Inhibition of TGF- β Signaling in SHED Enhances Endothelial Differentiation

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Abstract

Low efficiency of deriving endothelial cells (ECs) from adult stem cells hampers their utilization in tissue engineering studies. The purpose of this study was to investigate whether suppression of transforming growth factor beta (TGF- β) signaling could enhance the differentiation efficiency of dental pulp–derived stem cells into ECs. We initially used vascular endothelial growth factor A (VEGF-A) to stimulate 2 dental pulp–derived stem cells (dental pulp stem cells and stem cells from human exfoliated deciduous teeth [SHED]) and compared their differentiation capacity into ECs. We further evaluated whether the vascular endothelial growth factor receptor I (VEGF-R1)-specific ligand placental growth factor-1 (PLGF-1) could mediate endothelial differentiation. Finally, we investigated whether the TGF- β signaling inhibitor SB-431542 could enhance the inductive effect of VEGF-A on endothelial differentiation, as well as the underlying mechanisms involved. ECs differentiated from dental pulp–derived stem cells exhibited the typical phenotypes of primary ECs, with SHED possessing a higher endothelial differentiation potential than dental pulp stem cells. VEGFR1-specific ligand-PLGF exerted a negligible effect on SHED-ECs differentiation. Compared with VEGF-A alone, the combination of VEGF-A and SB-431542 significantly enhanced the endothelial differentiation of SHED. The presence of SB-431542 inhibited the phosphorylation of Suppressor of Mothers Against Decapentaplegic 2/3 (SMAD2/3), allowing for VEGF-A-dependent phosphorylation and upregulation of VEGFR2. Our results indicate that the combination of VEGF-A and SB-431542 could enhance the differentiation of dental pulp–derived stem cells into endothelial cells, and this process is mediated through enhancement of VEGF-A-VEGFR2 signaling and concomitant inhibition of TGF- β -SMAD2/3 signaling.

Keywords: cell differentiation, angiogenesis inducing agents, vascular endothelial growth factors, dental pulp, stem cells, signal transduction

Introduction

Prevascularization, which enhances rapid anastomosis with the host circulatory system, is critical for transportation of oxygen, nutrients, and metabolic waste within the large engineered tissue constructs (Laschke and Menger 2016). Currently, most studies utilize mature vascular cells, such as human umbilical vein endothelial cells (ECs) and primary pericytes/vascular smooth muscle cells (SMCs), in vascular tissue engineering (Sun et al. 2016). However, the slow expansion rate and low proliferative capacity of mature somatic cells, as well as the scarcity of human tissues for cell isolation, hinder their applications (Kim and von Recum 2008). Therefore, human embryonic stem cells, induced pluripotent stem cells, and adult stem cells have been widely explored in stem cell–based vascular regeneration (Yamashita et al. 2000; Oswald et al. 2004; Orlova et al. 2014).

As a subpopulation of mesenchymal stem cells, dental pulp–derived stem cells (stem cells from human exfoliated deciduous teeth [SHED] and dental pulp stem cells [DPSCs]) represent potentially promising candidate cell sources for tissue engineering due to their ready availability, ease of isolation with minimal invasiveness, and immunocompatibility arising from their autologous origin. These dental pulp–derived stem cells exhibit multipotent differentiation capacity, and their osteogenic/odontogenic, adipogenic, and neurogenic potential

is well documented (Huang et al. 2009). Moreover, our recent studies demonstrated that SHED also have the potential to differentiate into ECs (Gong et al. 2017) and functional vascular SMCs (Xu et al. 2017).

It has been demonstrated that the TGF- β signaling pathway plays an important role in numerous aspects of EC development, such as regulating the expression of VEGF (Clifford et al. 2008) and its receptor vascular endothelial growth factor receptor I (VEGF-R2) (Mandriota et al. 1996). Hence, modulating TGF- β signaling had been widely used in ECs differentiation, such as enhancing the expansion rate and long-term maintenance of stem cell–derived ECs through suppression of

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the TGF- β signaling pathway (James et al. 2010; Israely et al. 2014).

In this study, we found that ECs derived from dental pulp-derived stem cells exhibited typical EC-associated genes and proteins, with SHED possessing a higher endothelial differentiation potential than DPSCs. Suppression of the TGF- β pathway could significantly enhance the differentiation of SHED into ECs. These findings thus provide a new insight into the differentiation pathway of dental pulp-derived stem cells into the endothelial lineage.

