Original Article

Sonic hedgehog promotes endothelial differentiation of bone marrow mesenchymal stem cells via VEGF-D

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Background: Bone marrow-derived mesenchymal stem cells (BMSCs) have been proved to be capable of differentiating into endothelial cells (ECs), however, the differentiation efficiency is rather low. Sonic hedgehog (Shh), an important factor in vascular development and postnatal angiogenesis, exerted promotional effect on new vessel formation in the ischemic animal models. Therefore, the current study aims to investigate whether Shh could induce the endothelial differentiation of BMSCs both *in vitro* and *in vivo*, as well as the mechanism of differentiation induction.

Methods: The current study over-expressed Shh in BMSCs by lentivirus transduction. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis was performed to determine the angiogenic factors in both control BMSCs and Shh over-expressed BMSCs. Immunocytochemistry was also conducted to examine the EC markers. Angiogenesis was determined by *in vitro* tube-forming assay on Matrigel and *in vivo* Matrigel plug in severe combined immunodeficient (SCID) mice. Last, mRNA sequencing analysis was used to elaborate the underlying mechanisms. Loss of function study was performed by vascular endothelial growth factor D (VEGF-D) siRNA.

Results: Shh expression was increased by about 3,000-fold and 5,000-fold at 3 days-transfection and 7 days-transfection, respectively. Patched 1 (Ptch1), the receptor for Shh, had a two-fold increase after transduction. The angiogenic factors such as hepatocyte growth factor (HGF), angiopoietin-1 (Ang-1), insulin-like growth factor 1 (IGF1) and vascular endothelial growth factor A (VEGF-A) had at least a 1.5-fold increase after transduction. Expression of EC-lineage markers, CD31 and VE-cadherin, on Shh-overexpressed BMSCs were increasingly detected by immunocytostaining. Angiogenesis of BMSCs could be efficiently induced by Shh overexpression in the *in vitro* tube-formation assay and *in vivo* Matrigel plug. Additionally, mRNA sequencing analysis revealed that Shh activation upregulated the expression of several pro-angiogenic factors, like Angptl4, Egfl6, VEGF-D. Loss of function study by VEGF-D siRNA confirmed that Shh enhanced the angiogenic ability of BMSCs via VEGF-D.

Conclusions: This study demonstrated that Shh could promote endothelial differentiation of BMSCs via VEGF-D.

Keywords: Sonic hedgehog (Shh); bone marrow-derived mesenchymal stem cells (BMSCs); endothelial differentiation; angiogenesis; vascular endothelial growth factor D (VEGF-D)

Submitted Jun 12, 2018. Accepted for publication Sep 13, 2018.
doi: 10.21037/jtd.2018.09.50
View this article at: http://dx.doi.org/10.21037/jtd.2018.09.50

Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) have been proven effective for therapeutic angiogenesis in *in-vitro* and *in-vivo* animal experiments and in clinical settings (1-3). The angiogenic properties of BMSCs can be attributed to direct differentiation into endothelial cells (ECs) and the paracrine effect as well (4). It has been reported that only a very small fraction of BMSCs could differentiate into ECs *in vitro* (3). There have been few studies demonstrating the concrete EC oriented differentiation of MSCs *in vivo* (5).

Sonic hedgehog (Shh) gene is an angiogenic growth factor that specifically regulates vascular tube formation (6,7). Several studies have indicated that although Shh remained silent postnatally, this gene could be reactivated in response to tissue ischemia, exerting promotional effect on the angiogenic genes (8,9). Roncalli *et al.* (9) observed that bone-marrow-derived progenitor cells (BMPCs), cultured in conditioned medium collected from fibroblasts transfected with Shh-overexpressed plasmids, exhibited enhanced *in vitro* tube-forming ability, and up-regulated angiogenic genes expression. In addition, they presented more incorporation of BMPCs into vessels in myocardial infarction area after application of Shh-plasmids. In another study, researchers witnessed Shh-induced up-regulation of angiogenic growth factors in MSCs and improved blood vessel density in infarcted area (10). These studies both indicated the promoted pro-angiogenic ability of bone marrow-derived stem/progenitor cells with Shh gene overexpression; however, with no direct evidence of the endothelial differentiation in bone marrow-derived stem/progenitor cells induced by Shh.

Considering the role of Shh in postnatal angiogenesis, it may be effective to establish an endothelial differentiation method in MSCs by up-regulating Shh. We tested this hypothesis by *in vitro* tube formation assay and *in vivo* Matrigel assay. Furthermore, this study elaborated the mechanism of endothelial differentiation in Shh-overexpressed MSCs using mRNA sequencing analysis.

Methods

Animals

All animals received humane care in accordance with the Guidelines for the Care and Use of Research Animals established by Soochow University. Eight- week-old severe combined immunodeficient (SCID) mice and 3-week-old Sprague-Dawley (SD) rats were purchased from Model Animal Research Center of Nanjing University, China (11). SCID mice and SD rats were housed in standard polycarbonate cages in the Animal Facility of Soochow University. Animals were maintained on a 12-hour light/dark cycle as well as provided with free access to feed and water.

BMSCs isolation and characterization

Mesenchymal stem cells were isolated from bone marrow of 3-week-old SD rat femur and tibia as previously described (12). Briefly, rat femur and tibia were collected and flushed using DMEM/F12 medium (Gibco, China). Thereafter, cells were centrifuged at 200 RCF for 5 mins and re-suspended. Cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. MSC colonies appeared within 8–10 days. The phenotype of MSCs was identified by flow cytometry.

BMSCs were identified by flow cytometry as previously described (13). MSCs at passage 3 were stained with immunofluorescence antibodies against CD29, CD11b/c, MHCII, and CD90 (Abcam). The cells were detected by flow cytometry, and data were analyzed by FlowJo software (FlowJo, LCC, USA).

Reconstruction of Shh plasmid and lentivirus transfection

Lentiviral vector containing Shh was constructed using vector pCDH-CMV-MCS-EF1-copGFP as backbone. Shh fragments were amplified from rat genomic DNA using the primers as follows: sense, 5'-CGGAATTCGCCACC ATGCTGCTGCTGCTGGCCAG-3'; anti-sense, 5'-CGCGGATCC TCAGCTGGACTTGACTGCCATT-3'. Then Shh fragments were ligated with vector which was digested with EcoRI and BamHI (NEB, USA). The reconstructed plasmid was verified by Sanger sequencing. HEK293NT cells were used for lentiviral production. Briefly, HEK293NT cells were co-transfected with control vector or lentiviral plasmid carrying Shh fragments, along with lentiviral packaging mix. Culture medium were collected and incubated with polyethylene glycol 8000 (PEG 8000) overnight before centrifugation. Purified lentivirus was stored at –80 ℃.

