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Sry-type HMG box 18 Contributes to the differentiation of Bone Marrow-derived Mesenchymal Stem Cells to Endothelial Cells

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Abstract

Objective—Mesenchymal stem cells (MSC) have shown therapeutic potential to engraft and either differentiate into or support differentiation of vascular endothelial cells (EC), smooth muscle cells and cardiomyocytes in animal models of ischemic heart disease. Following intracoronary or transendocardial delivery of MSCs, however, only a small fraction of cells engraft and the majority of those persist as an immature cell phenotype. The goal of the current study was to decipher the molecular pathways and mechanisms that control MSC differentiation into ECs. Vascular endothelial growth factor (VEGF-165) treatment is known to enhance *in vitro* differentiation of MSCs into ECs. We tested the possible involvement of the Sry-type HMG box (Sox) family of transcription factors in this process.

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Competing Interests

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Authors' contributions

Izuagie Ikhapoh: Conception, design, execution of the experiments, analysis and interpretation of data and writing of the initial draft of the manuscript.

Christopher J. Pelham: Performed angiogenesis assay experiments. Analyzed data and critically evaluated and edited the original manuscript and the revised manuscript.

Devendra K. Agrawal: Directly supervised and mentored Ikhapoh Izuagie, involved in the discussion on developing the project, provided research support, and critically evaluated and edited the manuscript after each author provided the comments, finalized the manuscript for submission to the journal.

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Method and Results—MSCs were isolated from the bone marrow of Yucatan microswine and underwent a 10 day differentiation protocol. VEGF-165 (50 ng/ml) treatment of MSCs *in vitro* induced a significant increase in the protein expression of VEGFR-2, Sox9 and Sox18, in addition to the EC markers PECAM-1, VE-cadherin and vWF, as determined by Western blot or flow cytometry. siRNA-mediated knockdown of Sox18, as opposed to Sox9, in MSCs prevented VEGF-165-mediated induction of EC markers and capillary tube formation. Inhibition of VEGFR-2 signaling (SC-202850) reduced Sox18 and reduced VEGF-165-induced differentiation of MSCs to ECs.

Conclusion—Here we demonstrate that VEGF-165 mediates MSC differentiation into ECs via VEGFR-2-dependent induction of Sox18, which ultimately coordinates the transcriptional upregulation of specific markers of the EC phenotype.

Keywords

Differentiation; Endothelial cell; Mesenchymal stem cell; Sox9; Sox18; Growth Factor

1. Introduction

Cardiovascular disease is the leading cause of mortality in developed countries (Kibos et al., 2007 and Farooq et al., 2011). Angioplasty with stenting is the most widely adopted surgical intervention for ischemic heart disease (Farooq et al., 2011). During such procedures, the endothelial lining of coronary arteries is particularly susceptible to mechanical disruption (Kibos et al., 2007). Endothelial cells also succumb to anti-proliferative agents contained on drug-eluting stents (Hoffman et al., 2000). Loss of the endothelium predisposes the intima of coronary arteries to enhanced leukocyte and platelet adherence, as well as smooth muscle cell hyperplasia, resulting in restenosis (Meurice et al., 1996, Hoffman et al., 2000, and Puri et al., 2012). The endothelium is a key source of vasculoprotective factors including nitric oxide and prostacyclin that produce vasodilation and also prevent activation of platelets (Weyrich et al., 2002, Pelliccia et al., 2010 and Marx et al., 2011). As such, the loss of the endothelium can be a causative factor for in-stent thrombosis and potential myocardial infarction (Marx et al., 2011).

MSC-based therapies have been investigated as a means to increase vascularity in coronary and peripheral artery disease (Williams et al., 2011). MSCs are multipotent cells capable of differentiating into cells of the mesodermal lineage including osteocytes, chondrocytes, adipocytes, myocytes and neurons (Boyle et al., 2010 and Cashman et al., 2013). MSCs themselves secrete factors that induce endothelial cell proliferation including VEGF-165, Fibroblast growth factor-2 and -7 (Williams et al., 2011). In particular, VEGF-165 promotes vasculogenesis and angiogenesis by coordinating the regulation of transcription factors and other factors that control EC proliferation, migration and function (Guilherme et al., 2005, Coultas et al., 2005 and Yang et al., 2010). Targeted deletion of both alleles of VEGFR-2^(-/-) produces an embryonic lethal phenotype that is in part due to the failure of mesodermal and endodermal progenitors to differentiate into vascular endothelial cells (Cai et al., 2006, Olsson et al., 2006, Kiselyov et al., 2007, and Noma et al., 2014).