Materials and Methods

Cell Culture and Evaluation of Stem Cell Phenotype

SHED and DPSCs were kindly provided as a gift by Songtao Shi (School of Dental Medicine, University of Pennsylvania). SHED were extracted from the primary incisors of 7-y-old boys and DPSCs from the permanent premolars of 13-y-old boys undergoing orthodontic extraction. The cells were cultured separately in α -minimum essential medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin solution (Life Technologies).

SHED and DPSCs were evaluated for their “stemness” before induction to ECs. Flow cytometry was used to validate the expression of stem cell-associated phenotypic markers CD73, CD90, and CD105 and the absence of hematopoietic marker CD45 (all primary antibodies were from Santa Cruz Biotechnology). In addition, the multiple-lineage differentiation capacity of SHED and DPSCs was confirmed with culture in adipogenic, osteogenic, chondrogenic, and neurogenic induction media (all from Lonza), according to the manufacturer’s instructions.

Endothelial Induction

SHED and DPSCs were plated at a density of 3×10^3 cells/cm², and differentiation was induced in EGM (no. CC-3162, EGM-2 BulletKit; Lonza) supplemented with 50 ng/mL of rhVEGF₁₆₅ (Peprotech) alone or with 10 μ M SB-431542 (SB), an inhibitor of the TGF- β signaling pathway (Tocris Bioscience) for the indicated time durations, with culture medium being refreshed every 2 d. In evaluating the effects of placental growth factor-1 (PLGF-1) on the endothelial differentiation of SHED, ET binding motif (EBM) (no. CC-3156; Lonza) supplemented with 250 ng/mL of PIGF-1 (Peprotech) was used to induce differentiation. The control groups of SHED and DPSCs were cultured in α -minimum essential medium supplemented with 2% (v/v) FBS and 1% (v/v) penicillin-streptomycin solution (Life Technologies).

Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction

Real-time quantitative reverse transcriptase polymerase chain reaction was performed according to our previously described protocol (Yuan et al. 2015). Primer sequences are as followed:

GAPDH, 5'-TGCACCACCAACTGCTTAGC-3', 5'-GGCATG GACTGTGGTCATGAG-3'; CD31, 5'-AAGCTGCCGGTTC TTAAATCC-3', 5'-AACTTGGTGGGAAGGAGGGTATG-3'; VEGFR-2, 5'-CACAGTGGCCGACACCTAAA-3', 5'-TCTG ACCATGTTGGCCAGACT-3'; VEGFR-1, 5'-TCCACCAAG ATCTAAATCCAAACA-3', 5'-CTGTCACAGGTGGTTTG CGTAT-3'; Tie-2, 5'-AGAAGCGGCCTAGGACAGAAC-3', 5'-CCTTTGGCAGAGGCATGTTT-3'; Ephrinb2, 5'-TCCCT GGTCACCCGACTTT-3', 5'-ACTGTAACACCCCAATC CATAGAC-3'.

RNA Extraction and High-Throughput Sequencing

Total RNA from primary DPSCs and SHED was separately extracted and purified with the RNeasy Mini Kit (Qiagen). RNA concentration and quality were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). Concentration ≥ 80 ng/ μ L, RNA integrity number ≥ 7.0 , and 28S/18S ratio ≥ 1.0 were compulsory for further analysis. An equal amount of total RNA from 6 samples (3 for DPSCs and 3 for SHED) was pooled to construct cDNA libraries (HiSeq RNA-Seq as the library type). An Illumina HiSeq2000 platform was used to subsequently sequence the constructed libraries at BGI.

Western Blot

Western blot analyses of protein expression were also performed as previously described (Yuan et al. 2015). The following primary antibodies were used: mouse monoclonal anti- β -actin monoclonal antibody (sc-47778; Santa Cruz), rabbit polyclonal anti-CD31 antibody (ab32457; Abcam), and rabbit monoclonal anti-VEGFR2 antibody (ab134191; Abcam).

