Semaphorin 3A-hypoxia inducible factor 1 subunit alpha co-overexpression enhances the osteogenic differentiation of induced pluripotent stem cells-derived mesenchymal stem cells *in vitro*

Jing-Yi Li[1], Ting-Ting Wang[2], Chong Li[2], Zhi-Fang Wang[2], Shan Li[2], Li Ma[3], Li-Li Zheng[2]

[1]Department of Plastic Surgery, Capital Medical University Affiliated Beijing Tiantan Hospital, Beijing 100050, China;
[2]Department of Endocrinology and Metabolism, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450000, China;
[3]Department of Plastic Surgery, China-Japan Friendship Hospital, Beijing 100029, China.

Abstract

Background: Mesenchymal stem or stromal cells (MSCs) derived from the induced pluripotent stem cells (iPSCs) have uniform biological activity, which makes the clinical application of MSCs in bone repair possible. Culturing the iPSC-MSCs onto osteoconductive materials is a promising tissue engineering-based strategy in bone regeneration. The aim of this work was to evaluate the effects of semaphorin 3A (Sema3A) and hypoxia inducible factor 1 subunit alpha (HIF1α) co-overexpression on the survival and osteogenic differentiation of iPSC-MSCs.

Methods: Sema3A and HIF1α were linked together with the three (GGGGS; G, glycine; S, serine) peptide fragment, and their co-expression in iPSC-MSCs was mediated by a lentiviral vector. The fusion protein retained the immune reactivity for both Sema3A and HIF1α as determined with Western blotting. iPSC-MSCs were infected with overexpression lentivirus (oeLenti) as negative control, oeLenti-Sema3A, oeLenti-HIF1α or oeLenti-Sema3A-HIF1α lentiviruses.

Results: Sema3A overexpression alone promoted the osteogenic differentiation of iPSC-MSCs (the activity and/or expression of osteoblast markers, such as alkaline phosphatase, osteopontin, and osteocalcin, were upregulated), and suppressed cell survival. The Sema3A-HIF1α fusion protein showed a comparable osteoconductive effect to that of Sema3A without reducing cell survival. We further seeded iPSC-MSCs modified by SemaA-HIF1α overexpression onto hydroxyapatite (HA) scaffolds, and evaluated their growth and differentiation on this three-dimensional material. Additional data indicated that, as compared to iPSC-MSCs cultured in ordinary two-dimensional dishes, cells cultured in HA scaffolds grew (blank *vs.* HA scaffolds: 0.83 *vs.* 1.39 for survival) and differentiated better (blank *vs.* HA scaffolds: 11.29 *vs.* 16.62 for alkaline phosphatase activity).

Conclusion: Modifying iPSC-MSCs with pro-osteogenic (Sema3A) and pro-survival (HIF1α) factors may represent a promising strategy to optimize tissue engineering-based strategy in bone repair.

Keywords: Semaphorin 3A; Hypoxia inducible factor 1 subunit alpha; Induced pluripotent stem cells; Mesenchymal stems; Cell survival; Osteogenic differentiation; Hydroxyapatite scaffolds

Introduction

Mesenchymal stem or stromal cells (MSCs) are a population of cells with the capability to self-renew and differentiate into varied cell lineages,[1] which are majorly housed within the bone marrow.[2] The culture of MSCs onto osteoconductive materials, such as hydroxyapatite (HA) scaffolds, can induce osteogenic differentiation, which is considered as a promising tissue engineering-based strategy in orthopedics.[3] However, the limited proliferation potential of MSCs and the difficulty to prepare the bank of MSCs with uniform biological activity hinder the clinical application of MSCs.[4] Deriving functional MSCs from induced pluripotent stem cells (iPSCs) is a potential strategy developed to address these limitations.[5-7]

Xie *et al*[8] reported that the biomimetic nanofibrous scaffolds that consist of HA/collagen/chitosan enhanced the osteogenic differentiation of iPSC-MSCs, suggesting the application potential of iPSC-MSCs in bone regeneration. The aim of the present work was to evaluate whether the co-overexpression of semaphorin 3A (Sema3A) and hypoxia inducible factor 1 subunit alpha (HIF1α) can further magnify the osteoconductive effects of HA scaffolds on iPSC-MSCs.