BMSCs at passage 3 seeded in 6 cm dishes were used for lentivirus transfection. Cells were washed by PBS twice before transfection. Then, 2,000 μL of culture medium, 100 μL of purified lentivirus and 2 μL of polybrene were

Table 1 Primer sequences for RT-qPCR

Gene name	Forward primer (5'–3')	Reverse primer (5'–3')
Shh	TTCTCATCAACCGGGTGCTC	TCGCTTCCGCTACAGATTGC
Ptch1	TCCGCTTCTGTAACTGTCGC	ACCTTCGAGTCCCTCCTCTC
HGF	GTGAGGGAGATTATGGTGGCC	CAGGACGATTTGGGATGGCA
Ang-1	CGAGCTGATGGACTGGGAAG	CAAGCTGCTCTGTTTGCCTG
IGF1	AAGCCGCTTCCTTCACAGAAT	TTGCTTTCGAGGAGGCCAAA
VEGF-A	CTCTCTCTCCCAGATCGGTGA	CAAAGGAATGTGTGGTGGGGA
GAPDH	TTCCACCTTTGATGCTGGGG	CCACCACCCTGTTGCTGTAG
ANGPTL4	GTTGGAGATGCAGAGGGACC	TTGTGAGCTGTGCCTTGGAA
EGFL6	CCCTTCTGGCTTCTGTGGAC	CTGTGAAGGAGCAGGCTGAG
VEGF-D	CCTGTTGACATGCTGTGGGA	TTACAGACACACTCGCAGCG
PDE7B	TCCAATCAGCACAGACGCAG	AGGTGGCATTTCGAAACCCA
AMIGO2	CACAGGGGCCTAGCGTTTTA	GGTGAGGGTGTAGGGAGTGA
EDNRB	CGCATGGGAGAGGGATTGTT	GTAAGTGGGCTGTGAGTGCA
SOX9	TTTGCAGTGTTTTCCGCCAC	CCGTACACTCTCCAACCACAG
FBXO32	CTGACAAAGGGCAGCTGGAT	GGGGTGAAAGTGAGACGGAG
KANK4	TGCACAATACATTCCCGGCA	GGGCTGCAAAGACAACATCG
VSNL1	CTGCGTCACCATTCTCCTGT	CCAAAACAGCTCTTTCCAGATCG
CD31	AGGCTTGCATAGAGCTCCAG	TTCTTGGTTTCCAGCTATGG
VE-Cadherin	TCCTCTGCATCCTCACTATCACA	GTAAGTGACCAACTGCTCGTGAAT

RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

added into the cell dish. MSCs transfected with empty vector and Shh are refered as MSC^{NC} and MSC^{Shh}, respectively. MSC^{NC} and MSC^{Shh} are used for the following assays at 2 different time points post lentivirus transfection, 3- and 7-day.

mRNA expression level determined by real time reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Real-time RT-qPCR was used to determine the mRNA expression, namely Shh, Ptch, hepatocyte growth factor (HGF), angiopoietin-1 (Ang-1), IGF, and VEGF, in MSCs after transfected with empty lentivirus or Shh lentivirus. In addition, expression level of endothelial markers, CD31 and Ve-cadherin, in MSCs transfected with empty lentivirus or Shh lentivirus were detected using RT-qPCR. Cell samples were prepared as follows: MSCs transfected with empty lentivirus or Shh lentivirus were collected 3 and 7 days after transfection. Total RNA of cell samples was extracted using Trizol reagent (Ambion by Life Technologies, USA) and then purified according to the manufacturer's protocol (QIAGEN, USA) as previously described (14). Then, cDNA was converted from extracted total RNA using the PrimeScript RT reagent kit (TAKARA, Tokyo, Japan). Then, a total 2 μL of cDNA sample was used for qPCR, which was performed using Power Syber Green (Applied Biosystems, USA) under the StepOne-Plus real-time PCR system (Applied Biosystems, USA). GAPDH was used to normalize the results. The sequences of primers used in qPCR are presented in *Table 1*. The $2^{-\Delta\Delta CT}$ method was used to evaluate the relative quantification of changes in the expression of target genes.

Western blot

The expression of Shh protein in MSCs and culture medium of MSCs were determined using western blot.

Seven days after Shh-lentivirus or empty-lentivirus transfection, MSCs were washed twice by PBS and culture medium were changed. Three days later, the protein was extracted from MSCs and culture medium of MSCs. Then, protein was lysed with M-PER reagents and Halt Protease Inhibitor Cocktail kits (Pierce, USA). The concentration of extracted protein was measured by BCA Protein Assay Kit (Beyotime Biotechnology, China). Then, the extracted protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (Millipore, USA). The membranes were blocked using Tween 20/PBS including 5% skim milk, and then incubated with primary antibody against Shh (1:100, Abcam, USA) and β-actin (1:1,000, Beyotime Biotechnology, China) and followed by the HRP-conjugated secondary antibody (1:1,000; Beyotime Biotechnology, China). The protein labeled bands were determined using the enhanced chemiluminescent kit and analyzed using the Scion Image Software (Scion, USA). The intensity of protein bands was evaluated with Image J (National Institutes of Health, USA). The intensity of the protein bands was quantified relative to the β-actin bands from the same sample.

Tube formation assay

After the determination of Shh mRNA and its downstream mRNA expression, the *in vitro* angiogenic ability of MSCs was examined by tube formation assay as previously described (15). Briefly, MSCs were transfected with empty lentivirus or Shh lentivirus and cultured in DMEM/F12 medium supplemented with 10% FBS. Three days or seven days after transfection, 2×10^4 MSC^{NC} or MSC^{Shh} were suspended using 100 μL EBM-2 culture medium. Before MSCs were seeded on a 96-well plate, 50 μL Matrigel matrix (BD, Bedford, MA, USA) was added to each well beforehand, and incubated for 30 min at 37 ℃. After seeded on Matrigel, MSCs were incubated for 6 hours at 37 ℃. Tube length and mashes numbers were measured by Image J software. The tube length and mashes numbers in each high-power field (HPF) were manually calculated and presented as means ± SD.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 min and then blocked with 0.05% Tween and 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Cells were incubated with 1:100 diluted anti-PECAM antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-VE-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-vWF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 ℃. Cells were then washed three times with PBS to remove the unbound antibody and then incubated with 1:1,000 diluted anti-rat IgG Alexa Fluor 488 (Invitrogen, USA) or anti-goat IgG Alexa Fluor 594 (Invitrogen, USA) in dark for 1 h at room temperature. Cells were washed twice with PBS, and then stained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, USA) for 5min. Cells were then sealed and observed with a fluorescence microscope (Olympus, Japan).