Various transcription factors have been associated with MSC differentiation (Jager et al., 2011). Amongst these, Sox proteins are the high mobility group (HMG) transcription factors that remain active during all stages of embryonic development, and are selectively maintained in mesodermal tissue (Jager et al., 2011, Sarkar et al., 2012, and Kamachi et al., 2013). The goal of the present study was to determine if Sox transcription factors contribute to the differentiation of bone marrow (BM)-derived MSCs into ECs. Sox9 has been shown to promote the differentiation of MSCs into chondrocytes (Sarkar et al. 2012), while Sox10 has been demonstrated to regulate neuronal differentiation of embryonic cells (Bowles et al., 2000). Furthermore, expression of Sox9 and Sox 18 is detected in the developing cardiac system (Francois et al., and Bowles et al., 2000). Sox18 has a prominent role in developmental angiogenesis of the lymphatic vasculature (Francois et al., 2008). Mutations in Sox18 were discovered to underlie the cardiovascular and hair follicle defects in *ragged* mutant mice (Pennisi et al., 2000). Homozygous mutations in *ragged* mice are noted to cause peripheral blood vessels defects and edema, and these mice have very poor survival rates beyond weaning (Pennisi et al., 2000). There is limited information related to Sox18 as it applies to the differentiation of ECs for stem cell-based therapies.

However, a possible role of Sox18 in modulating the differentiation of MSCs into ECs is yet to be examined. Herein, we show that the activation of VEGFR-2 on differentiating MSCs upregulates the expression of Sox18. Additionally, we demonstrate that Sox18 is critical for the expression of EC markers on differentiating MSCs.

2. Materials and Methods

2.1 MSC isolation and differentiation

MSCs were isolated from the BM of Yucatan microswine femurs as previously described by our group (Pankajakshan et al., 2012). The animal research protocol was approved by the Institutional Animal Care and Use Committee of Creighton University. The growth media (GM) used to harvest and culture MSCs was Dulbecco modified eagle medium (DMEM) with 10% fetal bovine serum (FBS). Recombinant human VEGF-A isoform VEGF-165 (Peprotech, Rocky Hill, NJ) was used to induce EC differentiation. The differentiation media (DM) used for differentiation was Endothelial growth media-2 (EGM-2) containing 50 ng/ml VEGF-165. EGM-2 without VEGF-165 supplementation was used as a control compared to DM and is referred to as control differentiation media. Stimulation began when MSCs were at 60% confluency and continued for 10 days. The cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. MSCs between passages 2 to 5 were characterized as CD14⁻CD45⁻CD44⁺CD90⁺ and differentiated as previously described by our group (Pankajakshan et al., 2012). The fluorophore-conjugated antibodies used were CD14-FITC, CD45-PerCP-Cy5.5, CD44-APC and CD90-PE (eBioscience, San Diego, CA).

2.2 Tri-lineage mesoderm differentiation

To verify if the MSCs were capable of tri-lineage differentiation, freshly harvested MSCs were subjected to differentiation using STEMPRO Osteogenic, Adipogenic and Chondrogenic Differentiation kits according to the manufacturer's protocols (Life Technologies, Grand Island, NY). For the osteogenic lineage, differentiated cells were fixed

and analyzed for calcium deposits by Alizarin red stain at 21 days of differentiation. For the adipogenic lineage, differentiated cells were fixed and analyzed for lipids by Oil Red O stain at 21 days of differentiation. For the chondrogenic lineage, pellet cultures were maintained for 21 days then fixed and paraffin embedded. Paraffin sections (5 μ m thickness) were stained with Alcian Blue/Safranin O. In each case, the stained cells were imaged using standard microscopy.

2.3 Transfection

Expression plasmids for Sox9 or Sox18 and siRNAs directed against Sox9 or Sox18 were obtained from Origene (Rockville, MD). MSCs were transfected with plasmids or siRNA using the Amaxa Nucleofector II Device and the MSC Nucleofector Kit (Lonza, Basel, Switzerland) according to the manufacturer's Optimized Protocol for MSCs. Cells were counted using a Beckman Coulter cell counter (Beckman Coulter, Brea, CA), and $\sim 2.5 \times 10^5$ cells were used for each nucleofection sample. Briefly, MSCs were washed in DMEM supplemented with 200 U/ml penicillin G sulfate plus 200 mg/ml streptomycin sulfate. MSCs were suspended in Nucleofector medium (100 μ l) containing 50 nM of plasmid or siRNA in a 4 mm diameter cuvette and received a single electrical pulse. After electroporation, MSCs were washed in PBS three times. For plasmid transfection, the electroporated cells were left to equilibrate in growth medium at 37°C for 24 hours following electroporation. Next, at the 24 hour time point, the medium was changed to control differentiation media lacking VEGF-165 supplementation and incubated for either 12 hours or 24 hours. For plasmid overexpression experiments, cell cultures were harvested at 36 or 48 hours post-transfection and protein lysates were isolated for Western blot analysis. For siRNA-mediated knockdown, the cells were left to equilibrate for 24 hours following transfection. Next, at the 24 hours mark the medium was changed to either, control differentiation media lacking VEGF-165 supplementation or complete DM for 10 days (including media change at alternate days). For transfection control, an equivalent amount of scrambled siRNA was used. For siRNA experiments, cell cultures were harvested at 72 hours or 10 days post-transfection.

