-like domains 4-7 contain structural features that block receptor dimerization and vascular endothelial growth factor-induced signaling. *J Biol Chem*. 2001;276:21916–21923.

Investigative Ophthalmology & Visual Science

27. Meyer RD, Mohammadi M, Rahimi N. A single amino acid substitution in the activation loop defines the decoy characteristic of VEGFR-1/FLT-1. *J Biol Chem*. 2006;281:867–875.
28. Cebe-Suarez S, Zehnder-Fjallman A, Ballmer-Hofer K. The role of VEGF receptors in angiogenesis; complex partnerships. *Cell Mol Life Sci*. 2006;63:601–615.
29. Nowak G, Karrar A, Holmén C, et al. Expression of vascular endothelial growth factor receptor-2 or Tie-2 on peripheral blood cells defines functionally competent cell populations capable of reendothelialization. *Circulation*. 2004;110:3699–3707.
30. Haigh JJ. Role of VEGF in organogenesis. *Organogenesis* 2008;4:247–256.
31. Cao Y. Positive and negative modulation of angiogenesis by VEGFR1 ligands. *Sci Signal* 2009;2:re1.
32. Rahimi N, Golde TE, Meyer RD. Identification of ligand-induced proteolytic cleavage and ectodomain shedding of VEGFR-1/FLT1 in leukemic cancer cells. *Cancer Res* 2009;69:2607–2614.
33. Chang JH, Garg NK, Lunde E, Han KY, Jain S, Azar DT. Corneal neovascularization: an anti-VEGF therapy review. *Surv Ophthalmol* 2012;57:415–429.
34. Meek KM, Knupp C. Corneal structure and transparency. *Prog Retin Eye Res* 2015;49:1–16.
35. Ye HQ, Maeda M, Yu FS, Azar DT. Differential expression of MT1-MMP (MMP-14) and collagenase III (MMP-13) genes in normal and wounded rat corneas. *Invest Ophthalmol Vis Sci* 2000;41:2894–2899.
36. Han KY, Dugas-Ford J, Seiki M, Chang JH, Azar DT. Evidence for the involvement of MMP14 in MMP2 processing and recruitment in exosomes of corneal fibroblasts. *Invest Ophthalmol Vis Sci*. 2015;56:5323–5329.
37. Booth AM, Fang Y, Fallon JK, Yang JM, Hildreth JE, Gould SJ. Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. *J Cell Biol*. 2006;172:923–935.
38. Robinson DG, Ding Y, Jiang L. Unconventional protein secretion in plants: a critical assessment. *Protoplasma*. 2016;253:31–43.
39. Hakulinen J, Sankkila L, Sugiyama N, Lehti K, Keski-Oja J. Secretion of active membrane type 1 matrix metalloproteinase (MMP-14) into extracellular space in microvesicular exosomes. *J Cell Biochem*. 2008;105:1211–1218.
40. Han KY, Tran JA, Chang JH, Azar DT, Zieske JD. Potential role of corneal epithelial cell-derived exosomes in corneal wound healing and neovascularization. *Sci Rep*. 2017;7:40548.
41. Azar DT, Casanova FH, Mimura T, Jain S, Chang JH. Effect of MT1-MMP deficiency and overexpression in corneal keratocytes on vascular endothelial cell migration and proliferation. *Curr Eye Res*. 2008;33:954–962.
42. Holzer G, Obermair A, Koschat M, Preyer O, Kotz R, Trieb K. Concentration of vascular endothelial growth factor (VEGF) in the serum of patients with malignant bone tumors. *Med Pediatr Oncol*. 2001;36:601–604.
43. Kim I, Ryan AM, Rohan R, et al. Constitutive expression of VEGF, VEGFR-1, and VEGFR-2 in normal eyes. *Invest Ophthalmol Vis Sci*. 1999;40:2115–2121.
44. Sato T, del Carmen Ovejero M, Hou P, et al. Identification of the membrane-type matrix metalloproteinase MT1-MMP in osteoclasts. *J Cell Sci*. 1997;110(pt 5):589–596.
45. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A*. 1993;90:10705–10709.
46. Roeckl W, Hecht D, Sztajer H, Waltenberger J, Yayon A, Weich HA. Differential binding characteristics and cellular inhibition by soluble VEGF receptors 1 and 2. *Exp Cell Res*. 1998;241:161–170.
47. Kim A, Lakshman N, Karamichos D, Petroll WM. Growth factor regulation of corneal keratocyte differentiation and migration in compressed collagen matrices. *Invest Ophthalmol Vis Sci*. 2010;51:864–875.

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