In vivo endothelial differentiation of BMSCs

The neovascularization potential of MSCs was examined using Matrigel plug in immunodeficient SCID mice, in which dynamic and long-term neovascularization of implanted MSCs could be observed (16). Considering their stronger endothelial differentiation ability than 3-day MSC^{Shh}, 7-day MSC^{Shh} were used for the *in vivo* Matrigel plug model. Generally, a total of 1×10^4 7-day MSC^{NC} or MSC^{Shh} suspended in 100 μL DEME/F-12 medium were mixed with 100 μL Matrigel. Then 200 μL suspension was injected subcutaneously into dorsal region of nude mouse (16). Two weeks after injection, Matrigel plugs were harvested and stained with anti-rat CD31 antibody (Abcam, Catalog No. ab119339). In this assay, we used anti-CD31 antibody whose species reactivity only contains rat, human and cow, but not mouse.

mRNA sequencing by Illumina HiSeq

Total RNA of each sample was extracted using TRIzol Reagent (Invitrogen)/RNeasy Mini Kit (Qiagen)/other kits. Total RNA of each sample was quantified and qualified by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher Scientific Inc., MA, USA) and 1% agrose gel. 1 μg total RNA with RIN value above 7 was used for following library preparation. Next generation sequencing library preparations were constructed according to the manufacturer's protocol (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®). Then libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2×150 bp

paired-end (PE) configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. The sequences were processed and analyzed by JINGYUN. Differential expression analysis used the DESeq Bioconductor package, a model based on the negative binomial distribution. After adjusted by Benjamini and Hochberg's approach for controlling the false discovery rate, P value of genes were set <0.05 to detect differential expressed ones. GO-TermFinder was used to identify Gene Ontology (GO) terms that annotate a list of enriched genes with a significant P value less than 0.05. KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances (http://en.wikipedia.org/wiki/KEGG). We used scripts in house to enrich significant differential expression gene in KEGG pathways.

Gene knockdown by small interfering RNA (siRNA)

Vascular endothelial growth factor D (VEGF-D) siRNA (sense, 5'-GCUGUGGGAUAACACCAAATT-3'; antisense, 5'-UUUGGUGUUAUCCCACAGCTT-3') were designed and synthesized by GenePharma (Shanghai, China). MSC^{NC} or MSC^{Shh} were then transfected with scramble siRNA and VEGF-D siRNA for 24 h. Then the cells were harvested for real-time PCR or tube formation analysis. Each experiment was performed separately 3 times.

Statistical analyses

All results are expressed as mean ± SD. The data were analyzed using GraphPad Prism 6.0 software, via Student's unpaired *t* test for the difference between two groups or one-way ANOVA with Bonferroni correction for multiple group comparison. Differences were considered significant at P<0.05.

Results

MSCs culture and successful transfection of Shh lentivirus in MSCs

MSCs changed their morphologies into spindle-shape and were adherent to plastic dishes at day 3 (*Figure 1A*). Cell surface markers were identified by flow cytometry. Results presented that MSCs were positive for CD90 and CD29, and negative for CD11b/c and MHCII (*Figure 1B*).

About 95% of BMSCs expressed green fluorescent protein (GFP) after lentiviral infection, which suggests the success of packaging and cell infection (*Figure 1C*). Accordingly, RT-qPCR has proved the augmentation of Shh mRNA level in BMSCs. Shh mRNA expression level had increased by about 3,000-fold or 5,000-fold in 3-day MSC^{Shh} and 7-day MSC^{Shh} compared with MSC^{NC} (*Figure 1D*). Consistently, Shh protein expression in 7-day MSC^{Shh} presented about 1.2-fold increase compared with 7-day MSC^{NC} (*Figure 1E,1F*). However, no Shh protein level was observed in the culture medium of 7-day MSC^{Shh} and 7-day MSC^{NC} (*Figure 1G*).

Angiogenic factors were increased in MSCs by overexpressing Shh

We further determined the expression levels of several pro-angiogenic mRNA in BMSCs after 3 or 7 days of Shh transfection. The expression level of patched 1 (Ptch1), the ligand of Shh, has increased by more than 2-fold in MSC^{Shh} compared with MSC^{NC} at both times point of 3-day and 7-day post transfection (P<0.001, 3-day MSC^{Shh} *vs.* 3-day MSC^{NC} and 7-day MSC^{Shh} *vs.* 7-day MSC^{NC}), while Ptch1 expression level showed no statistical significance between day 3 and day 7 post transfaction (*Figure 2A*). The expression levels of HGF (*Figure 2B*) and Ang-1 (*Figure 2C*) augmented by approximately 2-fold (HGF; P<0.05, 3-day MSC^{Shh} *vs.* 3-day MSC^{NC}; P<0.001, 7-day MSC^{Shh} *vs.* 7-day MSC^{NC}) and 1.5-fold (Ang-1; P<0.001, 3-day MSC^{Shh} *vs.* 3-day MSC^{NC} and 7-day MSC^{Shh} *vs.* 7-day MSC^{NC}) post transfection, with no statistical significance between the two-time points post transfection. Insulin-like growth factor 1 (IGF1) and vascular endothelial growth factor A (VEGF-A) mRNA expression increased gradually after lentivirus transfection. IGF1 presented consistent augmentation after transfection, with 1.66-fold at day 3 and 2.18-fold at day 7, respectively (*Figure 2D*; P<0.05, 3-day MSC^{Shh} *vs.* 7-day MSC^{Shh}). VEGF-A mRNA expression (*Figure 2E*) manifested mild increase at day 3 (P<0.05, 3-day MSC^{Shh} *vs.* 3-day MSC^{NC}), and marked increase at day 7 after transfection (P<0.001, 7-day MSC^{Shh} *vs.* 7-day MSC^{NC}).