2.4 Flow cytometry characterization of EC differentiation

MSCs between passages 2 to 5 were characterized as CD14⁻CD45⁻CD44⁺CD90⁺ and differentiated as previously described by our group (Pankajakshan et al., 2012). The same pre-defined MSC type populations (>95% pure) were used for all subsequent FACS experiments. The same gating strategy was employed across all the FACS experiments. Briefly, cells (1×10^6 /ml) were washed with PBS containing 4% FBS and incubated with primary antibodies conjugated to fluorophores for 30 min at 4°C in the dark. The dilution and concentration of antibodies followed the specifications suggested by the manufacturer. The cells were further washed 3 times in PBS and re-suspended in 750 μ l FACS-FIX. FACS analysis was acquired on a BD FACS Aria I/II System (BD Biosciences, San Jose, CA, USA). As opposed to naïve MSCs, differentiated MSCs were highly positive for the following fluorophore-conjugated antibodies: vWF-PE (R&D Systems, Minneapolis, MN), VE-cadherin-FITC and PECAM-1-AP (eBioscience, San Diego, CA).

2.5 RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated using Trizol reagent (Sigma) from MSC cultures according to the manufacturer's instructions. The yield of RNA was quantified using a Nanodrop (Thermo-Scientific, Rockford, IL). First-strand cDNA synthesis was performed following the manufacturer's instructions (Improm II reverse transcription kit; Promega, Madison, WI) using oligo dT primers. Real-time qPCR was performed using SYBR Green Master Mix and a Real-time PCR system (CFX96; BioRad Laboratories, Hercules, CA). Primers were obtained from Integrated DNA Technologies (Coralville, IA). The thermocycler conditions were as follows: 5 min at 95°C for initial denaturation, 40 cycles of: 30 sec at 95°C, 30 sec at 55–60°C (depending upon the primer annealing temperatures), and 30 sec at 72°C. Fold expression of mRNA transcripts relative to controls was determined after normalizing to GAPDH.

2.6 VEGFR-2 inhibition

To analyze the role of VEGFR-2 on EC differentiation, DM containing 50 ng/ml VEGF-165 was supplemented with either 50 μ M or 100 μ M SC-202850 (Santa Cruz Biotechnology, Santa Cruz, CA), a VEGFR-2 kinase inhibitor. The differentiation experiments were carried out for 1 hour, 12 hours, 24 hours, or 10 days and cells were harvested. The 10 day treatment followed the same differentiation protocol as described above. The 1 hour, 12 hour and 24 hour time points were designed specifically to examine the phosphorylation status of VEGFR-2 and total protein levels of VEGFR-2, as described previously (Kiselyov et al., 2007, and Noma et al., 2014). In order to validate the selectivity of SC-202850, we also tested the effects of PD-161570, a FGF-1 receptor kinase inhibitor. The vehicle for SC-202850 and PD-161570 was DMSO.

2.7 Western blot

Nuclear and cytosolic protein fractions were isolated using the Active Motif Nuclear Extract kit (Active Motif, Carlsbad, CA). Total protein lysates were isolated using RIPA buffer containing phosphatase and protease inhibitors. The protein concentration of the lysates was quantified by Bradford assay. Proteins were separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and blocked overnight in blocking solution (10% 10x TBS PH7.6, 0.1% Tween-20, and 5% w/v of nonfat dry milk). The membrane was then incubated with antibodies specific for Sox9, (1:1000; Abcam ab76997), Sox18 (1:1000; Abcam ab66145) VEGFR-2 (1:1000; Abcam ab11939), and phospho-Y1214 VEGFR-2 (1:1000; Abcam ab131241). As a loading control for nuclear proteins, Lamin B (1:1000 Abcam ab16048) was probed on blots. As loading controls for p-VEGFR-2, GAPDH (1:2000, Abcam ab8245) or total VEGFR-2 was probed and visualized in all the blots. The nitrocellulose membrane was then incubated with a 1:1,000 dilution of HRP conjugated anti-mouse secondary antibody in blocking solution for 1 hour at room temp (ab131368). Proteins were detected by chemiluminescence kit (Pierce, Rockford, IL). Densitometric analysis was done using a UVP Bioimaging system (UVP, Minneapolis, MN).

