MMP-2 and MMP-14 Silencing Inhibits VEGFR2 Cleavage and Induces the Differentiation of Porcine Adipose-Derived Mesenchymal Stem Cells to Endothelial Cells

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Key Words. Adipose-derived mesenchymal stem cells • Matrix metalloproteinases • MMP-2 • MT1-MMP • MMP-14 • Endothelial cell differentiation • Vascular endothelial growth factor receptor type 2

ABSTRACT

The molecular mechanisms that control the ability of adipose-derived mesenchymal stem cells (AMSCs) to remodel three-dimensional extracellular matrix barriers during differentiation are not clearly understood. Herein, we studied the expression of matrix metalloproteinases (MMPs) during the differentiation of AMSCs to endothelial cells (ECs) in vitro. AMSCs were isolated from porcine abdominal adipose tissue, and characterized by immunopositivity to CD44, CD90, CD105, and immunonegativity to CD14 and CD45. Plasticity of AMSCs was confirmed by multilineage differentiation. The mRNA transcripts for MMP-14 and MMP-2 enzyme activity were analyzed by gelatin zymography, enzyme-linked immunosorbent assay (ELISA), and Western blot. The differentiation of AMSCs to ECs was confirmed by mRNA and protein expressions of the endothelial markers. The mRNA transcripts for MMP-2 and MMP-14 were significantly increased during the differentiation of MSCs into ECs. Findings revealed an elevated MMP-14 and MMP-2 expression, and MMP2 enzyme activity. Silencing of MMP-2 and MMP-14 significantly increased the expression of EC markers, formation of capillary tubes, and acetylated-low-density lipoprotein uptake, and decreased the cleavage of vascular endothelial growth factor receptor type 2 (VEGFR2). Inhibition of VEGFR2 significantly decreased the expression of EC markers. These novel findings demonstrate that the upregulation of MMP2 and MMP14 has an inhibitory effect on the differentiation of AMSCs to ECs, and silencing these MMPs inhibit the cleavage of VEGFR2 and stimulate the differentiation of AMSCs to ECs. These findings provide a potential mechanism for the regulatory role of MMP-2 and MMP-14 in the re-endothelialization of coronary arteries following intervention. Stem Cells Translational Medicine 2017;6:1385–1398

SIGNIFICANCE STATEMENT

We, for the first time, demonstrate that the upregulation of matrix metalloproteinase (MMP2) and MMP14 has an inhibitory effect on the differentiation of adipose-derived mesenchymal stem cells (AMSCs) to endothelial cells (ECs), and silencing these MMPs can inhibit the cleavage of vascular endothelial growth factor receptor type 2 (VEGFR2), and stimulate the differentiation of AMSCs to ECs. These findings provide a potential mechanism for the regulatory role of MMP-2 and MMP-14 in the re-endothelialization of coronary arteries following intervention.

INTRODUCTION

Mesenchymal stem cells (MSCs) were first identified as the population of bone marrow (BM) cells. They are characterized by their ability to differentiate into a wide range of mesodermal cell types such as adipocytes, chondrocytes, and osteoblasts [1]. The past decade has witnessed increasing development of cell-based therapeutics for vascular injuries with lead candidates being autologous whole BM and BM-derived MSCs (BM-MSCs) [2–4]. MSCs are particularly suitable for cell therapy because of easy isolation, high expansion potential giving unlimited pool of transplantable cells, low immunogenicity, amenability to ex vivo genetic modification, and multipotency [5–7].

Recent in vitro studies have shown that MSCs derived from adipose tissue have superior proliferative capacity compared to BM-MSCs [8]. Adult BM-MSCs have been successfully engrafted into
ischemic hearts, differentiating into the phenotype of smooth muscle cell, endothelial cell (EC), and cardiomyocytes [7]. However, the BM-MSCs contain only 0.001–0.01% of cell population, which makes their availability low in acute clinical conditions [9]. Adipose-derived MSCs (AMSCs) are promising alternative for regeneration therapy, since adipose tissue is abundant in most individuals and can be harvested easily with less invasive procedure, and least discomfort and donor-site damage. Moreover, adipose tissue has a significantly higher stem cell density than BM, suggesting that a small amount of adipose tissue can yield sufficient stem cells with proliferation and differentiation potential for autologous cell transplantation. Indeed, human AMSCs can differentiate into vascular cells, including ECs, cardiomyocytes, and smooth muscle cells, in vitro, suggesting that adipose-derived MSCs have the potential to regenerate the mono-endothelial layer of denuded arteries or form new capillaries [10–12].

The differentiation programs of MSCs can be affected by many factors such as extracellular matrix (ECM) composition, ligand density, and mechanical rigidity [13]. Even though matrix metalloproteinases (MMPs) initially reported to have an ability to degrade the ECM, MMPs are now known to regulate many biological processes via their involvement in physiological events and pathological processes [14]. MMPs and their inhibitors have an important role in mediating cell–cell adhesion, cell migration and invasion, cell proliferation, apoptosis, and tissue remodeling [15]. MMPs promote the release of ECM bound or cell-surface-bound cytokines which then regulate the differentiation of adipose-derived MSCs to ECs [16, 17]. It has been suggested that ECM signaling regulates EC morphogenesis, and induction of angiogenesis, therefore, can be coincident with the ECM degradation and exposure of ECs to type I collagen [15]. Previous studies demonstrated that MMP-2 and TIMP-2 have an essential effect on the differentiation of human MSCs, and demonstrated the role of MMP-14 in the differentiation of human MSCs to osteoblasts [13, 18]. However, the regulatory role of MMPs in dictating the commitment of AMSCs to ECs has not been clearly studied yet. In this article, we have examined porcine AMSCs for mRNA expression of different MMPs during the EC differentiation. Herein, we demonstrate that MMPs have a direct or indirect regulatory role on the differentiation of AMSCs to ECs.