In vitro angiogenic activity of MSCs were augmented by the overexpression of Shh

As shown in *Figure 3A*, MSC^{NC} presented limited tube-

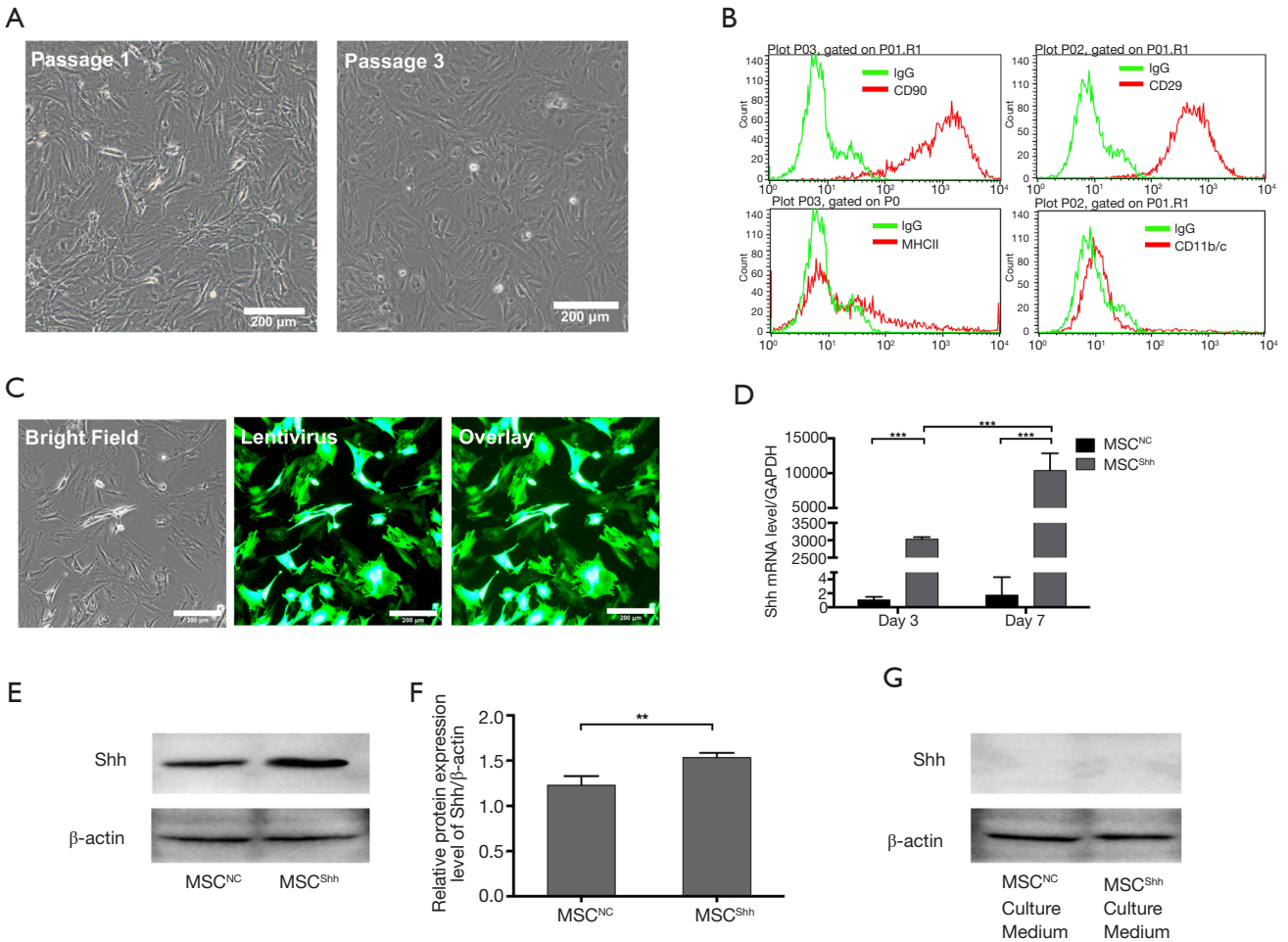

Figure 1 BMSCs culture and modulation of Shh in BMSCs. (A) Morphology of rat bone marrow-derived MSCs cultured at passage 1 and passage 3. Bar, 200 μm; (B) BMSCs cell surface marker was determined by flow cytometry. BMSCs were positive for CD90 and CD29, and negative for CD11b/c and MHCII; (C) infection of Shh in BMSCs. Bar, 200 μm; (D) Shh mRNA expression in BMSCs after lentivirus transfection was determined by RT-qPCR; (E,G) Western blot analysis of Shh protein expression level in 7-day MSC^{Shh} and MSC^{NC}, and the culture medium of 7-day MSC^{Shh} and MSC^{NC}, respectively; (F) quantitative analysis using image J software of protein band of 7-day MSC^{Shh} and MSC^{NC}. All results are presented as mean ± SD (n=3). ***P<0.001. BMSC, bone marrow-derived mesenchymal stem cell; Shh, sonic hedgehog; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; MSC, mesenchymal stem cell.

forming ability, while Shh transfection has successfully induced BMSCs to form tubes on Matrigel. Moreover, the enhanced angiogenic ability of MSC^{Shh} presented a time-dependent manner, with 20.64±2.29 mm tube formed by 3-day MSC^{Shh} *vs.* 39.39±3.68 mm formed by 7-day MSC^{Shh} (*Figure 3B*; P<0.001). Accordingly, mashes formed by BMSCs manifested similar tendency with tube length. 1.33±0.33 mashes and 0.67±0.33 mashes were formed by 3-day MSC^{NC} and 7-day MSC^{NC}, respectively. Mashes numbers were significantly increased after transduced with Shh, with 9.67±1.45 mashes and 55.33±3.53 mashes formed by 3-day MSC^{Shh} and 7-day MSC^{Shh}, respectively (*Figure 3C*; P<0.001).

Endothelial specific markers were highly expressed in Shh-overexpressed MSCs

Fluorescent microscopy analysis observed relatively low levels of CD31 and VE-cadherin in MSC^{NC} (*Figure 3D*). By contrast, CD31 and VE-cadherin displayed strong green fluorescent signal in MSC^{Shh} (*Figure 3D*), indicating that Shh-overexpressed MSCs underwent endothelial

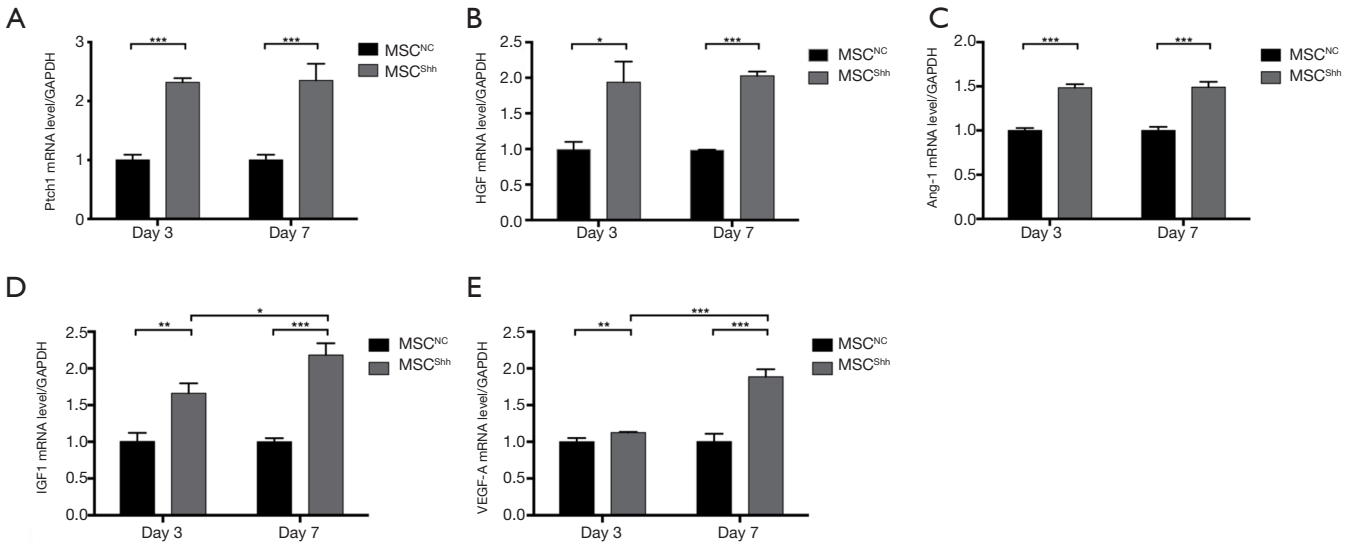

Figure 2 Real time RT-qPCR validation of angiogenic factors expression level. mRNA expression levels of Ptch1 (A), HGF (B), Ang-1 (C), IGF-1 (D) and VEGF-A (E) were determined in MSC^{Shh} and MS^{CNC} at 3- and 7-day post lentivirus transfection. All results are presented mean ± SD (n=3). *P<0.05; **P<0.01; ***P<0.001. RT-qPCR, reverse-transcription quantitative polymerase chain reaction; Ptch1, patched 1; HGF, hepatocyte growth factor; Ang-1, angiopoietin-1; IGF1, insulin-like growth factor 1; VEGF-A, vascular endothelial growth factor A; MSC, mesenchymal stem cell; Shh, sonic hedgehog.

differentiation. Consistently, RT-qPCR indicated that expression of CD31 and VE-cadherin in MSC^{Shh} increased by 1.58- and 1.55-fold compared with MSC^{NC}, respectively (*Figure 3E*).

