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ABSTRACT

The discovery that dental pulp stem cells are capable of differentiating into endothelial cells raises the exciting possibility that these cells can be a single source of odontoblasts and vascular networks in dental tissue engineering. The purpose of this study was to begin to define signaling pathways that regulate endothelial differentiation of SHED. Stem cells from exfoliated deciduous teeth (SHED) exposed to endothelial growth medium (EGM-2MV) supplemented with vascular endothelial growth factor (VEGF) differentiated into VEGFR2-positive and CD31-positive endothelial cells *in vitro*. *In vivo*, VEGFR1-silenced SHED seeded in tooth slice/scaffolds and transplanted into immunodeficient mice showed a reduction in human CD31-positive blood vessels as compared with controls ($p = 0.02$). Exposure of SHED to EGM2-MV supplemented with VEGF induced potent activation of ERK and Akt signaling, while it inhibited phosphorylation of STAT3. Notably, genetic (MEK1 silencing) or chemical (U0126) inhibition of ERK signaling restored constitutive STAT3 phosphorylation and inhibited the differentiation of SHED into endothelial cells. Collectively, analysis of these data unveiled the VEGF/MEK1/ERK signaling pathway as a key regulator of the endothelial differentiation of dental pulp stem cells.

KEY WORDS: angiogenesis, tissue engineering, stemness, endodontics, dental pulp stem cells, blood vessels.

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Endothelial Differentiation of SHED Requires MEK1/ERK Signaling

INTRODUCTION

Dental pulp tissue engineering is emerging as a potential treatment alternative for necrotic immature permanent teeth. Engineering of dental pulp requires the generation of a complex tissue, composed of odontoblasts, sensory cells, supportive fibroblasts, and a functional vascular network. Stem cells from exfoliated deciduous teeth (SHED) are capable of differentiating into functional odontoblasts that secrete mineralizable dentin matrices (Casagrande *et al.*, 2010; Sakai *et al.*, 2010). Dental pulp stem cells can also differentiate into vascular endothelial cells that form functional blood vessels that anastomize with the host vasculature (d'Aquino *et al.*, 2007; Iohara *et al.*, 2008; Cordeiro *et al.*, 2008; Sakai *et al.*, 2010). Therefore, SHED are uniquely suited for dental pulp engineering, since they generate functional dental pulp while providing a vascular network that supplies the oxygen and nutrients required for the high metabolic demands of cells that are engaged in tissue regeneration (Casagrande *et al.*, 2011). An understanding of the signaling pathways involved in the endothelial differentiation of dental pulp stem cells is critical for the optimization of methods to translate dental tissue engineering to the clinic.

Vascular endothelial growth factor (VEGF) is a potent inducer of angiogenesis (Hoeben *et al.*, 2004). Emerging evidence demonstrates that VEGF can function as an inducer of differentiation of human adipose-derived stem cells into endothelial cells (Cao *et al.*, 2005; Behr *et al.*, 2011). However, the effect of VEGF on the differentiation of dental pulp stem cells is unknown. We have recently shown that SHED constitutively express the membrane-bound vascular endothelial growth factor receptor (VEGFR)-1 and its co-receptor neuropilin (NP)-1 (Sakai *et al.*, 2010). VEGFR1 is widely expressed in normal and malignant tissues (Fan *et al.*, 2005; Roy *et al.*, 2006; Cao, 2009) and modulates the angiogenic potential of cells exposed to VEGF (Kearney *et al.*, 2004; Zhang *et al.*, 2010). Interestingly, VEGF induced VEGFR2 up-regulation in SHED, which was correlated with other evidence of endothelial differentiation of these cells. Analysis of our data, collectively, suggested that VEGFR1 is the receptor responsible for VEGF-induced endothelial differentiation, which is demonstrated by an increase in the expression of VEGFR2 along with CD31 and VE-cadherin, and an increase in capillary sprout formation in 3-D matrices (Sakai *et al.*, 2010). Here, we will focus on the downstream signaling events triggered by VEGF signaling in SHED.

It has been shown that the MAPK pathway is critical for endothelial differentiation of bone marrow stem cells (Xu *et al.*, 2008). Further, in

adipose-derived stem cells, it is the PI3K-Akt pathway that regulates endothelial differentiation (Cao *et al.*, 2005; Zhang *et al.*, 2011). We know that both Akt and ERK signaling pathways are induced by VEGF in SHED (Sakai *et al.*, 2010), but we do not know which pathways are required for this process. Therefore, the overall goal of the project is to understand the signaling pathways that regulate the differentiation of SHED into endothelial cells.