**Isolation and Culture of Porcine AMSCs**

MSCs were harvested from porcine abdominal fat with slight differences in the isolation procedure from previous published studies [19–22]. The adipose tissue from the abdominal fat was collected from the slaughterhouse and transferred to the laboratory in sterile conditions in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Grand Island, NY, http://www.invitrogen.com) with antibiotics 100 mg/ml penicillin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), and 100 mg/ml streptomycin (Sigma-Aldrich). The transferred adipose tissue was washed extensively with excess amount of phosphate-buffered saline (PBS), and minced into 4–8 mm pieces with sterile scissors. Thereafter, the adipose tissue was digested with 0.2% collagenase type 1 (Sigma-Aldrich) for 30 minutes at 37°C. Enzyme activity was terminated by dilution with DMEM containing 10% fetal bovine serum (FBS) (Gibco, Laboratories, Gaithersburg, MD, http://www.thermofisher.com/us/en/home/brands/gibco.html). The floating cells were separated from the mesenchymal cell fraction by centrifugation at 400g for 10 minutes. The pellets were resuspended in normal culture medium (DMEM, 10% FBS, and 5% penicillin/streptomycin), and filtered through a 100 μm nylon mesh to remove undigested tissue. The cells were then centrifuged in a 1.077 g/ml histopaque (Sigma-Aldrich) density gradient at 400g for 30 minutes. The cells in the interface layer were then collected, washed with DMEM, and resuspended in DMEM containing 10% MSC-specific FBS (MSC-FBS, Gibco), 100 mg/ml penicillin (Sigma-Aldrich), 100 mg/ml streptomycin (Sigma-Aldrich), and 2 mM Glutamax (Invitrogen). The primary cells were cultured in 25 cm² flask at 37°C with 5% CO₂/95% air, and 90% relative humidity. The medium was changed after 24 hours, and then every 3–4 days until the cells reached confluency in about 8–10 days. The cells were then passaged and used in subsequent experiments between passage 3 and 6. Human umbilical vein ECs (HUVECs, Lonza, Walkersville, MD, http://www.lonza.com) were cultured in endothelial growth medium (EGM-2, Lonza).

**Characterization of AMSCs**

**Immunostaining.** Immunofluorescence staining for CD14, CD45, CD44, and CD105 was performed and examined under fluorescence microscopy to observe the AMSC surface markers. Immunofluorescence staining for vascular endothelial growth factor receptor type 2 (VEGFR2) and phospo-VEGFR2 was also performed. AMSCs were seeded in four chamber slides, and left in at 37°C till they reached 60%–70% confluency. The cells were then fixed with 3.7% formaldehyde in PBS for 10 minutes, rinsed with PBS three times, and permeabilized by incubating with 0.1% Triton-X 100 in PBS for 10 minutes. Thereafter, the cells on the slides were incubated with blocking solution containing 0.1% BSA in PBS for 1 hour, and further incubated with CD14, CD45, CD44, CD90, and CD105 antibodies (1:100 dilution) for 1 hour at room temperature. This step was followed by PBS washing, and the cells on the slides were further incubated with fluorescent tagged-secondary antibody for 30 minutes. The cells on the slides were then washed with PBS, and mounted in Vectashield with 4’,6-Diamidino-2-Phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, https://vectorlabs.com). The stained cells were examined and photographed using an upright fluorescent microscope.

**Immunophenotyping.** Flow cytometric analysis was done to identify the MSC markers CD14, and CD90, macrophage marker CD14, and hematopoietic stem cell marker CD45. MSCs at passage 3–6 were washed with PBS, and detached from the monolayer with 0.25% trypsin-ethylenediamine tetra-acetic acid (trypsin-EDTA) (Sigma-Aldrich). The dissociated cells were centrifuged and washed with PBS supplemented with 4% FBS, and incubated for 1 hour at 4°C in the dark with conjugated antigen-specific antibodies that were diluted to the appropriate concentrations. Direct conjugated antigen-specific antibodies were used against CD14, CD45, CD44, and CD90 (eBioscience, CA, http://www.ebioscience.com/). The cells were then washed three times in PBS, and resuspended in 500 μl PBS. Flow cytometry was performed on a fluorescence-activated cell sorting (FACS) Aria Flow Cytometry System (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com/).
chamber slide, and incubated with DMEM containing 10% MSC-specific FBS (MSC-FBS, Gibco), 100 mg/ml penicillin (Sigma-Aldrich), 100 mg/ml streptomycin (Sigma-Aldrich), and 2 mM Glutamax (Invitrogen). The incubation with adipogenesis differentiation medium was started at 80%–90% confluency. The medium was replaced every 3 days, and the cells were maintained in a CO2 incubator at 37°C and 5% CO2. The cells were analyzed for adipogenic differentiation by oil red O staining after 20 days of stimulation, and observed under a bright field microscope.

AMSCs were also stimulated for chondrogenic differentiation by STEMPRO chondrogenesis differentiation kit (Gibco). Cells were seeded, and incubated for 20 days. Differentiation analysis was then done using Alcian Blue, and observed under a bright field microscope.

Similarly, osteogenic differentiation was done using STEMPRO osteogenesis differentiation kit (Gibco). The cells were analyzed for osteogenic differentiation by alizarin red S staining after 15 days of stimulation, and observed under a bright field microscope.

