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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.
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.

1. Introduction

Cardiovascular disease is the leading cause of mortality in developed countries (Kibos et al., 2007; 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 (Hoffmann and Mintz, 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; Hoffmann and Mintz, 2000; 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 and Cianfrocca, 2010;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 and Hare, 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; Cashman et al., 2013). MSCs themselves secrete factors that induce endothelial cell proliferation including VEGF-165, Fibroblast growth factor-2 and -7 (Williams and Hare, 2011). In particular, VEGF-165 promotes

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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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; 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; 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 and Hochedlinger, 2013; Kamachi and Kondoh, 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 and Hochedlinger, 2013), while Sox10 has been demonstrated to regulate neuronal differentiation of embryonic cells (Bowles et al., 2000). Furthermore, expression of Sox9 and Sox18 is detected in the developing cardiac system (Francois et al., 2008; Bowles et al., 2000). Sox18 has a prominent role in developmental angiogenesis of the lymphatic vasculature (François 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., 2013). 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% CO_2. MSCs between passages 2 and 5 were characterized as $CD14^-CD45^-CD44^+CD90^+$ and differentiated as previously described by our group (Pankajakshan et al., 2013). 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 differentation

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 maintain for 21 days then fixed and paraffin embedded. Paraffin sections (5 μm 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 μl) 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 equilibrate in growth medium at 37 °C for 24 h following electroporation. Next, at the 24 h time point, the medium was changed to control differentiation media lacking VEGF-165 supplementation and incubated for either 12 h or 24 h. For plasmid overexpression experiments, cell cultures were harvested at 36 or 48 h post-transfection and protein lysates were isolated for Western blot analysis. For siRNA-mediated knockdown, the cells were left to equilibrate for 24 h following transfection. Next, at the 24 h 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 h or 10 days post-transfection.

2.4. Flow cytometry characterization of EC differentiation

MSCs between passages 2 and 5 were characterized as $CD14^-CD45^-CD44^+CD90^+$ and differentiated as previously described by our group (Pankajakshan et al., 2013). 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 μl FACS-FIX. FACS analysis was acquired on a BD FACSAria 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,