MATERIALS & METHODS

Cell Culture

Human Stem cells from Exfoliated Deciduous Teeth (SHED) were isolated from primary teeth and fully characterized, as we described previously (Miura *et al.*, 2003). SHED were cultured in alpha-MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO₂. We induced endothelial differentiation by culturing SHED cells with endothelial cell growth medium (EGM-2MV; Lonza, Walkersville, MD, USA) supplemented with 50 ng/mL rhVEGF₁₆₅ (R&D Systems, Minneapolis, MN, USA), referred to as 'differentiation medium' throughout this manuscript. The cell culture medium was changed every 2 days in all experiments included here. We assessed endothelial differentiation *in vitro* by determining the expression of 2 endothelial markers (VEGFR2 and CD31) by Western blot and RT-PCR. Human dermal microvascular endothelial cells (HDMEC; Lonza) were used as positive control. To evaluate cell morphology, we cultured SHED cells in 8-well chamber slides (Fisher, Rochester, NY, USA) in alpha-MEM or EGM2-MV medium with or without rhVEGF supplementation. Alexa Fluor 488 phalloidin (Invitrogen) was used for visualization of the cytoskeleton (F-Actin; green), and nuclei were stained with DAPI (Prolong Gold; Invitrogen).

Semi-quantitative RT-PCR

Total RNA was extracted with TRIzol Reagent (Invitrogen), and PCR reactions were performed with Superscript™ III Platinum Two-Step qRT-PCR kit (Invitrogen) according to the manufacturer's instructions. Primers were the following: human VEGFR2 (sense 5'-gctgtctcagtgacaacccat-3' and anti-sense 5'-ctcccacatggattggcagagg-3'; size = 373 bp); human CD31 (sense 5'-gagtctgctgaccctctg and anti-sense 5'-acagttgacctcagatcc-3'; size = 416 bp); and human GAPDH (sense 5'-gaccccttcattgacctcaact-3' and anti-sense 5'-accacctcttgatgcatc-3'; size = 683 bp).

Lentiviral-mediated Gene Silencing

Gene silencing was performed with lentiviral vectors encoding shRNA constructs, as described previously (Sakai *et al.*, 2010). Briefly, 293T cells were transiently co-transfected with lentivirus packaging vector psPAX2, pMD2.G, and shRNA-C (control), shRNA-VEGFR1 or shRNA-MEK1 (Vector Core, University of Michigan) with calcium phosphate. We used supernatants containing lentiviruses to infect SHED overnight, and then cells were selected with 1 µg/mL puromycin (InvivoGen,

San Diego, CA, USA) for at least 1 wk. Infection efficiency was determined by fluorescence, and gene silencing by Western blot.

Tooth Slice/Scaffold Assay

To evaluate the role of VEGFR1 in the differentiation of SHED into endothelial cells *in vivo*, we seeded SHED stably transduced with shRNA-VEGFR1 (SHED-shRNA-VEGFR1) or control shRNA (SHED-shRNA-C) in a human tooth slice/scaffold, as described previously (Cordeiro *et al.*, 2008). Briefly, 1-mm-thick tooth slices were obtained from healthy human third molars. The pulp chamber of the tooth slice was filled with sieved NaCl, and a PLLA/chloroform solution was gently dropped, soaking the salt. After 24 hrs, tooth slice/scaffolds were washed with Milli-Q water to remove the salt, and scaffolds were re-hydrated/disinfected with decreasing concentrations of ethanol (100%-70%). Cells (6 x 10⁵) were seeded in each tooth slice/scaffold that was transplanted subcutaneously into the dorsum of severe combined immunodeficient mice (CB.17 SCID; Charles River, Wilmington, MA, USA). After 28 days, specimens were retrieved and fixed with IHC Zinc Fixative (BD Pharmingen, Franklin Lakes, NJ, USA) for 24 hrs at 4°C and demineralized with Decalcifier II (Surgipath; Richmond, IL, USA) for 24 hrs at room temperature. Hematoxylin-eosin staining and immunohistochemistry with rabbit anti-human CD31 (Bethyl Laboratories, Montgomery, TX, USA) were used to evaluate the morphology and microvessel density. Vessels were counted in 5 fields *per* tooth slice by a calibrated evaluator (ICC = 0.95) in a blinded fashion. This work was done under a protocol reviewed and approved by the appropriate institutional committee.