**EC Characterization of Differentiating AMSCs**

**EC Differentiation.** AMSCs at passage 3–6 were used for EC differentiation. The stimulation was started when the cells were 50%–60% confluent. The MSCs were stimulated with endothelial basal medium (EBM) containing growth supplements and 2% FBS (EGM: EGM-2 Bullet kit, Lonza), and 50 ng/ml of VEGF (Peprotech, Rocky Hill, NJ, https://www.peprotech.com). The medium was replaced every 3 days, and the cells were maintained in a CO2 incubator at 37°C and 5% CO2. The EC differentiation was analyzed after 10 days of stimulation.

**RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction**

Total RNA was isolated from MSCs at passage 3–6 using TRIzol reagent per the manufacturer’s protocol (Sigma). Briefly, 1 ml of TRIzol reagent was added for T25 flask, and 1.5 ml for T75 flask. The separation phase was performed by adding chloroform, and then samples were shaken for 20 seconds and incubated for 5 minutes at room temperature. Samples were then centrifuged at 12,000 g for 15 minutes. RNA was precipitated by mixing the aqueous phase with isopropyl alcohol followed by 10 minutes incubation at room temperature. Samples were centrifuged again and the remaining RNA pellet was washed with 75% ethanol. The obtained samples were dissolved in water, and the RNA pellet was left to air dry for 10 minutes. The RNA quantification was done using Nanodrop (Thermo Scientific, Waltham, MA, http://www.thermoscientific.com/). Reverse transcriptase-polymerase chain reaction (RT-PCR) was done for the identification of the EC markers, VEGF, and MMPs. First-strand cDNA synthesis was done using MgCl2, dNTP mix, and RNAse inhibitor, ImProm II reverse transcriptase, 1 µg total RNA with oligo dT 1 µg per Impron reverse transcription kit (Promega, Fitchburg, WI, http://www.promega.com/). Using a RT-PCR System (CFX96, Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com/), RT-PCR was done using 8 µl cDNA, 10 µl SYBER Green Master Mix (BioRad Laboratories), and 10 picomol/µl forward and reverse primers. The PCR cycling condition were 5 minutes at 95°C for initial denaturation, 40 cycles of 30 seconds at 95°C, 30 seconds at 52–58°C, and 30 seconds at 72°C. Data were analyzed using BioRad CFX manager software based on ΔΔCq method. Expression of the genes was normalized to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Two-tailed t test was performed to evaluate significant differences between groups for the statistical analysis of real-time quantitative PCR results. Fold-expression of mRNA transcripts relative to controls was determined after normalizing to GAPDH.

**Gelatin Zymography**

To determine MMP2 activity during endothelial differentiation, conditioned medium was collected at different time points (0, 3, 6, 10 days), and replaced with fresh serum-free medium for 24 hours. The samples were then tested for their ability to cleave MMP2 substrate. The collected media were centrifuged at 12,000 g for 5 minutes to remove cellular debris. Zymogram gels were loaded with equal protein amounts, and separated under non-reducing conditions. Samples were run on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing gelatin (1.0 mg/ml). After electrophoresis, the gels were washed in Triton X-100 and incubated for 18 hours in 50 mmol/l Tris-HCl buffer (pH 7.5; containing 0.2 mol/l NaCl and 10 mmol/l CaCl2). Gels were stained with Brilliant Blue R250 and destained. MMP-2 band for each sample were identified by running a MMP-2 and MMP-9 standard (Chemicon, Billerica, MA, http://www.emdmillipore.com/). Band intensities were obtained by imaging the gel using the Image Lab. Software (Bio-Rad) [23].

**ELISA**

Protein was collected at different time points (0, 3, 6, 10 days) during the EC differentiation of AMSCs. Cell pellet were resuspended in 100 µl of radioimmunoprecipitation assay (RIPA) buffer (Sigma) and 1 µl of protease inhibitor for T25 flasks. The cell lysate was then vortexed, and incubated in ice for 10 minutes. This step was repeated three times. The supernatants containing extracted proteins were collected by centrifugation at 16,000g. Total protein lysates were prepared by Bradford assay, and quantified by Enspire Manager Software (PerkinElmer Enspire, Waltham, MA, http://www.perkinelmer.com/). Levels of MMP-14 were analyzed by using commercially available Quantikine ELISA kit (R&D Systems, Minneapolis, MN, https://www.rndsystems.com/). The MMP-14 ELISA kit recognizes native and recombinant MMP-14 protein. Total MMP-14 was analyzed per the manufacturer’s protocol.

**Western Blot**

Protein extraction was done at different time points (0, 3, 6, 10 days) during the EC differentiation of AMSCs as described above. Proteins were separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and blocked overnight in blocking solution (10% ×10 TBS pH7.6, 0.1% Tween-20, and 5% w/v of nonfat dry milk). The membrane was then incubated with primary antibodies specific for MMP-2 and MMP-14 (ab110186, and ab38971; Abcam, Cambridge, MA, http://www.abcam.com). The loading control GAPDH (1:2000, ab8245, Abcam) was probed and visualized in all the blots. The nitrocellulose membrane was then incubated with a horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody in blocking solution for 1 hour at RT (NB7160, Novus, Biologicals, Littleton, CO, https://www.novusbio.com). Proteins were detected by chemiluminescence kit (Thermo Scientific). Imaging and densitometric analysis were done using Image Lab. Software (Bio-Rad).