For the phosphorylation experiments, primary SHED were subjected to serum starvation for 12 h and then stimulated with TGF- β 1 (10 ng/mL, 60 min) and/or TGF- β inhibitor (10 μ M SB, 30 min) and/or rhVEGF₁₆₅ (100 ng/mL, 5 min). The cells were then lysed with either RIPA Lysis and Extraction Buffer or M-PER Mammalian Protein Extraction Reagent (nos. 89900 and 78501, respectively; Thermo Fisher Scientific Inc.) and then analyzed for expression of phosphorylated Suppressor of Mothers Against Decapentaplegic 2/3 (SMAD2/3) and phosphorylated VEGFR2. The following phosphorylation antibody kits were utilized: Smad2/3 Antibody Kit and Phospho-VEGF Receptor 2 Antibody Sampler Kit (nos. 12747 and 12599, respectively; Cell Signaling Technology).

In Vitro Sprouting Assay

In vitro Matrigel sprouting assay was performed according to an established protocol (Arnaoutova and Kleinman 2010). Briefly, different groups of cells (group 1, SHED; group 2, SHED-derived ECs with VEGF only; group 3, SHED-derived ECs with VEGF combined with SB) were seeded at a density of 36,000 cells per well of 48-well plates that had been pre-coated with Growth Factor Reduced Matrigel (354230; BD Biosciences). After 1-h incubation at 37 °C and 5% CO₂, images of each group were captured every 2 h.

Figure 1. Comparison of the endothelial differentiation potential of SHED and DPSCs. **(A)** SHED possessed a higher endothelial differentiation potential than DPSCs. There were significant differences between SHED and DPSCs in the expression of endothelial cell-specific marker genes (*CD31* and *VEGFR2*; $P < 0.05$). Values are presented as mean $\pm$ SD. * $P < 0.05$. **(B)** SHED exhibited a stronger endothelial differentiation transcriptome signature than DPSCs. All experiments were performed 3 times ($n = 3$). DPSC, dental pulp stem cell; SHED, stem cells from human exfoliated deciduous teeth.

Matrigel Plug Assay

All animal experiment protocols were approved by the Committee on the Use of Live Animals in Teaching and Research of Anhui Medical University. The formation of blood vessel structures in vivo was evaluated with a Matrigel plug assay as previously described (Yuan et al. 2016). Briefly, a total of 1.5×10^6 cells (group 1, SHED; group 2, SHED-derived ECs with VEGF only; group 3, SHED-derived ECs with VEGF combined with SB) was resuspended in 250 μ L (200 μ L per plug plus 25% extra) of Phenol Red-Free Matrigel (356231; BD Bioscience). The mixture was implanted evenly and slowly with a 25-gauge needle into the subcutaneous space of the dorsum of 6-wk-old female severe combined immune-deficient mice (CB.17). Implants of Matrigel alone served as controls. Two implants were bilaterally placed in each mouse. Two mice were allocated to each experimental condition. After 2 wk, the implants were retrieved, fixed in 10% (w/v) buffered formalin overnight, and then embedded in paraffin for sectioning (7- μ m-thickness for each section).

Hematoxylin and eosin staining was performed to examine the presence of luminal structures and evaluate the microvessel density. Immunohistochemistry with rabbit anti-human CD31 (ab32457; Abcam) was used to detect the presence of transplanted SHED-derived ECs in regenerated tissues. To quantify the density of human-derived microvessels, we manually counted the number of luminal structures surrounded by

human CD31-positive cells (expressed as number of vessels/ mm^2). Luminal structures containing red blood cells were considered as perfused lumens. Three sections were cut from 1 implant, and 3 randomized areas were analyzed for 1 section.

Statistical Analysis

All experimental groups were performed in triplicates. All numerical data are expressed as mean $\pm$ SD and were tested for normal distribution with SPSS 20.0 for Windows (IBM). Statistical significance of the differences in quantitative data were determined with 1-way analysis of variance, and the threshold of statistical significance was set at $P < 0.05$.

Results

Expression of Mesenchymal Stem Cell Markers and Multilineage Differentiation Potential

The flow cytometry analysis (Appendix Fig. 1A) showed that CD90 was strongly expressed by DPSCs ($99\% \pm 0.46\%$) and SHED ($99\% \pm 0.40\%$). DPSCs displayed a lower expression level ($89\% \pm 3.33\%$) of CD73 as compared with SHED ($100\% \pm 0.56\%$). Moreover, CD105, which was highly expressed by SHED ($89\% \pm 4.23\%$), was moderately expressed by DPSCs ($69\% \pm 3.36\%$). DPSCs and SHED did not express the hematopoietic marker CD45.