2.8 Angiogenesis assay

The angiogenesis assay was utilized to assess functional endothelial capillary tube formation *in vitro* according to the manufacturer's instructions (Chemicon, Temecula, CA). The cells used in the assay were HUVECs, naïve MSCs, and MSCs that were differentiated for 10 days according to the above protocols. First, ECMatrix gel (100 µl per well) was polymerized in individual wells of a 24-well plate by incubation at 37°C for 1 hour. Next, cells (1×10^4) were seeded onto the ECMatrix gel. Cells were incubated for 6 hours in EGM-2 medium (300 µl per well) at 37°C and 5% carbon dioxide. Images of capillary tube formation were taken using an inverted phase contrast microscope (Model CKK41; Olympus, Tokyo, Japan).

2.8 Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD). Data were analyzed using GraphPad Prism. Multiple group comparisons were performed by Bonferroni's multiple comparison test using one-way ANOVA. Probability (P) value < 0.05 or less was accepted as statistically significant.

3. Results

3.1. Characterization of BM-MSCs

Primary cultures of BM-MSCs were isolated from Yucatan microswine. Adherent cells exhibited fibroblastoid morphology typical of MSCs. FACS analysis confirmed that MSCs at passages 2 to 5 stained negatively for CD14 (monocyte marker) and CD45 (hematopoietic marker) and positive for CD44 (hyaluronic acid receptor) and CD90 (Thy-1) (Figure 1).

3.2. VEGF-165 induces Sox9 and Sox18 mRNA and protein during MSC differentiation

We examined the potential of VEGF-165 to regulate the transcription of a panel of Sox transcription factors. Sox2, Sox4, Sox9, Sox10, Sox11 and Sox18 were selected based on their association with mesodermal lineage differentiation, as opposed to pluripotent or hematopoietic lineage differentiation. Sox9 and Sox18 mRNA were significantly upregulated following incubation of MSCs with VEGF-165 (50ng/ml) (Figure 2). VEGF-165 did not affect mRNA expression Sox2, Sox4 or Sox11. Sox10 expression was significantly higher than other Sox genes at the Day 0 control time point, and Sox 10 was also became significantly downregulated through the course of MSC differentiation (Figure 2). Sox10 has been demonstrated to act as a potent regulator of peripheral nerve differentiation (Stolt et al., 2002 and Kim et. al., 2003). We did not examine the potential role of Sox10 in EC differentiation any further. Next, we measured the protein levels of Sox9 and Sox18 by Western blot using nuclear lysates from VEGF-165-treated MSCs. Sox9 and Sox18 both increased from day 0 to day 1 and remained elevated to the end of the differentiation protocol at day 10 (Figure 3A–B). In the absence of VEGF-165 stimulation, control naïve MSCs displayed a minimal amount of Sox9 and Sox18 protein levels as examined by Western blot analysis (Figure 3A–B). Sox9 and Sox18 are transported between the cytoplasm and nucleus and are functional as transcription factors within the nucleus. Therefore, immunocytofluorescence was utilized to reveal the expression and cellular localization of Sox9 and Sox18 on control naïve MSCs and on MSCs harvested on day 5 of

differentiation in the presence of VEGF-165 (Figure 3C). Sox9 and Sox18 levels were induced by VEGF-165 treatment. Sox9 and Sox18 both localized to the nucleus and cytoplasm. Sox9 appeared to exhibit a more perinuclear staining pattern compared to Sox18 (Figure 3C). These results support the notion that Sox9 and Sox18 could be serving functional roles in EC differentiation.

3.3. Sox 18 knockdown decreases EC marker expression

Following 10 days of VEGF-165 treatment, differentiated MSCs showed a marked induction in the percentage of cells immunopositive for the EC markers vWF, PECAM-1 and VE-cadherin (Figure 4). The data are quantified in graph format and representative FACs plots are shown in Figure 4. Naïve MSCs cultured in control media demonstrated no expression of these EC markers (Figure 4A, 4J) and HUVEC positive control cells expressed >90% for each of the EC markers (Figure 4B, 4J). Control MSCs or VEGF-165-treated MSCs exhibited >95% cell viability, whereas cells transfected with each of the different siRNAs exhibited ~85–90% cell viability as quantified by annexin V/PI assay (data not shown). The slight reduction in cell viability likely explains why scrambled siRNA caused a small but non-significant reduction in the percentage of cells immunopositive for each of the EC markers (Figure 4C–D, J). Efficient knockdown of Sox9 or Sox18 at the protein level was verified by Western blot (Figure 4H–I). siRNA-mediated knockdown of Sox18, as opposed to Sox9, significantly decreased the percentage of VEGF-165-treated MSCs that were immunopositive for EC markers when compared to scrambled siRNA (Figure 4E–F, I). There was no additive effects of simultaneous knockdown of both Sox9 and Sox18 (Figure 4G, I).