Increased in vivo neovascularization potential of MSCs by overexpression of Shh

Matrigel plug was harvested 14 days after subcutaneous injection and immunochemistry staining using anti-rat CD31 antibody was performed. As presented in *Figure 3F*, the functional vessels were increasingly formed by 7-day MSC^{Shh} compared with MSC^{NC} (*Figure 3G*; P<0.001), indicating that Shh transduction could induce MSCs differentiating into ECs *in vivo*.

Analysis of differentially-expressed mRNA between MSC^{NC} and MSC^{Shh}

We obtained total mRNA of MSC^{NC} and MSC^{Shh} for high-throughput sequencing. Heatmap analysis showed the distribution of differentially-expressed mRNA in both MSC^{NC} and MSC^{Shh} (*Figure S1* and *Figure 4A*). Three hundred and thirty differentially-expressed mRNA were detected (fold change >2.0, P value <0.05) (*Figure S1*). One hundred and thirty-eight mRNA were up-regulated and 192 mRNA were down-regulated compared with MSC^{NC}. Among these, 100 top differentially-expressed mRNA were manifested for clear observation (*Figure 4A*). Interestingly, from the heat map we observed that the expression of genes related with angiogenesis in MSC^{Shh}, such as Vegfd, Angptl4, Egfl6, Pde7b, Amigo2 and Ednrb, were much higher than those in MSC^{NC}.

The GO analysis was classified into three parts: biological process (BP), cellular component (CC) and molecular function (MF). We have listed 3 parts of the top 10 generally changed GO terms (*Figure 4B*). Those meaningful BP terms, CC terms, and MF terms were related to response to stress, response to oxygen levels, receptor binding, cargo receptor activity and CX3C chemokine binding. Moreover, 10 pathways were significantly enriched by the 330 differentially expressed genes (*Figure 4C*), including PPAR signaling pathway, PI3K-Akt signaling pathway, p53 signaling pathway and cytokine-cytokine receptor interaction.

Validation of differentially-expressed genes by real time PCR

To verify the molecular signature between MSC^{NC} and MSC^{Shh}, a total of 10 genes were selected for validation

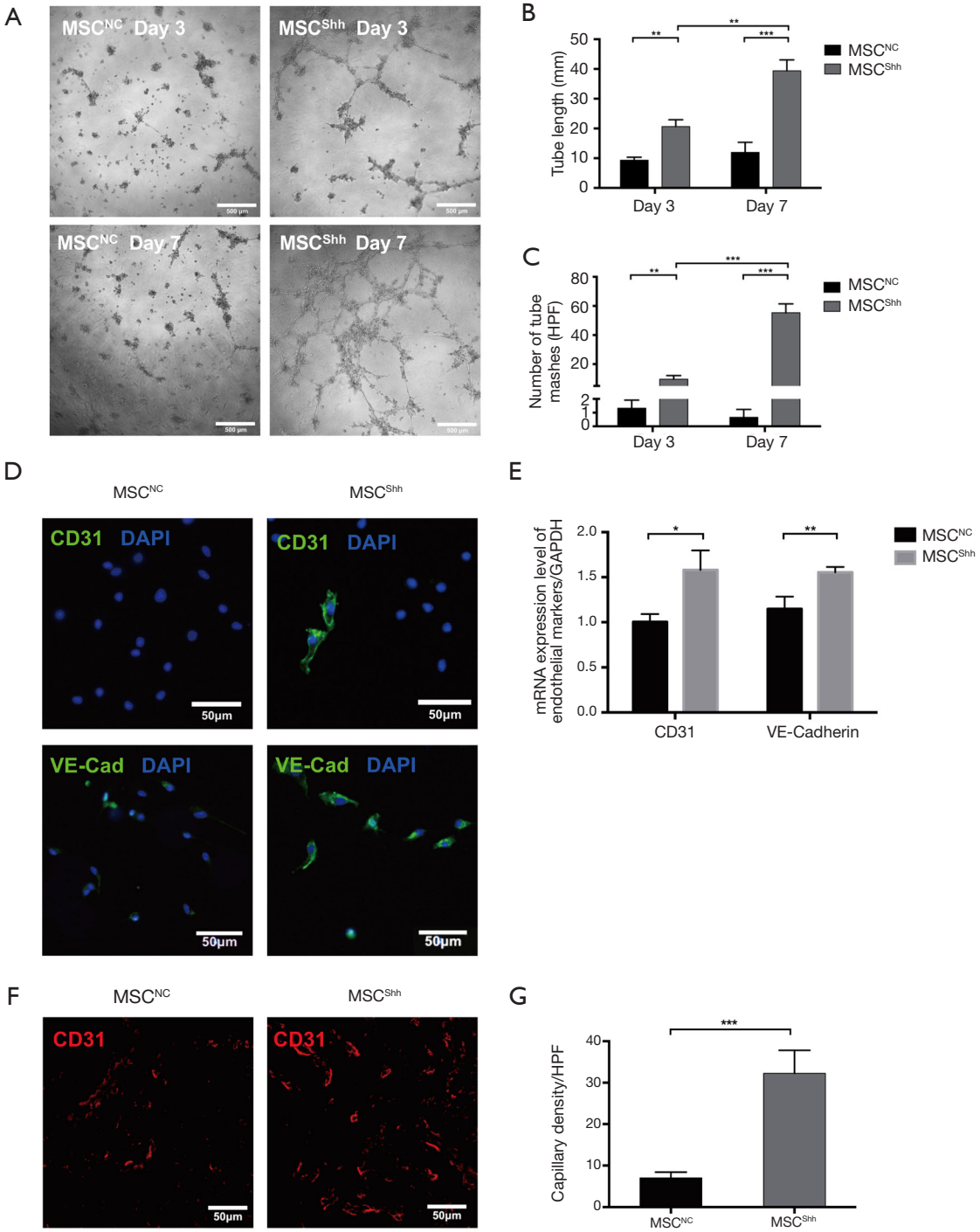

Figure 3 Endothelial differentiation ability of BMSCs was augmented by Shh transfection. (A) *In vitro* tube forming capacity of MSC^{NC} and MSC^{Shh}. Both tube length (B) and mash numbers (C) formed by MSC^{Shh} were significantly higher than MSC^{NC}. (D) Assessment of endothelial markers, CD31 and VE-cadherin, in cultured MSCs by immunocytostaining indicated that Shh could induce the endothelial differentiation of MSCs. (E) mRNA expression level of endothelial markers, CD 31 and VE-Cadherin, in MSCs transfected with Shh or empty vector. (F) Immunofluorescence staining of vessels in the sections of Matrigel plug presented red-labelled CD31 positive neo-formed vessels. (G) Capillaries formed by MSC^{Shh} were significantly higher than MSC^{NC} per high power field. All results are presented mean ± SD (n=6). **P<0.01; ***P<0.001. BM^{SC}, bone marrow-derived mesenchymal stem cell; MSC, mesenchymal stem cell; Shh, sonic hedgehog.