The multiple-differentiation capacity of SHED and DPSCs was further confirmed through osteogenic, adipogenic, chondrogenic, and neurogenic induction (Appendix Fig. 1B–E).

SHED Possessed Higher Endothelial Differentiation Potential Than DPSCs

As shown in Figure 1A, rhVEGF₁₆₅ upregulated the mRNA expression levels of EC-associated markers (CD31 and VEGFR2) after 7 d of induction, with significant differences between SHED and DPSCs. The results (Fig. 1A) thus showed that SHED possessed a higher endothelial differentiation potential than DPSCs.

RNA sequencing was further used to analyze the global gene expression profile of DPSCs and SHED (Appendix Fig. 2). We constructed an endothelial differentiation-related genes library through accessing database and searching literatures (Appendix Table) and compared the genes profile of DPSCs and SHED with this library. The results showed that SHED exhibited a stronger endothelial differentiation transcriptome signature than DPSCs (Fig. 1B). Hence, only SHED were utilized in our further studies.

VEGFR1-Specific Ligand PLGF-1 Had No Effect on Endothelial Differentiation of SHED

To evaluate whether the vascular endothelial growth factor receptor I (VEGF-R1)-specific ligand PLGF-1 alone or combined with rhVEGF₁₆₅ could enhance endothelial differentiation of SHED, we exposed SHED to EBM with 2% (v/v) FBS supplement with 250 ng/mL of PLGF-1 and/or 50 ng/mL of rhVEGF₁₆₅. The gene expression data indicated that the VEGFR1-specific ligand PLGF-1 had no effect on the endothelial differentiation of SHED (data not shown).

SB, a Specific Inhibitor of TGF- β 1 Signaling, Promoted Endothelial Differentiation of SHED

To assess whether SB, a specific inhibitor of TGF- β 1 signaling, could enhance VEGF-mediated differentiation of ECs, we

Figure 2. SB-431542 (SB) promotes rhVEGF₁₆₅-mediated endothelial differentiation of SHED. **(A)** Expression levels of EC-specific gene expression relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at different induction time points. After 7 d of induction, SHED cultured in EGM supplemented with rhVEGF₁₆₅ and SB displayed a higher vascular endothelial growth factor receptor-2 (VEGFR2) expression as compared with the rhVEGF₁₆₅-only group ($P < 0.05$). The continued culture of SHED in EGM supplemented with rhVEGF₁₆₅ and SB for 14 d yielded a significant increase in the expression of other EC-associated genes (VEGFR1, EphrinB2, Tie-2), as compared with the rhVEGF₁₆₅-only group ($P < 0.05$). However, the expression of another mature EC marker (CD31) in the rhVEGF₁₆₅-only group is higher than the rhVEGF₁₆₅-with-SB group ($P < 0.05$). Values are presented as mean $\pm$ SD. * $P < 0.05$. **(B)** The protein expression levels of VEGFR2 and CD31 were analyzed by Western blot with β -actin as the internal marker. Numbers depict the band density normalized against the untreated controls and β -actin. All experiments were performed 3 times ($n = 3$). DPSC, dental pulp stem cell; EC, endothelial cell; SHED, stem cells from human exfoliated deciduous teeth.

utilized a combination of 50 ng/mL of rhVEGF₁₆₅ and 10 μ M SB to induce SHED differentiation into ECs and analyzed the mRNA (Fig. 2A) and protein (Fig. 2B) expression levels of EC-specific markers on days 7 and 14.

The results, as presented in Figure 2A show that VEGF combined with SB significantly upregulated the expression of EC-associated genes. After 7 d of induction, SHED cultured in EGM supplement with rhVEGF₁₆₅ and SB displayed a higher expression of VEGFR2, as compared with the rhVEGF₁₆₅-only group. The continuous culture of SHED in EGM supplement with rhVEGF₁₆₅ and SB for 14 d yielded a significant increase in the expression of other EC-associated genes (VEGFR1, EphrinB2, and Tie-2) as compared with the rhVEGF₁₆₅-only group. However, the expression of another mature EC marker (CD31) in the rhVEGF₁₆₅-only group was higher than that in the rhVEGF₁₆₅-with-SB group.