3.4. VEGFR-2 kinase inhibitor decreases EC marker expression

Classic VEGF-165-mediated activation of VEGFR-2 occurs through the phosphorylation of Tyr1214. We demonstrated that treatment of naïve MSCs with VEGF-165 for 1 hour induces phosphorylation of VEGFR-2 at Tyr1214 and this occurs in a dose-dependent manner (Figure 5A, C). Next, we utilized SC-202850, a selective VEGFR-2 kinase inhibitor, to test its effect on the phosphorylation of VEGFR-2 (Figure 5B, 5C). In preliminary experiments, titration was performed for the SC-202850 compound (20–120 μ M). MSCs were treated for 24 hours, and cell viability was examined via annexin V/PI assay to determine the optimal concentrations. FACS analysis revealed ~85% cell viability at concentrations between 20 μ M and 100 μ M of SC-202850 and >95% cell viability in non-treated MSCs (data not shown). The results demonstrated that SC-202850 pre-treatment for 1 hour prevented the induction of phospho-Tyr1214 VEGFR-2 levels by VEGF-165 treatment for an additional 1 hour (Figure 5B–C). Importantly, immunophenotyping of MSCs pre-treated with SC-202850 (50–100 μ M) and differentiated with VEGF-165 (50 ng/ml) for 10 days revealed a significant decrease the percentage of cells immunopositive for vWF, PECAM-1 and VE-cadherin that was dose dependent (Figure 5E–H). In order to validate the selectivity of SC-202850, we also tested the effects of PD-161570, a FGF-1 receptor kinase inhibitor. PD-161570 had no effect on phospho-Tyr1214 VEGFR-2 levels (data not shown). The vehicle for SC-202850 and PD-161570 was DMSO. Control experiments demonstrated that treatment with the vehicle DMSO had no effect on phospho-VEGFR-2 levels (data not shown).

3.5. Roles of Sox18 and VEGFR-2 in capillary tube formation

It is important to evaluate whether or not differentiated “EC-like” cells that express EC markers display functional properties of mature ECs. To address this point, we performed an *in vitro* angiogenesis assay to determine if differentiated cells were capable of forming capillary tubes. Cells were seeded onto ECMatrix gel to assess tube formation (Figure 6A–G). HUVECs were used as a positive control to demonstrate characteristic capillary tube structures (Figure 6A). Naïve MSCs failed to form any tube structures (Figure 6F). The effect of Sox9 or Sox18 knockdown on angiogenesis was examined using MSCs differentiated in the presence of VEGF-165 for 10 days. MSCs treated with VEGF-165 and transfected with scrambled siRNA were capable of forming characteristic capillary structures similar to those formed by HUVECs (Figure 6B). Therefore, in addition to expressing EC markers, the differentiated “EC-like” cells also display the characteristic morphology of ECs and the functional capacity to form capillary tubes in the angiogenesis assay. Transfection of MSCs with Sox9 siRNA did not affect VEGF-165-induced capillary tube formation (Figure 6C). In contrast, transfection of MSCs with Sox18 siRNA significantly decreased VEGF-165-induced capillary tube formation compared to scrambled siRNA transfection (Figure 6D). Furthermore, inhibition of VEGFR-2 by treating cells with SC-202850 abrogated VEGF-165-induced capillary tube formation (Figure 6E). Capillary tube formation is quantified in Figure 6G. The results indicate that the functional property of capillary tube formation in these differentiated ECs is both Sox18- and VEGFR-2-dependent.

3.6. Regulation of VEGFR-2 and Sox18 expression

We found that VEGF-165 induces the expression of Sox9 and Sox18 transcription factors. Next, we sought to determine if Sox9 or Sox18 has a direct effect on expression of VEGFR-2. To examine this possibility, naïve MSCs were transfected with plasmids over-expressing either Sox9 or Sox18, then cultured in control differentiation media lacking VEGF-165 supplementation. Over-expression of Sox18, but not Sox9, was sufficient to induce the protein expression of VEGFR-2 in naïve MSCs (Figure 7A). For positive control, we utilized DM containing VEGF-165 (50 ng/ml), which significantly increased VEGFR-2 expression. As negative control, naïve MSCs were transfected with an empty plasmid, which did not induce VEGFR-2 (Figure 7A).