**Cell Transfection**

Knockdown of MMP-2 and MMP-14 in AMSCs was performed by siRNA transfection per the manufacturer’s protocol. Briefly, 1 day
before transfection, AMSCs were plated in DMEM complete medium into T25 flasks, and allowed to reach 40%–50% confluence after 24 hours of incubation. MMP-2 or MMP-14 siRNA (4313, 4323; Thermo Scientific) at a final concentration of 50 nM was combined with 10 μl Lipofectamine (Thermo Scientific) in a total volume of 4 ml, and incubated for 20 minutes at room temperature. The transfection mixture was then applied to the AMSCs and incubated at 37°C and 5% CO2. Cell viability and the capacity for differentiation along the mesodermal lineage were not affected under these conditions.

Immunophenotyping

AMSCs were stimulated for EC differentiation for 10 days, and then harvested for Flow cytometry analysis to identify the EC markers CD31, and CD144. Direct conjugated antigen-specific antibodies were used against CD31, and CD144 (17-0319, 25-1449, eBioscience).

Angiogenesis Assay

After 10 days of stimulation for EC differentiation, an angiogenesis assay was done per the manufacturer’s protocol (EMD Millipore, Billerica, MA, http://www.emdmillipore.com/). ECM gel matrix solution (100 μl) was added into each well of 24-well plate, and incubated for 1 hour at 37°C and 5% CO2. The stimulated cells were then seeded on EC matrices at a concentration of 1 × 104 cells, and incubated in EGM medium for 8 hours at 37°C and 5% CO2. The formation of capillary-like structures was observed using an inverted phase-contrast microscope. Angiogenesis progression was analyzed by defining the visual patterns on a scale of 1–5, and counting the capillary tube branch points per the manufacturer protocol (ECM625, Millipore).

Acetylated Low-Density Lipoprotein (Ac-LDL) Uptake Assay

AMSCs were stimulated for EC differentiation for 10 days, and then transferred to 4-well chamber slides for 24 hours. The cells
were then incubated with 10 μg/ml Acetylated low-density lipoprotein LDL (Ac-LDL; Molecular Probes - Thermo Fisher) in serum-free medium for 4 hours at 37°C and 5% CO2. The cells were then washed three times with PBS, and fixed with 4% formaldehyde in PBS. The Ac-LDL uptake was observed using an inverted fluorescent microscope. Fluorescence intensity of Ac-LDL uptake in cultured AMSCs with EBM, EGM, EGM plus MMP-2 siRNA, and EGM plus MMP-14 siRNA were measured using ImageJ software.

VEGFR2 Inhibition

VEGFR2 was inhibited by VEGFR 2 Tyrosine Kinase Inhibitor (676499, Calbiochem - EMD Millipore) to analyze its role on the differentiation of AMSCs to ECs before and after MMP2 and MMP14 silencing. AMSCs were plated in DMEM complete medium into T25 flasks, and allowed to reach 40%-50% confluence. VEGFR2 kinase inhibitor was applied to the AMSCs in EGM at a final concentration of 5 μM. To examine the phosphorylation status of VEGFR2, differentiated AMSCs were incubated in EGM and VEGFR2 inhibitor for 3 hours, and tested for VEGFR2 activity by immunofluorescence. Cell viability was not affected under these conditions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism. Multiple group comparisons were performed by Bonferroni’s multiple comparison test using one-way analysis of variance (ANOVA). Descriptive data are presented as the mean ± SD. Differences were considered significant at p values of < .05.

RESULTS

Characterization of AMSCs

Primary cultures of AMSCs were isolated from the abdominal fat of microswine. Adherent cells exhibited fibroblastoid (long-shaped) morphology typical of MSCs. To classify MSCs, they need to fall into three criteria. They must adhere to plastic surfaces, show a group of positive MSC markers and negative hematopoietic stem cell markers, and can differentiate into different lineages. In this study, we isolated and characterized porcine AMSCs, and demonstrated the effect of MMPs on the differentiation to ECs in vitro.

Immunostaining

AMSCs were isolated by collagenase type 1 digestion and gradient centrifugation from porcine adipose tissue from slaughter house. The adherent cells showed fibroblastoid morphology (Fig. 1I-A) and stained negatively for CD14 (Fig. 1I-B) and CD45 (Fig. 1I-C), which indicate the absence of macrophages and hematopoietic stem cells markers. The adherent cells stained positively for CD44 (Fig. 1I-D), and CD90 (Fig. 1I-E).
Multilineage Differentiation of AMSCs

The multilineage potential of the isolated cells was tested by stimulating AMSCs for adipogenic, chondrogenic, and osteogenic lineages. The cells were cultured on chamber slides and the differentiation medium was added at 60%–70% confluency (Fig. 1III-A). We tested the ability of isolated cells to differentiate into adipocytes, chondrocytes, and osteocytes using specific differentiation medium for each lineage. The cells were differentiated into chondrogenic micromass pellets. They were stained positively for Alcian Blue which indicates the synthesis of proteoglycans in the differentiated cells (Fig. 1III-B). The cells were also analyzed for osteogenesis by staining the cells with Alizarin Red S. Morphologically, the cells were grouped together forming bone nodules. They were positively stained with Alizarin Red S, which stains for calcific deposition by the cells (Fig. 1III-C). Successful differentiation into adipocytes was initially characterized morphologically by the formation of round lipid droplets within the cells. The cells were positively stained for Oil Red O, which stains the neutral triglycerides and lipids (Fig. 1III-D). There was no differentiation observed for the untreated cells.