The role of SB in SHED-endothelial differentiation was further confirmed by analyzing the expression of the EC-specific proteins CD31 and VEGFR2. The results (Fig. 2B) show that SB significantly enhanced the protein expression level of VEGFR2.

Figure 3. The results of the in vitro sprouting assay. (A–D) The capillary-like structures were not detected in the SHED control group. (E–L) SHED-derived ECs with vascular endothelial growth factor (VEGF) combined with SB-431542 displayed a higher capacity in generating capillary-like structures than with VEGF only. Group 1, SHED; group 2, SHED-derived ECs with VEGF only; group 3, SHED-derived ECs with VEGF combined with SB-431542. All experiments were performed 3 times ($n = 3$). EC, endothelial cell; SHED, stem cells from human exfoliated deciduous teeth.

The results of the in vitro sprouting assay (Fig. 3) revealed that the capillary-like structures were detected only in the SHED-derived ECs with the VEGF-combined-with-SB group and the SHED-derived ECs with the VEGF-only group but not in the SHED control group. As shown in Figure 3E–L, SHED-derived ECs with VEGF combined with SB possessed a higher tube formation capacity than SHED-derived ECs with VEGF only.

The in vivo Matrigel plug assay showed that vascular structures were not observed in the implants of Matrigel alone (Fig. 4A) and that only a few host cells infiltrated into the margin of the Matrigel implants (Fig. 4F). Hematoxylin and eosin staining in Figure 4C showed a typical low-magnification view of the entire implant structure, including the inner Matrigel, the middle host adipose tissue, and the outer host skin. Figure 4D and E showed a high-magnification view of a luminal structure containing red blood cells. The immunohistochemistry results (Fig. 4G–I) showed that the luminal structures were positively stained for human CD31, which revealed that these luminal structures arose from vasculogenesis of the implanted SHED-derived ECs. Although quantitative analyses of the numbers of perfused vessels showed that there were no significant differences among all 3 groups (data not shown), the diameters and red blood cell perfusion were consistently higher in the SHED-derived ECs groups (Fig. 4H, SHED-derived ECs with VEGF only; 4I, SHED-derived ECs with VEGF combined with SB) versus the SHED control group (Fig. 4G).

TGF- β Inhibition Enhances VEGFR2 Signaling in SHED-ECs Differentiation

To assess the role of TGF- β signaling in the differentiation of SHED into ECs, we first examined the effects of TGF- β

treatment on SHED. As shown in Figure 5A, the phosphorylation of SMAD2/3 occurred in primary SHED, with or without stimulation by exogenous TGF- β (lanes 2, 4), and this effect was apparently inhibited by SB (lanes 3, 5). To further explore the relationship between VEGF signaling and TGF- β signaling during the course of SHED-ECs differentiation, we investigated the phosphorylation of VEGFR2 on SHED stimulated with TGF- β and/or VEGF-A. The results showed that the phosphorylation of VEGFR2 was inversely correlated to the phosphorylation of SMAD2/3 (Fig. 5A, lanes 4, 5; Fig. 5B, lanes 3, 4). Notably, despite treatment with exogenous TGF- β , the presence of SB prevented the phosphorylation of SMAD2/3, allowing for VEGF-A-dependent phosphorylation and

upregulation of VEGFR2 (Fig. 5A, lane 5; Fig. 5B, lane 4). By using different types of protein extraction buffer, we further found a significant presence of phosphorylated VEGFR2 within the cytoplasm and nucleus (Fig. 5C).

Discussion

In a previous study related to the endothelial differentiation of dental stem cells, which investigated SHED and DPSCs as potential candidate cell sources, Zhang et al. (2016) found that VEGF induced SHED/DPSCs-ECs differentiation through the canonical Wnt/ β -catenin pathway. In this study, we initially compared the endothelial differentiation capacity of DPSCs versus SHED. The results demonstrated that SHED-derived ECs exhibited a genes profile more closely with primary ECs than DPSCs. Although both cell types are isolated from dental pulp tissues, SHED originating from deciduous teeth differ significantly from DPSCs of permanent teeth, in terms of colony-forming potential, proliferation rate, and differentiation capacity (Miura et al. 2003). SHED are accordingly regarded as being at a more developmentally immature stage as compared with DPSCs. Through RNA sequencing, we constructed and compared the endothelial differentiation-related genes profile of DPSCs and SHED. The results confirmed that SHED possessed a higher endothelial differentiation potential than DPSCs. Among these genes, the ETS family of transcriptional regulators is regarded as the most important in endothelial differentiation. ETS transcriptional regulators are thought to directly regulate most endothelial genes expression because almost all characterized endothelial promoters possess ETS binding sites (Lammerts van Bueren and Black 2012). Our study indicated that the expression level of most members of the ETS family (e.g., ETS

translocation variant 1 [ETV2] and Friend leukemia integration 1 [FLI1]) in SHED was higher than DPSCs. Hence, only SHED were utilized in our further studies.