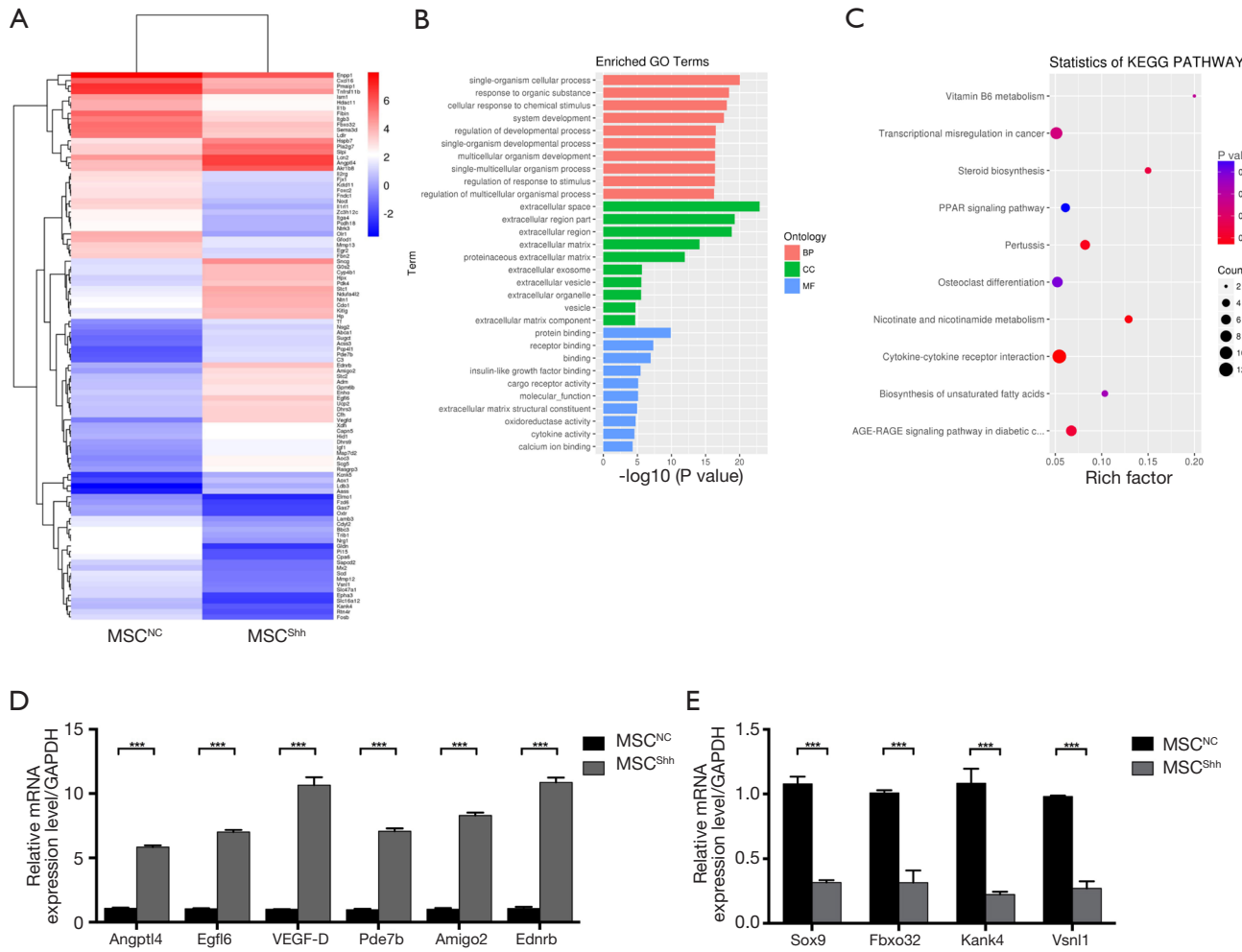

Figure 4 Gene expression profiling and functional analysis between MSC^{Shh} and MSC^{NC}. (A) Hierarchical clustering of the top 100 differentially expressed genes was generated between MSC^{Shh} and MSC^{NC}. The expression levels were visualized and the scale from least abundant to highest range is from –4.0 to 8.0. Their phylogenetic relationships were shown on the left tree; (B) the distributions are summarized in three main categories: biological process (BP), molecular function (MF), and cellular component (CC). The x-axis indicates the number of genes in each category and the y-axis indicates different GO terms; (C) rich factor is the ratio of the differentially expressed gene number to the total gene number in a certain pathway; (D,E) RT-qPCR confirmed that angptl4, Egfl6, VEGF-D, Pde7b, Amigo2 and Ednrb were elevated in MSCShh, and Sox9, Fbox32, Kank4 and Vsnl1 were down-regulated in MSCShh. Q-value is corrected P value ranging from 0.01–0.04. The color and size of the dots represent the range of the Q-value and the number of differentially expressed genes mapped to the indicated pathways, respectively. Top 10 enriched pathways are shown in the figure. MSC, mesenchymal stem cell; Shh, sonic hedgehog.

by qPCR (*Figure 4D,4E*). The genes were selected based on fold change differences, participation in migration, cell proliferation and/or involvement in vasculogenesis. All tested genes exhibited a high agreement with the high-throughput sequencing output. The expressions of Angptl4, Egfl6, VEGF-D, Pde7b, Amigo2 and Ednrb were significantly higher in MSC^{Shh} compared to MSC^{NC} (*Figure 4D*; P<0.001). Sox9, Fbxo32, Kank4 and Vsnl1 expressions were significantly lower in MSC^{Shh} compared to MSC^{NC} (*Figure 4E*; P<0.001).