EC Characterization of AMSCs

The isolated AMSCs were grown in EGM medium containing 50 ng/ml (1.75 nM) of VEGF. After 10 days of stimulation, the morphology of AMSCs was changed to the morphology similar to ECs (Fig. 2A, 2B).

mRNA Expression of EC Markers

The mRNA expression level of VE-Cadherin was increased after the stimulation of AMSCs for EC differentiation for 10 days. RT-PCR was done to analyze the mRNA expression of PECAM and VE-Cadherin. There was no significant increase in the expression of PECAM-1 in relative to the control (adipose-derived mesenchymal stem cells with Dulbecco’s modified Eagle medium). However, VE-Cadherin expression showed a significant increase by approximately four-folds relative to the control after incubating the cells with EGM and 50 ng/ml of VEGF (Fig. 2C). The expression of EC markers was normalized to the housekeeping gene GAPDH.

mRNA Expression of MMPs

We evaluated the mRNA expression of a panel of MMPs and TIMPs during the differentiation of AMSCs to ECs at different time points during stimulation with EGM for endothelial differentiation (Fig. 2D). The expression of EC markers was normalized to the housekeeping gene GAPDH.
The purpose of this experiment was to check for significant upregulation or downregulation in the mRNA expression of MMP1, 2, 3, 7, 9, 10, 11, 14, TIMP1, and TIMP2 during the differentiation process. RT-PCR data showed no significant increase in the expression of MMP1, 3, 7, 9, 10, 11 (Fig. 3A, 3C–3F). However, there were downregulations at day 3 for MMP1 and MMP7, and at day 10 for MMP3. The downregulation of these three MMPs was not constant with the other time points in each one (Fig. 3A, 3C, 3D). Moreover, there was a constant and significant downregulation for TIMP2 at day 3, 6, and 10 (Fig. 3J).

More importantly, the mRNA expression of MMP-2, MMP-14, and TIMP1 were significantly increased during the differentiation process, and the upregulation was indicated at the three time points (Fig. 3B, 3H, 3I). The quantification cycle (Cq) value reflected higher mRNA levels of MMP-2 and MMP-14 than the other MMPs. MMP-2 and MMP-14 were expressed at approximate Cq values of 15 and 18, respectively, which indicates a higher level of mRNA expression for these two MMPs (Fig. 3K).

Enzymatic Activity of MMP-2, and Protein Expression of MMP-2 and MMP-14

Collected media from cell culture at day 3, 6, and 10 were used to examine the enzymatic activity of MMP2 and MMP9 during EC differentiation. The latent and active forms of MMP-9 were not detected by gelatin zymography during the EC differentiation of AMSCs at day 3, 6, and 10. However, gelatin zymography showed a gradual increase in MMP2 activity during the differentiation process at day 3, 6, and 10 in comparison to control cells with DMEM (Fig. 4A).

The protein expression of MMP-14 was evaluated by ELISA. The data showed a significant increase in the protein expression of MMP-14 during the stimulation of AMSCs for EC differentiation at day 3, 6, and 10 in comparison to the AMSCs with DMEM (Fig. 4B).

Cell lysate was also analyzed by Western blot for protein expression of MMP-2 and MMP-14. The blots showed bigger bands after stimulating the cells with EGM for EC differentiation, which indicate increases in MMP-2 and MMP-14 expression during the EC differentiation. The graphs also showed significant increases in the expression of MMP-2 and MMP-14 after 3, 6, and 10 days of EC differentiation (Fig. 4C, 4D).

Cell Transfection

To determine the concentration of siRNA to silence MMP-2 and MMP-14, we used three different concentrations between 10 - 50 nM per the manufacturer’s protocol. All three doses, 10, 35 and 50 nM of siRNA for MMP2 and MMP14 reduced the protein expression of MMP2 and MMP14. However, 50 nM of siRNA for MMP2 and MMP14 showed the highest inhibition among all the three different concentrations (Supporting Information). MMP-2 and MMP-14 were then silenced with 50 nM of siRNA, and the protein was isolated. Western blot data showed that the inhibition...
was successfully done in comparison to the EGM and EGM with scrambled siRNA samples. The expression of MMP-2 and MMP-14 after siRNA transfections resulted in more than 50% inhibition compared to AMSCs with EGM and EGM plus scrambled siRNA (negative control). GAPDH was used as a housekeeping gene (*, p < .05; **, p < .01; ***, p < .001). (II): Flow cytometric analysis of PECAM1 (CD31) in five different groups; control group was the undifferentiated cells with EBM (A), AMSCs with differentiation medium EGM (B), AMSCs with differentiation medium EGM and MMP-2 siRNA (C), AMSCs with differentiation medium EGM and MMP-14 siRNA (D), and HUVECs as the positive control (E). Flow cytometry data were analyzed to show the significant differences between the groups (F). (III): Flow cytometric analysis of VE-Cadherin (CD144) in five different groups; control group was the undifferentiated cells with EBM (A), AMSCs with differentiation medium EGM (B), AMSCs with differentiation medium EGM and MMP-2 siRNA (C), AMSCs with differentiation medium EGM and MMP-14 siRNA (D), and HUVECs as the positive control (E). Flow cytometry data were analyzed to show the significant differences between the groups (F). (*, p < .05; **, p < .01; ***, p < .001). Abbreviations: CD, cluster of differentiation; EBM, endothelial basal medium; EGM, endothelial growth medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVECs, human umbilical vein endothelial cells; MMP, matrix metalloproteinases.

Figure 5. siRNA transfection and immunophenotyping of differentiated adipose-derived mesenchymal stem cells (AMSCs); (I): MMP-2 (A) and MMP-14 (B) silencing by siRNA transfection with EGM compared to AMSCs with EGM and EGM plus scrambled siRNA (negative control). Cell viability was tested using automated hemocytometer (Countess, Invitrogen) with trypan blue staining, and the results showed high cell viability rate (96%–97%) after MMP-2 and MMP-14 siRNAs transfection (Supporting Information).