Statistical Analyses

We performed a *t* test to compare the numbers of CD31-positive vessels in pulps generated with SHED-shRNA-VEGFR1 vs. SHED-shRNA-C cells, using the SigmaStat 2.0 software (SPSS, Chicago, IL, USA). Significance was determined at *p* < 0.05.

RESULTS

Time-course of the Differentiation of SHED into Endothelial Cells

To evaluate the effect of VEGF on endothelial differentiation, we exposed SHED to EGM-2MV supplemented with rhVEGF₁₆₅ and observed cells with acquired mRNA (Fig. 1A) and protein (Fig. 1B) expression of endothelial markers (VEGFR2 and CD31). In contrast, SHED cultured in α-MEM without rhVEGF₁₆₅ did not express endothelial markers for up to 28 days (data not shown). VEGFR2 and CD31 mRNA expression was highest 1 day after exposure to the differentiation medium and then tapered off (Fig. 1A). Interestingly, VEGFR2 protein expression was observed after 7 days of treatment and lasted for 28 days, while CD31 expression was strong from day 7 through day 21 (Fig. 1B). Qualitative morphological evaluation revealed that SHED exposed to EGM-2MV, whether containing VEGF or not, became elongated, when compared with controls cultured in alpha-MEM (Fig. 1C). Analysis of these data, collectively, characterizes culture conditions with EGM2-MV supplemented

Figure 1. Time-course of the differentiation of SHED into endothelial cells. **(A)** Time-course of mRNA and **(B)** protein expression of endothelial cell markers VEGFR2 and CD31 upon culture of SHED cells with EGM2-MV + 50 ng/mL rhVEGF₁₆₅. **(C)** Cell morphology over time upon culture of SHED cells with basal medium (alpha-MEM), endothelial growth medium (EGM2-MV), or EGM2-MV supplemented with 50 ng/mL rhVEGF₁₆₅ (bar = 20 μ m).

with rhVEGF₁₆₅ that induce the differentiation of dental pulp stem cells into endothelial cells.

VEGFR1 Silencing Inhibits Endothelial Differentiation of SHED *in vivo*

We have previously reported that VEGFR1 is constitutively expressed in SHED (Sakai *et al.*, 2010). We have also observed that SHED-LacZ differentiate into human blood vessels when seeded in tooth slice/scaffolds and transplanted into immunodeficient mice, as determined by the consistent presence of B-galactosidase-positive blood vessels in the resulting pulp chambers (Cordeiro *et al.*, 2008). However, the role of VEGFR1 signaling in the endothelial differentiation of SHED *in vivo* is unknown. Here, VEGFR1-silenced SHED or SHED transduced with control lentiviral vector (shRNA-C) (Fig. 2E) were seeded

into tooth slice/scaffolds and transplanted into immunodeficient mice. After 28 days, the tooth slice/scaffolds were retrieved, and pulp-like tissues were observed in the pulp chambers (Figs. 2A, 2B). Microvessel density was evaluated with an anti-human CD31 antibody that does not cross-react with mouse blood vessels. A decrease in the density of anti-human CD31-positive cells ($p = 0.02$) was observed in the pulps generated with SHED-shRNA-VEGFR1 cells (Figs. 2C, 2F) as compared with pulps generated with control SHED-shRNA-C cells (Figs. 2D, 2F).

MEK1/ERK Signaling is Required for Endothelial Differentiation of SHED *in vitro*

To begin to evaluate the signaling events involved in the endothelial differentiation of SHED, we exposed cells to EGM2-MV supplemented with VEGF and evaluated phosphorylation of key

Figure 2. VEGFR1 silencing inhibits endothelial differentiation of SHED *in vivo*. (A, C) Tooth slice/scaffolds seeded with VEGFR1-silenced SHED (SHED-shRNA-VEGFR1) and (B, D) vector control SHED (SHED-shRNA-C) were transplanted into the subcutaneous space of immunodeficient mice. After 28 days, tooth slice/scaffolds were retrieved, fixed, and analyzed by hematoxylin-eosin staining immunohistochemistry with anti-human CD31. Black arrows indicate CD31-positive blood vessels. (E) Western blot to evaluate the effectiveness of VEGFR1 knockdown in SHED cells. (F) Microvessel density analysis from 4 tooth slice/scaffolds and 5 microscopic fields/specimen (at 200x) *per* experimental condition.