Tube formation abilities were inhibited in Shh-overexpressed MSCs by knocking down of VEGF-D

We first confirmed the knocking down efficiency of VEGF-D siRNA by qPCR. It showed that VEGF-D

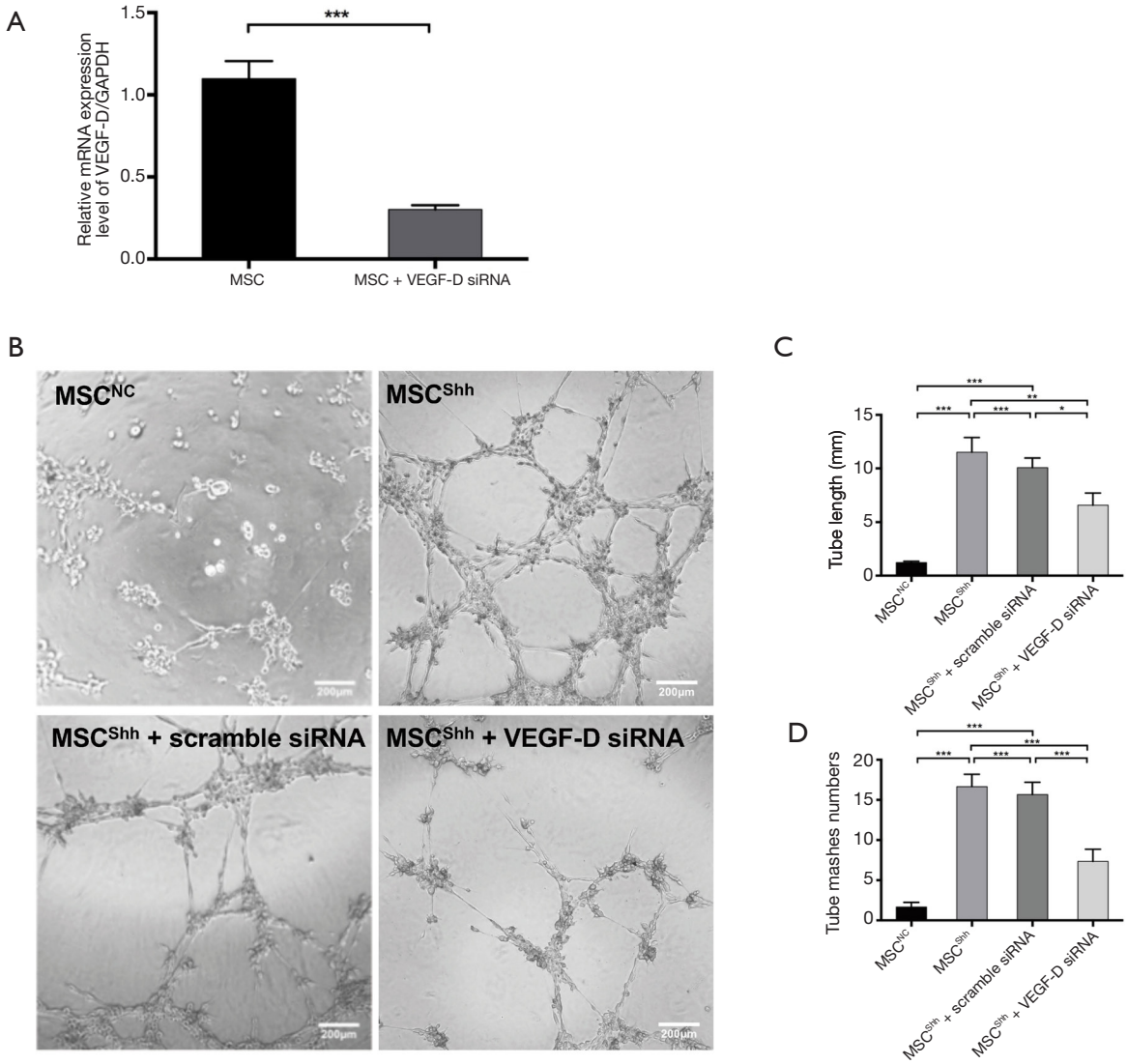

Figure 5 Knocking down of VEGF-D diminished endothelial differentiation ability of MSC^{Shh}. (A) mRNA expression levels of VEGF-D in MSC were significantly lower after VEGF-D siRNA treatment. (B) Tube formation assay of MSC^{NC}, MSC^{Shh}, MSC^{Shh} pretreated with scramble siRNA or VEGF-D siRNA was performed. Bar: 200 μm. Quantitative assessment of total number of meshes (C) and tube length (D) was carried out using ImageJ. n=6. All results are presented mean ± SD (n=6). *P<0.05; **P<0.01; ***P<0.001. VEGF-D, vascular endothelial growth factor D; MSC, mesenchymal stem cell; Shh, sonic hedgehog.

siRNA could inhibit the expression of VEGF-D by about 70% (*Figure 5A*). After transfecting MSC^{Shh} with VEGF-D siRNA, the cells presented attenuated tube-forming ability (*Figure 5B*). Accordingly, mashes formed by MSC^{Shh} with VEGF-D siRNA transfection manifested the tube length of 6.58±1.13 mashes compared with MSC^{Shh} 11.51±1.38 mashes, respectively (*Figure 5C*, P<0.01). Mashes numbers had been significantly decreased after Shh-overexpressed MSCs were transduced with VEGF-D siRNA, with 7.33±1.53 mashes *vs.* 16.67±1.53 mashes formed by MSC^{Shh} with or without VEGF-D siRNA treatment, respectively (*Figure 5D*, P<0.001).

Discussion

This study presents several new findings. First, it gives solid

evidence that bone marrow mesenchymal stem cells with upregulated Shh could differentiate into ECs both *in vitro* and *in vivo*. Second, it demonstrates that Shh could enhance the mRNA expression level of VEGF-D, which is important for endothelial differentiation in mesenchymal stem cells. In addition, it revealed that Shh activates several pathways that are highly related to endothelial differentiation ability of mesenchymal stem cells, such as PPAR signaling pathway, PI3K-Akt signaling pathway, p53 signaling pathway and cytokine-cytokine receptor interaction.

BMSCs represent a great hope for ischemic diseases. However, their therapeutic usage is still restricted because of the limited differentiation ability. Researchers have been struggling to establish a reliable endothelial differentiation, but often have gotten controversial conclusions (17). Various culture conditions such as VEGF addition (18-20), serum deprivation (21), high cell plating density (22) and mechanical stimulation (23) could induce endothelial differentiation of MSCs *in vitro*. Dr. Oswald induced the differentiation of MSCs into endothelial-like cells by addition of 50 ng/mL of VEGF, showing a strongly increased expression of kinase insert domain receptor (KDR) and fms related tyrosine kinase 1 (Flt-1) (18). Further study proved that combined stimulation of shear stress and VEGF resulted in more profound EC oriented differentiation of MSCs in comparison to any individual stimulation (23). There are also a few studies demonstrating that engineered biomaterials can enhance endothelial differentiation of MSCs (24). Very recently, Dr. Liu genetically engineered a protein containing VEGF mimicking peptide, which effectively promoted endothelial differentiation of MSCs (25). However, very rare reports detected differentiation of MSCs into ECs *in vivo*. Patrick Au *et al.* proved that MSCs couldn't differentiate into ECs *in vivo*, and could merely function as perivascular cell precursors (26).