Immunophenotyping for EC Markers

Flow cytometric analysis was done to examine and compare the expression of EC markers for AMSCs with EBM, EGM, EGM plus MMP-2 siRNA, and EGM plus MMP-14 siRNA. The AMSCs...
incubated with EBM showed no expression for both PECAM and VE-Cadherin (Fig. 5II-A, 5III-A). AMSCs stimulated with EGM for endothelial differentiation showed little, but significant, increases in the expression of both markers (Fig. 5II-B, 5II-F, 5III-B, 5III-F). Surprisingly, siRNA silencing of MMP-2 with EGM resulted in significant increases in the expression of PECAM and VE-Cadherin by about 13%–15% in comparison to the EGM only group (Fig. 5II-C, 5II-F, 5III-C, 5III-F). Likewise, MMP14 siRNA silencing with EGM resulted in greater increases in the expression of both markers by 28%–31% in comparison to the EGM group (Fig. 5II-D, 5II-F, 5III-D, 5III-F).

**Formation of Capillary-Like Structures (Angiogenesis)**

After 10 days of stimulation with EGM, EGM plus MMP-2 siRNA, and EGM plus MMP-14 siRNA, cells were seeded on ECM gel to examine the capability of those cells to form capillary-like structures. These data support the findings of immunophenotyping data. AMSCs with EBM did not show any formation of capillary tubes (Fig. 6I-A). Stimulated AMSCs for endothelial differentiation with EGM showed some formation of capillary-like tubes (Fig. 6I-B). More interestingly, AMSCs stimulated with EGM plus MMP-2 or MMP-14 siRNAs demonstrated higher formation of capillary-like structures (Fig. 6I-C, 6I-D). HUVECs were also tested for angiogenesis as a positive control, and they showed a clear formation of capillary-like tubes (Fig. 6I-E). Moreover, angiogenesis progression measured for the average visual patterns (angiogenic index) (Fig. 6I-F) and average branch points (Fig. 6I-G) showed significant increases in AMSCs with EGM, EGM plus MMP-2 siRNA, and EGM plus MMP-14 siRNA compared to AMSCs in EBM only.

**Ac-LDL Uptake**

To further support the differentiation of AMSCs into ECs, another EC function was evaluated for the AMSCs with EBM, EGM, EGM plus MMP-2 siRNA, and EGM plus MMP-14 siRNA groups. Similarly, AMSCs cultured with EBM showed no Ac-LDL uptake (Fig. 6I-A), while there was a significant increase in the Ac-LDL uptake when the cells were cultured with EGM alone (Fig. 6I-B, 6I-E). However, AMSCs cultured with EGM and MMP-2 siRNA showed significantly higher Ac-LDL uptake in comparison to the EGM and EBM groups (Fig. 6I-C, 6I-I-E). AMSCs cultured with EGM and MMP-14 siRNA showed an increased percentage of Ac-LDL uptake compared to that of EGM, EGM and EGM plus MMP2 siRNA groups (Fig. 6I-II, 6I-I-E).

**Immunostaining for VEGFR2**

To examine the role of VEGFR2 on the differentiation of AMSCs to ECs, immunofluorescence staining was performed for VEGFR2 in AMSCs cultured with EBM, EGM, EGM plus MMP-2 siRNA, and EGM plus MMP-14 siRNA groups. After 10 days, AMSCs in EGM showed significant increases in the staining of VEGFR2 (Fig. 7I-B, 7I-E) in comparison to EBM group (Fig. 7I-A, 7I-E). Interestingly, AMSCs cultured with EGM and MMP-2 siRNA showed significantly higher positive staining of VEGFR2 compared to the EGM cultured cells (Fig. 7I-C, 7I-E). AMSCs cultured with EGM and MMP-14 siRNA showed greater positive staining of VEGFR2 compared to that of EGM and EGM plus MMP2 siRNA (Fig. 7I-D, 7I-E).

**Immunophenotyping for EC Markers After VEGFR2 Inhibition**

Flow cytometric analysis was done to examine and compare the expression of EC markers for the AMSCs with EBM, EGM, EGM plus MMP-2 siRNA, and EGM plus MMP-14 siRNA groups. Similarly, AMSCs cultured with EBM showed no changes in the expression of both PECAM and VE-Cadherin (Fig. 7II-A, 7III-A). AMSCs cultured with EBM showed no changes in the expression of both PECAM and VE-Cadherin (Fig. 7II-B, 7II-F, 7III-B, 7III-F). Surprisingly, siRNA silencing of MMP-2 with EGM resulted in significant decreases in the expression of PECAM and VE-Cadherin by about 13%–15% in comparison to the EGM only group (Fig. 7II-C, 7II-F, 7III-C, 7III-F). Likewise, MMP14 siRNA silencing with EGM resulted in greater decreases in the expression of both markers by 28%–31% in comparison to the EGM group (Fig. 7II-D, 7II-F, 7III-D, 7III-F).
In this study, we demonstrate an efficient method to isolate pure population of porcine AMSCs, and evaluated the mRNA and protein expression of different MMPs during the differentiation of AMSCs to ECs. Moreover, this is the first report providing evidence that the silencing of MMP-2 and MMP-14, also known as membrane type 1-MMP (MT1-MMP), can increase the expression of EC markers and improve EC functionality after stimulating AMSCs with a specific endothelial differentiation medium in vitro. The importance of MSCs arises from their anti-inflammatory effect, ease of isolation, expansion potential, multilineage differentiation, and ability to migrate to the sites of injury [5–7, 24, 25]. MSCs have been isolated from different sources including adult...
Figure 7. Immunostaining of VEGFR2 and immunophenotyping of differentiated adipose-derived mesenchymal stem cells (AMSCs) after VEGFR2 kinase inhibition; (I): Immunofluorescence staining for VEGFR2. AMSCs in EGM showed significant increases in the expression of VEGFR2 (B & E) compared to endothelial basal medium group (A & E). AMSCs cultured with EGM and MMP-2 siRNA showed significantly higher fluorescence intensity of VEGFR2 in comparison to the EGM cultured cells (C & E). AMSCs cultured with EGM and MMP-14 siRNA showed the greatest positive staining of VEGFR2 compared to that of EGM and EGM plus MMP2 siRNA (D & E). Fluorescence intensity was measured to show the significant differences between the groups using ImageJ software (E).