In most studies, the strategies for *in vitro* differentiation of ECs are based on recapitulating the sequential stages of cardiovascular development. Several signaling pathways are known to be crucial for the development of ECs, such as bone morphogenetic protein-4 (BMP-4) signaling (Olson 2004) and VEGF signaling (Olsson et al. 2006). BMP-4 is regarded as a crucial cytokine in the commitment of pluripotent stem cells to mesodermal progenitors (Ivan and Adam 2015) and is often used for a short time span during the early stage of EC differentiation (Goldman et al. 2009). Considering that SHED are already a mesodermal subtype, we chose the VEGF signaling pathway to modulate SHED-ECs differentiation in our study.

Among VEGF signaling pathways, VEGFR2 signaling plays key roles in regulating vascular-EC biology, and VEGFR1 is regarded as a negative regulator of VEGFR2 signaling (Olsson et al. 2006). However, a previous study related to dental stem cells (Bento et al. 2013) demonstrated that it is through VEGFR1 that VEGFA initiates SHED-ECs differentiation and that VEGFR1-silenced SHED cannot differentiate into ECs. In our study, we chose to examine 2 members of the VEGF family, VEGFA and PLGF, during induction of SHED to ECs. The results showed that the VEGFR1-specific ligand-PLGF exerted a negligible effect on SHED-ECs differentiation whenever used alone or combined with VEGFA. Based on a previous study that showed that PLGF and VEGFA affected different phosphorylation sites on VEGFR1 (Autiero et al. 2003), we hypothesized that this differential binding made the “decoy” effect of PLGF to VEGFA negligible in SHED-ECs differentiation.

The TGF- β signaling pathway has been reported to be involved in embryonic stem cell-ECs differentiation. Inhibition of TGF- β signaling enhanced the expansion rate and long-term maintenance of the vascular identity of embryonic stem cell-derived ECs, and this effect could be explained by Akt or Id1 upregulation upon inhibition of TGF- β signaling during the

Figure 4. The results of the *in vivo* Matrigel plug assay. (A, B) Macroscopic view of the Matrigel plug. (C) Hematoxylin and eosin (H&E) staining showed a typical low-magnification view of the entire implant structure, including the inner Matrigel, the middle host adipose tissue, and the outer host skin. (D, E) A high-magnification view of a typical perfused luminal structure (yellow arrow) containing red blood cells (blue arrow). (F) Few host cells infiltrated into the margin of the Matrigel-alone implant. (G–I) Immunohistochemical staining with human-specific CD31 antibody showed typical luminal structures (arrows) formed by SHED-derived ECs. All experiments were performed 3 times ($n = 3$). EC, endothelial cell; IHC, immunohistochemistry; SHED, stem cells from human exfoliated deciduous teeth.

process of embryonic stem cell-ECs differentiation (James et al. 2010; Israely et al. 2014). In our study, we chose SB to inhibit TGF- β signaling during the process of SHED-ECs differentiation.

Our *in vivo* study initially confirmed that vascular structures were not detectable within implants of Matrigel alone (without SHED or SHED-derived ECs). This is crucial as the results would indicate that Matrigel itself could not induce the formation of blood vessels. The results further showed that primary SHED seeded within Matrigel could also form blood vessels. However, SHED-derived ECs possess strong capacity in forming large-diameter blood vessels with more red blood cell perfusion. We speculated that the complex constituents of Matrigel could also induce differentiation of SHED into ECs, as indicated by vascular formation in the SHED group. However, the predifferentiated ECs are ready building blocks of blood vessels, unlike SHED, which require a series of differentiation steps into EC after transplantation. Hence, the generation of new blood vessels could be earlier, and anastomosis with the host circulation could be quicker with SHED-derived ECs.