Lastly, we examined the potential that VEGFR-2 activation modulates Sox18 expression. Co-treatment with the VEGFR-2 kinase inhibitor, SC-202850, significantly decreased VEGF-165-induced expression of Sox18 (Figure 7B).

4. Discussion

Despite the current advances in clinical management, cardiovascular disease remains the leading cause of death in the United States (Sumi et al., 2001 and McKeller et al., 2009). Atherosclerosis is the most common type of occlusive cardiovascular disease (Schillinger et al., 2002, and Charo et al., 2011). Current therapies aimed at retarding atherosclerosis are centered on hypolipidemic, hypoglycemic and anti-hypertensive medications (Bjelajac et al., 1996). Nonetheless, when advanced plaques ultimately become occlusive or rupture,

interventional procedures are required. Balloon angioplasty with stenting is the leading procedure used to widen calcified blood vessels (Danielsson et al., 2000, and Stefanini et al., 2013). However, restenosis after successful stent placement ranges from ~5–30% of total patients and increases the likelihood that patients will need further surgical procedures (Forte et al., 2009). Endothelial cell wasting is a significant problem that contributes to restenosis (Dangas et al., 2002). The morphology of the endothelium after stent placement typically reveals microinjuries and exposed basement membrane tissue (Oettgen et al., 2006). Endothelial damage induces inflammatory cascades and allows for increased platelet adhesion and thrombus formation (Varani et al., 1994 and Schillinger et al., 2002).

Repair and/or regeneration of damaged endothelium is a conceivable mechanism that could prevent restenosis and the recurrence of myocardial infarction. The heart has a limited ability to renew its vasculature and myocardium. Thus, stem cell therapy has been proposed as a way to kick-start the repair process. For instance, autologous MSCs can be differentiated into ECs and then delivered distal to the angioplasty balloon via intracoronary infusion (Forte et al., 2012). Yet, clinical trials based on the therapeutic applications of MSCs in patients with cardiovascular disease have yielded inconclusive results (Forte et al., 2009). Re-endothelialization rates and final clinical outcomes are reported to be widely varied (Forte et al., 2009). A major concern is our lack of understanding for a standardized method to characterize the “EC-like” phenotype of MSCs differentiated *in vitro* (Tyndall et al., 2008, William 2011 et al., 2011, Franquesa et al., and Pedro et al., 2013). A pre-defined mechanism of EC differentiation could be useful in investigating divergent clinical outcomes. In the current study, we proposed and investigated a novel pathway that underlies differentiation of MSCs into ECs.

Sox proteins are HMG box transcription factors that are expressed in mesodermal tissues during all stages of embryonic development (Sarkar et al., 2013, Kamachi et al., 2013 and Jager et al., 2011). Members of the Sox family in undifferentiated cells contribute to the regulation of cardiovascular lineage specification (Bowles et al., 2000). More specifically, expression of Sox9 and Sox18 is detected in the developing cardiac system (Francois et al., 2008 and Bowles et al., 2000).

Sox18 is known to positively regulate developmental angiogenesis of the lymphatic vasculature (Francois et al., 2008). Results from our present study indicate that Sox18 is essential for the induction of EC markers by VEGF-165 treatment of MSCs. The very recent study by Fontijn and colleagues shows that ectopic expression of Sox18 is sufficient to induce the differentiation of adipose-derived stromal cells into “EC-like” cells (Fontijn et al., 2014). In this study, we illustrate a VEGF-165/VEGFR-2/Sox18 axis that controls VEGF-165-mediated induction of BM-MSC differentiation into ECs.

The mechanisms contributing to VEGF-165-mediated induction of MSC differentiation into ECs include: (i) an increase in phospho-Tyr1214 VEGFR-2 and total VEGFR-2 protein levels; (ii) upregulation of Sox18 mRNA and protein; (iii) induction of EC markers vWF, PECAM-1 and VE-cadherin; and (iv) functional gain of the ability to form capillary tubes as examined *in vitro* by angiogenesis assay. Although VEGF-165 induced the expression of both Sox18 and Sox9, siRNA-mediated knockdown experiments demonstrated that Sox18

was essential for EC differentiation, whereas Sox9 was dispensable. Silencing of Sox18 abrogated VEGF-165-mediated induction of EC marker expression as well as capillary tube formation. VEGFR-2 kinase inhibition prevented VEGF-165-mediated upregulation of Sox18. Furthermore, we showed that ectopic expression of Sox18 in naïve MSCs caused upregulation of VEGFR-2. Sox18-mediated upregulation of VEGFR-2 supports a positive feedback loop promoting EC differentiation.