II: Flow cytometric analysis of PECAM1 (CD31) in three different groups; control group was the differentiated cells with EGM and 5 μM of VEGFR2 inhibitor (A), AMSCs with differentiation medium EGM, MMP-2 siRNA and 5 μM of VEGFR2 inhibitor (B) and AMSCs with EGM, MMP-14 siRNA and 5 μM of VEGFR2 inhibitor (C). Flow cytometry data were analyzed to show the significant differences between the groups in comparison to the same groups without VEGFR2 inhibitor (D).

III: Flow cytometric analysis of VE-Cadherin (CD144) in three different groups; the differentiated cells with EGM and 5 μM of VEGFR2 inhibitor (A), AMSCs with differentiation medium EGM, MMP-2 siRNA and 5 μM of VEGFR2 inhibitor (B) and AMSCs with EGM, MMP-14 siRNA and 5 μM of VEGFR2 inhibitor (C). Flow cytometry data were analyzed to show the significant differences between the groups in comparison to the same groups without VEGFR2 inhibitor (D).

Abbreviations: CD, cluster of differentiation; DMEM, Dulbecco’s modified Eagle medium; EGM, endothelial growth medium; HUVECs, human umbilical vein endothelial cells; MMP, matrix metalloproteinases; VEGFR2, vascular endothelial growth factor receptor type 2.
human BM. However, there is an increased interest in AMSCs because of their abundance and less invasive methods to harvest the tissue. Several studies have shown the ability to harvest MSCs from adipose tissues, and differentiate them into multiple cell lineages including osteocytes, chondrocytes, and adipocytes [20–22, 26, 27].

Kumar et al. [21] have successfully harvested AMSCs expressing CD44, CD90, and CD105. Further immunophenotyping of AMSCs revealed the expression of CD29, CD44, and CD105 [26]. The isolated AMSCs were carefully identified by performing different methods to characterize these cells. Attachment to the plastic surface, capability to differentiate into different lineages, immunostaining, and immunophenotyping for MSC surface molecules were all consistent with MSC characteristics. The characterized cells expressed CD44 and CD90, and they were negative for CD14 and CD45.

MMPs have been identified as their ability to translocate from the membrane or cytosol to the nucleus to induce proteolytic degradation of transcription factors, and thus playing important role in cell development, morphogenesis, proliferation, and differentiation [28]. Different studies have shown important role and functions of MMPs and TIMPs in the differentiation of MSCs to myocytes, chondrocytes, osteocytes, ECs, and adipocytes [26–28]. We studied a group of MMPs and TIMPs to evaluate their role in the differentiation of AMSCs to ECs. Protein and mRNA expression of MMP-2 and MMP-14 were significantly increased during the endothelial differentiation. Ghajar et al. [29] demonstrated that MSCs enhance the angiogenesis in mechanically viable prevascularized tissue via the upregulation of MMP-14, which indicates a particularly important role in the angiogenic response among different MMPs. During the differentiation of AMSCs to ECs, the mRNA expression of TIMP-1 and TIMP-2 were significantly downregulated and upregulated, respectively. The upregulation of TIMP-1 corresponded with the downregulation of MMP-9, and the downregulation of TIMP-2 corresponded with the increase in MMP-2 and MMP-14 in which they work as inhibitors for those MMPs.

Embryonic and BM-derived MSCs were reported to have the ability to differentiate into ECs [1, 30]. Although EC differentiation of AMSCs has not been well studied, some studies have demonstrated the ability of AMSCs to differentiate into ECs [26, 30]. In this study, we showed that the morphology of AMSCs was significantly changed after 10 days of stimulation with EC differentiation medium. Moreover, the mRNA expression of the EC marker VE-Cadherin was also elevated after 10 days of stimulation. Protein and mRNA expression of MMP-2 and MMP-14 were upregulated during the endothelial differentiation. Protein expression of MMP-2 and MMP-14 were shown to increase in cardiac fibroblasts in response to hypoxia after coculture with MSCs [31]. Unexpectedly, siRNA-induced silencing of MMP-2 and MMP-14 showed an increase in the expression of EC markers compared to the cells stimulated with EGM only. Furthermore, the endothelial functions of differentiated AMSCs were greater after silencing MMP-2 and MMP-14. The expression of EC marker was elevated from approximately 4% to 15% after MMP-2 silencing, and to approximately 30% after MMP-14 silencing. Moreover, the formation of capillary-
like structures and Ac-LDL-uptake was greater after silencing MMP-2 in comparison to EGM treatment, and much greater after silencing MMP-14. These results suggest an important inhibitory role for these two proteases on the EC differentiation, and that the role of MMP-14 has a greater effect on the differentiation process.