Figure 5. The phosphorylation of VEGFR2 and SMAD2/3. **(A)** Smad2/3 became phosphorylated within 30 min of exposure to 2% (v/v) FBS (lane 2) or TGF- β 1 (lane 4), and SB-431542 inhibited this phosphorylation (lanes 3, 5). Numbers depict the band density normalized against the 2% (v/v) FBS-treated controls and β -actin. **(B)** VEGFR2 became phosphorylated within 5 min of exposure to 100 ng/mL of rhVEGF₁₆₅ (lane 2), and the phosphorylation of VEGFR2 was downregulated in SHED pretreated with 5 ng/mL of TGF- β 1 before exposure to rhVEGF₁₆₅ (lane 3), and SB-431542 inhibited this effect (lane 4). Numbers depict the band density normalized against the VEGF-treated controls and β -actin. **(C)** The nuclear and cytoplasmic protein-specific extraction buffer (M-PER) yielded a better result in detecting the phosphorylation of VEGFR2 than nonspecific extraction buffer (RIPA). Numbers depict the band density normalized against the M-PER group and β -actin. **(D)** Schematic diagram of the TGF- β signaling and VEGFR2 signaling pathways. SHED displayed multipotent differentiation capacity. SHED could be differentiated into smooth muscle cells under the stimulation of TGF- β and could also be induced into endothelial cells under the stimulation of VEGF. In the presence of the TGF- β signaling pathway inhibitor SB-431542, more SHED could be induced into endothelial cells. This could be explained from 2 perspectives: 1) addition of SB-431542 inhibited the smooth muscle cell (SMC) differentiation potential of SHED; 2) SB-431542 prevented the phosphorylation of Smad 2/3, which in turn upregulated expression of the VEGF ligand receptor (VEGFR2) that is phosphorylated upon binding to VEGFA. All experiments were performed 3 times ($n = 3$). SHED, stem cells from human exfoliated deciduous teeth.

The results of our in vitro studies demonstrated that SB significantly enhanced the capacity of rhVEGF₁₆₅ to induce SHED differentiation into ECs. The process could be explained from 2 aspects. First, addition of SB inhibited the SMC differentiation potential of SHED. In our previous study (Xu et al. 2017), SHED could be differentiated into functional SMCs under stimulation with TGF- β 1, and this course was mediated

by the TGF- β 1-ALK5-Smad2/3 signaling pathway. The results of this study showed that even without stimulation by exogenous TGF- β ligand, there was still phosphorylation of Smad2/3 in SHED under normal culture condition. We hypothesize that after overnight nutrient starvation, the sudden supply of extra nutrition could induce SHED to generate enough autogenous TGF- β ligand within a short time that

could further promote Smad2/3 phosphorylation. Second, besides inhibiting the SMC differentiation potential of SHED, SB prevented the phosphorylation of Smad 2/3, which in turn upregulated expression of the VEGF ligand receptor (VEGFR2) that is phosphorylated upon binding to VEGFA. A study by Mandriota et al. (1996) showed that TGF- β 1 reduced the VEGFR2 mRNA levels and the VEGF binding capacity in ECs in a dose-dependent manner. In this study, mild phosphorylation of Smad 2/3 was detected in SHED when exposed to culture medium with 10% (v/v) FBS, and this process was prevented upon treatment with SB. A further study of the relationship between TGF- β -Smad2/3 signaling and VEGFA-VEGFR2 signaling demonstrated that phosphorylation of Smad 2/3 was inhibited under stimulation with VEGFA combined with SB, which is accompanied by upregulation of VEGFR2 phosphorylation.

Collectively, treatment with SB, with VEGFA, might be a simple method to increase the endothelial differentiation potential of SHED. This finding will provide us a better understanding of the differentiation of dental stem cells to ECs.

Author Contributions

J.G. Xu, contributed to conception, design, and data acquisition, drafted and critically revised the manuscript; T. Gong, contributed to design and data acquisition, critically revised the manuscript; Y.Y. Wang, contributed to design and data interpretation, critically revised the manuscript; T. Zou, contributed to conception and data acquisition, critically revised the manuscript; B.C. Heng, contributed to conception and data interpretation, critically revised the manuscript; Y.Q. Yang, contributed to conception, data analysis, and interpretation, critically revised the manuscript; C.F. Zhang, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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