In summary, our research demonstrates the novel finding of a VEGF-165/VEGF-R2/Sox18 pathway that promotes differentiation of BM-MSCs into ECs (Figure 8). These results provide greater insight into the processes that guide MSC differentiation into ECs.

The consistent drawbacks to the use of stem cell transplants in the treatment of occlusive cardiovascular diseases are that: (i) only a small fraction of cells engraft and remain viable; (ii) the majority of engrafted cells persist as an immature cell phenotype; and (iii) differentiated “EC-like” cells lose their EC phenotype. We envision that protocols designed to enhance the differentiation of MSC-derived ECs could lead to improved strategies that circumvent these problems. Further *in vivo* testing on intracoronary or transendocardial delivery of MSC-derived ECs in animal models of occlusive cardiovascular disease is required. With continued improvements, MSC-based therapies may prove to have vital applications in human cardiovascular diseases.

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Abbreviations

DMEM	Dulbecco's modified Eagle medium
EC	Endothelial cell
FBS	Fetal bovine serum
PECAM	Platelet endothelial cell adhesion molecule
MAPK	Mitogen-activated protein kinase
MSC	Mesenchymal stem cell
SMC	Smooth muscle cell
Sox	Sry-type HMG protein
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial cell growth factor
VEGFR-2	Vascular endothelial cell growth receptor-2
vWF	von Willebrand factor

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Highlights

- VEGF-165 stimulation of MSCs induced prolonged upregulation of Sox9 and Sox18 mRNA and protein levels.
- siRNA-mediated knockdown of Sox18, as opposed to Sox9, diminished VEGF-165-mediated induction of the EC markers vWF, PECAM-1, and VE cadherin.
- Exogenous expression of Sox18 in MSCs caused upregulation of VEGFR-2.
- Pharmacological inhibition of VEGFR-2 prevented VEGF-165-mediated induction of Sox18 and differentiation of MSCs into ECs.
- The VEGF-165—VEGFR—Sox18 axis plays a critical role in MSC to EC differentiation.

Figure 1. Characterization of BM-MSCs

BM-MSCs used in this study were between passages 2 and 5. Figure 1A represents the initial undefined population of cells isolated by plastic adherence. Forward scatter (FSC) was plotted against side scatter (SSC) and revealed a dense agranular population of cells in quadrant 4. BM-MSCs stained negatively for CD14 (monocyte marker) and CD45 (hematopoietic marker), and stained positively for CD44 (hyaluronic acid receptor) and CD90 (Thy-1) (B–C). Inverted microscopy demonstrated that isolated BM-MSCs possessed adherent character (D). Tri-lineage mesoderm differentiation experiments were conducted as outlined in the Methods. BM-MSCs were differentiated into osteogenic, adipogenic and chondrogenic lineages. Osteogenesis was verified by Alizarin red staining of cells (E). Adipogenesis was verified by Oil Red O staining of cells (F). Chondrogenesis was verified by Alcian Blue/Safranin O staining of paraffin sections taken from pellet cultures (G). Data are representative of independent experiments performed from independent preparations of cultures from swine bone marrow (n=3).

Figure 2. Gene expression of Sox family transcription factors during MSC to EC differentiation
 Naïve MSCs were cultured in DM containing VEGF-165 (50ng/ml) for the indicated number of days. The mRNA expression of Sox family members Sox2, Sox4, Sox9, Sox10, Sox11 and Sox18 was normalized to GAPDH. Relative mRNA expression is shown as fold-change of Sox18 Day 0 time point (set as 1). Day 0 refers to control non-treated naïve MSCs. * $p < 0.05$ Sox target Day 0 vs. Sox18 Day 0 time point, # $p < 0.05$ Sox target Day 1–10 course vs. respective Day 0 time point ($n=3$).

Figure 3. Sox9 and Sox18 protein levels and cellular localization during MSC to EC differentiation

Naïve MSCs were cultured in DM containing VEGF-165 (50ng/ml) for the indicated number of days. Nuclear protein lysates were isolated from the cells. The protein levels of Sox9 and Sox18 were measured by Western blot and normalized to Lamin B protein (A–B). * $p < 0.05$ vs. Day 0 naïve MSCs, (n=3). Immunofluorescence experiments were performed to detect Sox9 and Sox18 expression and cellular localization in naïve MSCs and MSCs cultured in DM containing VEGF-165 (50ng/ml) for 5 days (C). Day 0 refers to control non-treated naïve MSCs. Data are representative of independent experiments performed from independent preparations of cultures from swine bone marrow (n=3).