Although TIMPs have been known to inhibit MMPs, the interaction of proMMP-2 with TIMP-2 is required for the activation of proMMP-2. TIMP-2 binds the catalytic domain of MMP-14 on the cell surface, and the C-terminal of TIMP-2 recruits the endogenous proMMP-2 to the cell surface and binds to it, forming proMMP-2/TIMP-2/MMP-14 complex. Once this complex is formed, an active MMP-14 closely located to the complex, cleaves the prodomain, and activates proMMP-2 [32]. The activation of proMMP-2 requires the action of active MMP-14. This may explain why MMP-14 silencing has a greater effect than MMP-2 silencing on the expression of EC markers, angiogenic activity, and Ac-LDL uptake after 10 days of stimulation for EC differentiation. Therefore, silencing MMP-14 would not only result in the inhibition of MMP-14 expression, but also in the inhibition of the activation of proMMP-2 by active MMP-14. Lu et al. [13] reported the importance of MMP-14 (MT1-MMP) in the differentiation of human MSCs to osteocytes. Silencing MMP-14 resulted in inability of human MSCs to differentiate to osteocytes, suggesting the role of MMP-14 in modulating the cell shape and pericellular ECM rigidity by proteolysis of the surrounding matrix during the osteogenic differentiation [13]. It was also found that MMP-2 and MMP-14 silencing impaired human MSCs invasion, whereas silencing TIMP-1 enhanced cell migration [14]. In another study, the upregulation of MMP-2 and MMP-14 was found to play an important role in angiogenic response [29]. However, our data for the first time suggest an inhibitory role of MMP-2 and MMP-14 in the differentiation of AMSCs to ECs.

The inhibitory effect of MMP-2 and MMP-14 in EC differentiation of AMSCs is expected to be primarily indirect, and potentially through VEGFR2 signaling. VEGF induces MSCs differentiation to ECs via the activation of VEGFR2 [33]. The relationship between elevated MMPs activity and cleavage of VEGFR2 has been demonstrated [34, 35]. The increased activity of MMP-2 was reported to enhance the cleavage of VEGFR2 and VE-cadherin in aortic ECs [36] indicating a possible negative effect of MMP-2 on EC differentiation of MSCs. VEGF can bind to VEGFR2, and induces the activation of different signaling pathways including mitogen-activated protein kinase (MAPK) [37]. MMP-14, on the other hand, can activate proMMP-2 by degrading β1-integrin-ligated collagen allowing proMMP-2 to be released from its binding site [38, 39]. This activation involves a complex formation of MMP-14, proMMP-2, and TIMP-2 [40], and suggests the involvement of MMP-14 with MMP-2 in the cleavage of VEGFR-2 and thus decreasing the differentiation of AMSCs into ECs. Our data showed that siRNA-induced silencing of MMP-2 and MMP-14 resulted in an increased expression of VEGFR2 compared to the cells stimulated with EGM only, indicating a cleavage activity associated with the increase in the expression and activity of MMP-2 and MMP-14 on VEGFR2. Moreover, the inhibition of VEGFR2 resulted in significant decrease in the expression of EC markers compared to same groups without VEGFR2 inhibitor. Therefore, the knockdown of MMP-2 and MMP-14 inhibits the cleavage of VEGFR2 and allows more signals to be transmitted to stimulate the expression of EC markers.

The increase in the expression of MMP-2 and MMP-14 during AMSCs differentiation to ECs does not necessarily indicate a positive regulatory effect on this process. The EC differentiation medium is comprised of various growth factors and other supplements including VEGF that may potentially stimulate the expression of MMP-2 and MMP-14 via the activation of different signaling pathways. This effect is currently under investigation in our laboratory to define the responsible transmembrane signaling pathway that led to the increase in the expression of EC markers, angiogenesis, and Ac-LDL uptake post siRNA treatments for MMP-2 and MMP-14. Although several studies have emphasized the role of MMP-2 and MMP-14 in cell trafficking, migration, and differentiation through modulating the cell shape and pericellular ECM rigidity, MMP-2, and MMP-14 activities need not to be restricted to these roles.

CONCLUSION

This is the first time demonstrating significant upregulation for MMP-2 and MMP-14 expression during the endothelial differentiation in porcine ADMCs, and silencing of these two proteases inhibits the cleavage of VEGFR2, and results in higher expression of EC markers and functionality. Hence, further in vivo studies using knockdown MMP-2 and MMP-14 animal model would advance our knowledge on the significant role of MMP-2 and MMP-14 in regulating signaling pathways that control the transcription of certain genes in the endothelial differentiation of AMSCs. The role of MMP-2 and MMP-14 in the differentiation of AMSCs to ECs that we outline inhere may well serve as a general paradigm for the biological activity of these two proteases in future experiments.

ACKNOWLEDGMENTS

This work was supported by research grants R01 HL116042, R01 HL112597, and R01 HL120659 to Devendra K. Agrawal from the National Heart, Lung and Blood Institute, National Institutes of Health, USA. The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Sami Almalki is financially supported by Majmaah University in Saudi Arabia to pursue Ph.D. program.

AUTHOR CONTRIBUTIONS

S.G.A.: performed all experiments, conception and design, acquisition of data, analysis and interpretation of data and wrote initial manuscript; Y.L.V.: performed some of the isolation as well as experiments; S.G.A.: performed all experiments, conception and design, acquisition of data, analysis and interpretation of data and wrote initial manuscript; D.K.A.: directly mentored Sami, approved idea, research support, edited the original and revised manuscript, has given final approval of the revised version for submission.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.
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