Shh is an angiogenic growth factor that specifically regulates vascular tube formation (6,7). Although being silenced in post-natal life, reactivation of the Shh signaling pathway plays a crucial regulatory role on injury-induced angiogenesis (10). Shh gene therapy could enhance angiogenesis in peripheral ischemia (27) and myocardial ischemia model (9), through upregulation of angiogenic and vasculogenic factors, such as VEGF, Ang-1 and SDF-1α. More recently, Dr. Ashraf proved that genetic modification of MSCs with Shh improves their survival and angiogenic potential in the ischemic heart via iNOS/netrin/PKC pathway (10), but this study didn't investigate the real trace of transplanted MSCs in the ischemic myocardium. Thus, evidence for reliable differentiation of MSCs into ECs *in vivo* is urgently needed. In this study, we over-expressed Shh in MSCs to induce cell differentiation towards the endothelial lineage. We have found that MSCs with Shh overexpression could form tubes in Matrigel *in vitro* by themselves, and grow into vessels in Matrigel plug underneath the skin of nude mice. Importantly, upregulation of Shh in MSCs activates multiple angiogenic pathways and affects cell fate, helping endothelial commitment.

As presented in *Figure 2*, Shh-overexpressed cells exhibited statistically higher Ptch1 compared to negative groups. We observed increased gene expression levels of VEGF-A, IGF-1, HGF and Ang-1 at 3 and 7 days of differentiation compared to negative control groups. In addition, there was significant difference in VEGF-A and IGF-1 gene expression between the 3 and 7 days. MSC^{Shh} could form remarkable tube structures in Matrigel, while MSC^{NC} just sprouted several branches , which provides reliable and solid evidence of endothelial differentiation of BMSCs *in vitro*. Consistent with the expression pattern of angiogenic genes, the number of mashes and tube length were significantly higher in 7-day MSC^{Shh} *vs.* 3-day MSC^{Shh}. Therefore, seven days of differentiation may be right time point for endothelial induction of BMSCs. We used 7-day induced BMSCs for further experiments. Shh-overexpressed MSCs formed neovessels in Matrigel plug after subcutaneous transplantation in nude mice, while few vessels were detected in the negative control group. This provides the direct evidence of endothelial differentiation of BMSCs *in vivo*, which is rarely demonstrated before.

Activation of Shh signaling pathway contributed much to improve functions of MSCs, including survival and neovascularization. However, limited studies have been conducted on the mechanism how Shh signaling pathway affected MSCs. This study further explored the mRNA expression profiling of BMSCs upon Shh overexpression. We identified 330 differentially-expressed mRNAs in Shh-overexpressed MSCs, with enriched functions of response to stress and receptor binding by GO analysis and enriched PPAR signaling pathway, PI3K-Akt signaling pathway and p53 signaling pathway by pathway analysis. Among these genes, the most highly-upregulated gene is VEGF-D, with the fold change of 16.9. Reviewing the literatures on the relationship of Shh and VEGFs, we could find reports on Shh/VEGF-A (28-30) and Shh/VEGF-C (31), but there is no report regarding Shh and VEGF-D. To further validate our discovery, we performed qPCR first. And we found a 10-fold change of VEGF-D after Shh overexpression.

Then, we performed tube formation assay using MSC^{Shh} and MSC^{NC} after knocking down of VEGF-D. The tube formation ability of MSC^{Shh} was partially abolished by VEGF-D siRNA, which reflected that Shh promoted angiogenic function of MSCs also via VEGF-D.

Except for VEGF-D, others genes like Angptl4 (32,33), Egfl6 (34), Pde7b (35), Amigo2 (36) and Ednrb (37,38) were also reported to be involved in the process of angiogenesis. These were all detected to be upregulated in Shh-overexpressed MSCs. Angptl4 (Angiopoietin-like 4) plays a prominent role in promoting the angiogenesis and vessel permeability (32,33). Moreover, EGFL6 promotes angiogenesis via ERK, STAT3, and integrin signaling cascades (34), and also acts as an angiogenic switch that is involved in tumor angiogenesis (39).

Conclusions

We can conclude that Shh could promote endothelial differentiation of bone marrow mesenchymal stem cells by Shh/VEGF-D axis.

Acknowledgements

Funding: This work was supported by National Natural Science Foundation of China (No. 81770260), Jiangsu Province's Key Discipline/Laboratory of Medicine (XK201118), National Clinical Key Specialty of Cardiovascular Surgery, Jiangsu Clinical Research Center for Cardiovascular Surgery (BL201451), and Natural Science Foundation of Jiangsu Province (BK20151212).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The experiment protocols were approved by the Ethic Committee of Soochow University (reference number: SZUM2008031233).

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Cite this article as: Shi S, Sun J, Meng Q, Yu Y, Huang H, Ma T, Yang Z, Liu X, Yang J, Shen Z. Sonic hedgehog promotes endothelial differentiation of bone marrow mesenchymal stem cells via VEGF-D. J Thorac Dis 2018;10(9):5476-5488. doi: 10.21037/jtd.2018.09.50

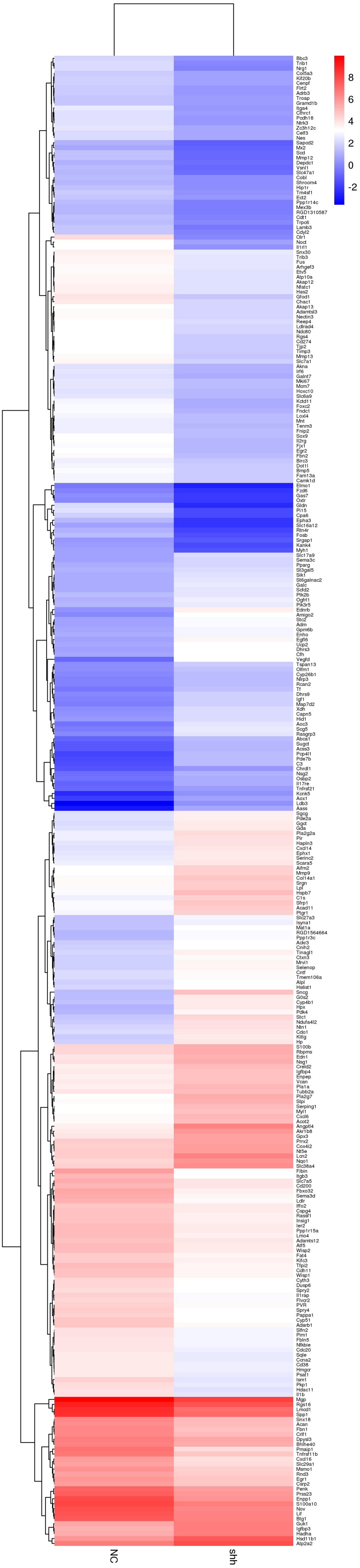

Figure S1 Cluster analysis of total gene expression profiles in MSC^{Shh} and MSC^{NC}. The cluster analysis of gene expression profiles. High expression is indicated in red, whereas low expression is coded in green. Each column corresponds to a gene. MSC, mesenchymal stem cell; Shh, sonic hedgehog.