Figure 4. Silencing of Sox18 inhibits VEGF-165-induced EC differentiation

The percentage of immunopositive cells for EC markers (PECAM-1, vWF, and VE-cadherin) was assessed at the end of the 10 day VEGF-165 stimulation protocol (A–G, J). Representative vWF⁺ FACS plots are shown for naïve MSCs (A), HUVECs (B), VEGF-165-stimulated MSCs (C), as well as VEGF-165-stimulated MSCs transfected with Sox9 siRNA, Sox18 siRNA or scrambled siRNA (D–G). siRNA-mediated knockdown of Sox9 or Sox18 was confirmed by Western blot using nuclear protein lysates from MSCs harvested 72 hours post-transfection (H–I). * $p < 0.05$ vs. scrambled siRNA ($n = 3$). Summary of the FACS analysis results for the EC markers (J). * $p < 0.05$ naïve MSCs vs. MSCs treated with VEGF-165, # $p < 0.05$ Sox9 and/or Sox18 siRNA vs. scrambled siRNA ($n = 4$).

Figure 5. VEGFR-2 kinase inhibitor prevents VEGF-165-induced EC differentiation

Western blot analysis was performed to measure the levels of phospho-Tyr1214 VEGFR-2 and total VEGFR-2 from control naïve MSCs and MSCs stimulated with VEGF-165 plus or minus SC-202850 for 1 hour (A–C). *p<0.05 VEGF-165 treatment alone vs. naïve MSCs, #p<0.05 SC-202850 plus VEGF-165 treatment vs. VEGF-165 treatment alone (n=3). The percentage of immunopositive cells for EC markers (PECAM-1, vWF, and VE-cadherin) was assessed at the end of the 10 day VEGF-165 stimulation protocol (D–G). Representative vWF⁺ FACS plots are shown for VEGF-165-stimulated MSCs plus or minus SC-202850 (D–F). Summary of the FACS analysis results for the EC markers (G). #p<0.05 vs. VEGF-165 treatment alone (n=4).

Figure 6. Sox18 and VEGFR-2 are critical for endothelial capillary tube formation
 Angiogenesis assay was performed to assess capillary tube formation on ECMatrix gel *in vitro* using HUVECs, naïve MSCs, and MSCs that were differentiated according to the 10 day protocol. Representative images are shown for HUVECs (A), VEGF-165-stimulated MSCs transfected with siRNA (B–D), VEGF-165-stimulated MSCs treated with SC-202850 (E), and naïve MSCs (F). The number of “tube-like” structures per arbitrary unit of area is quantified (G). *p<0.05 MSCs vs. MSCs treated with VEGF-165 and scrambled siRNA (n=3).

Figure 7. Regulation of VEGFR-2 and Sox18 expression

The effects of ectopic expression of Sox9 or Sox18 on VEGFR-2 protein levels were measured by Western blot. Following plasmid transfection, cells were incubated in GM for 24 hours. Then the media was changed to control differentiation media (EGM-2) lacking VEGF-165 supplementation and cells were incubated an additional 12 hours or 24 hours before harvesting total protein lysates. Total VEGFR-2 protein levels were normalized to GAPDH (A). * $p < 0.05$ MSCs treated with VEGF-165 vs. control non-treated naïve MSCs. # $p < 0.05$ Sox9 or Sox18 plasmid vs. empty plasmid ($n=3$). The effects of VEGFR-2 kinase inhibitor on Sox18 protein levels were measured by Western blot. MSCs were stimulated with VEGF-165 (50 ng/ml) plus or minus SC-202850 (50–100 μ M) for 24 hours before harvesting nuclear protein lysates. Sox18 protein levels were normalized to Lamin B (B). * $p < 0.05$ VEGF-165 treatment alone vs. naïve MSCs, # $p < 0.05$ SC-202850 plus VEGF-165 treatment vs. VEGF-165 treatment alone ($n=3$)

Figure 8. VEGF-165—VEGFR-2—Sox18 signaling pathway regulates EC differentiation
 Schematic diagram of the VEGF-165—VEGFR-2—Sox18 signaling pathway in differentiating ECs. Activation of the pathway leads to induction of EC-specific markers. Extracellular signal-regulated kinase 1/2 (ERK1/2), Early growth response protein 1 (EGR-1) and Myocyte enhancer factor 2C (MEF2C) are potential mediators of EC differentiation via the VEGF-165—VEGFR-2—Sox18 signaling pathway.