signaling molecules. We observed that ERK and Akt became phosphorylated within 15 min of exposure to the differentiation medium (Fig. 3A). This was followed by de-phosphorylation of STAT3, which is constitutively active in SHED (Fig. 3A). To evaluate a potential crosstalk between these pathways, we exposed SHED to the differentiation medium in the presence of well-characterized chemical inhibitors of STAT3 (Stattic V), PI3k/Akt (LY294002), or MEK/ERK (U0126). This experiment revealed that inhibition of ERK signaling with U0126 prevented differentiation medium-induced de-phosphorylation of STAT3 (Fig. 3B). Analysis of these data suggests the existence of crosstalk between ERK and STAT3 in SHED cells exposed to the differentiation medium. Notably, while inhibition of STAT3 or PI3k/Akt pathway inhibited SHED proliferation, inhibition of MEK/ERK did not have such an effect under the experimental conditions used here (Appendix Fig.).

To define a functional role for ERK signaling in the acquisition of an endothelial phenotype by SHED cells, we inhibited this pathway using 2 complementary strategies, a chemical (U0126) and a genetic (shRNA-MEK1) approach. SHED exposed to differentiation medium in the presence of U0126 no longer presented an elongated shape suggestive of endothelial

differentiation (Fig. 4A). Notably, induction of the endothelial cell marker (VEGFR2) by the differentiation medium was completely inhibited by treatment with the ERK inhibitor U0126 (Fig. 4C). Similar results were observed when ERK signaling was inhibited by silencing MEK1 (Fig. 4D), the upstream kinase that phosphorylates ERK. SHED stably transduced shRNA-MEK1 and, exposed to differentiation medium, no longer expressed VEGFR2 (Fig. 4E). In contrast, inhibition of PI3k/Akt signaling with LY294002 did not affect SHED morphology (Fig. 4A), nor did it prevent expression of the endothelial marker VEGFR2 induced by the differentiation medium (Fig. 4B). Collectively, these results demonstrate that specific signaling through MEK1/ERK is required for the endothelial differentiation of SHED.

DISCUSSION

We have recently demonstrated that human dental pulp stem cells differentiate into functional human blood vessels in immunodeficient mice, broadly recapitulating the differentiation steps observed in embryonic vasculogenesis (Sakai *et al.*, 2010). To generate functional blood vessels, dental pulp stem cells had to

Figure 3. Activation of signaling upon exposure of SHED to differentiation medium. **(A)** Western blot depicting effect of differentiation medium (EGM2-MV + rhVEGF₁₆₅) on phosphorylated and total ERK, Akt, and STAT3. **(B)** Western blot showing the effects of inhibitors for ERK (U0126), Akt (LY294002), or STAT3 (Stattic V) on signaling induced by the differentiation medium.

undergo differentiation into endothelial cells, organize themselves into capillary structures, and anastomize with the host vasculature. While the cellular events leading to the differentiation of dental pulp stem cells into endothelial cells have been partially characterized, the molecular mechanisms and signaling pathways underlying the acquisition of the vasculogenic fate are not known. Such knowledge may accelerate the translation of dental pulp stem-cell-based therapies to the clinic.

We have shown that SHED cultured in tooth slice scaffolds in α -MEM supplemented with rhVEGF₁₆₅ express endothelial markers suggesting their differentiation into endothelial cells (Sakai *et al.*, 2010). We hypothesized that angiogenic factors residing in the dentin matrix (Roberts-Clark and Smith, 2000; Zhang *et al.*, 2011) or stromal cells (Tran-Hung *et al.*, 2008), together with rhVEGF₁₆₅, triggered signaling pathways that were sufficient to induce endothelial differentiation of SHED. Here, we explored the possibility of creating differentiation conditions that were sufficient to induce endothelial differentiation of dental pulp stem cells without the tooth slice, which would facilitate mechanistic signaling studies. We discovered that the endothelial growth medium (EGM2-MV), which contains rhEGF, hydrocortisone, 5% fetal bovine serum, rhbFGF, R3-IGF-1, ascorbic acid, and 2 ng/mL rhVEGF supplemented with an additional 50 ng/mL rhVEGF, provides an inductive stimulus that allows for endothelial differentiation of SHED in monolayer culture. This culture condition ('differentiation medium') was used thereafter for the mechanistic studies included here.

VEGFR2 and CD31 are expressed primarily by vascular endothelial cells (Albelda *et al.*, 1991; Yamaguchi *et al.*, 1993). Both have been used as markers of endothelial differentiation of bone-marrow-derived mesenchymal stem cells (Quirici *et al.*, 2001; Xu *et al.*, 2008). In contrast, VEGFR1 is widely expressed in normal and malignant tissues (Fan *et al.*, 2005; Roy *et al.*, 2006; Cao, 2009) and modulate the angiogenic potential of cells exposed to VEGF (Kearney *et al.*, 2004; Zhang *et al.*, 2010).

Indeed, we have observed that VEGFR1 is constitutively expressed by undifferentiated SHED cells (Sakai *et al.*, 2010). Here, it was observed that VEGFR1-silenced SHED showed less angiogenic potential *in vivo* than controls, suggesting that VEGFR1 signaling plays an important role in endothelial differentiation of dental pulp stem cells. We postulate that VEGFR1 signaling allows for the differentiation of dental pulp stem cells into endothelial cells, as demonstrated by the acquisition of VEGFR2 and CD31 expression over time.

STAT3 phosphorylation is sufficient to maintain stem cells in an undifferentiated state (Matsuda *et al.*, 1999). In contrast, unstimulated stem cells express low levels of phosphorylated ERK and AKT, while cells that are induced to undergo differentiation exhibit an increase in ERK and Akt phosphorylation (Cao *et al.*, 2005; Xu *et al.*, 2008; Zhang *et al.*, 2011). Here, we observed that unstimulated SHED express high levels of phosphorylated STAT3 and that exposure of these cells to the differentiation medium quickly inhibits (within 30 min) STAT3 activity, which is in line with the observation that STAT3 activity correlates with stemness. Surprisingly, the inhibition of STAT3 phosphorylation with Stattic V enhanced ERK, but not Akt phosphorylation, beyond what was achieved with the differentiation medium. Further, inhibition of ERK with U0126 allowed for recovery of STAT3 phosphorylation in SHED cells that were induced to differentiate. To characterize the functional relevance of ERK signaling, we inhibited ERK with U0126 or by silencing MEK1 expression and observed that SHED cells no longer differentiated into endothelial cells. Finally, we observed that inhibition of PI3K/Akt resulted in slowdown in cell proliferation and/or induction of cell death, but had no effect on the regulation of SHED stemness/differentiation. In contrast, inhibition of ERK had no effect on cell proliferation/survival, but had a profound effect on cell differentiation. These findings suggest a cause-effect relationship between ERK inhibition and maintenance of STAT3 phosphorylation, which is consistent with

Figure 4. MEK1/ERK signaling is required for endothelial differentiation of SHED *in vitro*. **(A)** Qualitative analysis of the morphology of SHED cells treated with differentiation medium in the presence of UO126 or LY294002. **(B)** Western blot showing the effect of Akt inhibition with LY294002 or **(C)** the effect of ERK inhibition with UO126 on endothelial cell differentiation of SHED, as determined by VEGFR2 expression. **(D)** Effects of 2 different shRNA-MEK1 sequences (a,b) on silencing of MEK1 in SHED cells. **(E)** Effect of MEK1 silencing on endothelial cell differentiation of SHED, as determined by VEGFR2 expression. **(F)** Schematic diagram depicting the signaling pathways studied here.

ERK's role in the regulation of SHED stemness. Collectively, these results demonstrate the existence of bi-directional cross-talk between STAT3 and ERK signaling that plays a critical role in the regulation of dental pulp stem cell fate.

In conclusion, this work unveiled a pathway triggered by VEGF/MEK1 signaling that results in the inverse and reciprocal regulation of STAT3 and ERK activity that results, in turn, in the differentiation of primary tooth pulp stem cells into endothelial cells and the importance of VEGF signaling through VEGFR1 for this process. Such studies may offer clues into the mechanisms regulating cell differentiation during odontogenesis. In addition, the understanding of signaling pathways will be critical to exploit the differentiation potential of dental pulp stem cells in regenerative medicine.

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