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000000612

Correspondence to: Dr. Li-Li Zheng, Department of Endocrinology and Metabolism, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450000, China
E-Mail: Zhengli030733@163.com

Chinese Medical Journal 2020;133(3)

Received: 11-07-2019 **Edited by:** Li-Shao Guo

Sema3A is a secreted protein that belongs to the semaphorin family.[9] Semaphorins display pleiotropic biologic functions through their receptors, plexins.[10] The regulatory roles of Sema3A in the angiogenesis,[11] myogenic regeneration,[12] and synaptic connectivity[13] have been reported before. Recently, this factor has been suggested as an osteoprotective factor because of its ability to reduce bone resorption and enhance bone formation, and shift adipose MSCs towards osteogenic phenotype.[14,15] On the basis of these previous studies, to promote osteogenic differentiation of iPSC-MSCs, our group initially decided to modify the iPSC-MSCs by overexpressing Sema3A. It is noteworthy that, besides the pro-osteogenic role of Sema3A, Sema3A can trigger a pro-apoptotic program that sensitizes cancer cells or chondrocytes to apoptosis.[5,16]

HIF1α forms a heterodimer with the β-subunit.[17] Hypoxia stabilizes HIF1α from constitutive degradation largely by suppressing the activity of oxygen-dependent prolyl hydroxylases.[18] In contrast to pro-apoptotic Sema3A, HIF1α is a potent pro-survival factor, although this conclusion is deduced majorly from studies on cancer.[19,20] Increasing evidence also validates the pro-survival role of HIF1α in none-cancer cells, such as vascular endothelial cells.[21] Interestingly, we noted that MSCs overexpressing HIF1α displayed stronger proliferation as evidenced by a remarkable increase in 5-Bromo-2'-deoxyuridine (BrdU) incorporation in a study from Ciria *et al*.[22] Considering the potential apoptosis may result from Sema3A overexpression alone, we decided to co-overexpress HIF1α with Sema3A in iPSC-MSCs *in vitro*.

In the present study, iPSC-MSCs were infected with Sema3A, HIF1α, or Sema3A-HIF1α overexpression lentiviruses, and their survival and osteogenic differentiation *in vitro* were determined.

Methods

Ethical approval

C57BL/6 mice were obtained from the ChangSheng Biotechnology (Benxi, China). Experiments on animals were performed in agreement with the Guide for the Care and Use of Laboratory Animals (Eighth edition), and approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Isolation of mouse embryo fibroblasts (MEFs)

MEFs were isolated from mouse embryos according to protocols reported by Hached *et al*[23] with minor modification. In short, the pregnant mice were sacrificed by cervical dislocation 10 to 13 days post the appearance of copulation plug, and the embryos were separated from the uterus under aseptic condition. After removing the head, tail, limbs, and organs, the rest embryo was cut into small pieces and treated with 0.25% trypsin at 37°C for 1 to 2 h. Then the MEFs were collected by filtration and centrifugation, and passaged for three generations in Dulbecco modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA) at 37°C and 5% CO_2 incubator.

Establishment of iPSC-MSCs

Lentivirus particles expressing Oct4, Sox2, c-Myc, and Klf4 (Addgene, Watertown, MA, USA) were used to infect MEFs along with polybrene (Addgene) according to the supplier's protocols. Four or 6 h later, the culture medium was replaced with fresh medium. To guarantee the infection efficiency, MEFs were infected with the above-mentioned lentivirus particles for additional two times. Six days post the last infection, MEFs were harvested and seeded onto mitomycin C-treated feeder layer, and 24 h later, the culture medium was switched into iPSC culture medium, which consisted of DMEM/F12, 15% knockout serum replacement (Gibco), 0.11 mmol/L β-mercaptoethanol (Gibco), 1× non-essential amino acids (Solarbio, Beijing, China), 2 mmol/L L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA), and 1000 U/mL leukemia inhibitory factor (Sino Biological, Beijing, China). The cells were allowed to grow until cell clones appeared. Cell clones were picked, and seeded into new culture plates. To induce the transformation of iPSCs into MSCs, iPSCs were cultured in MSC medium, which consisted of low glucose DMEM, 10% FBS, and 2 mmol/L L-glutamine.[6,24] The iPSC-MSCs from passage 3 to 6 were used in the following experiments.

Identification of iPSC-MSCs via flow cytometry

The iPSC-MSCs were analyzed via flow cytometry using following antibodies: fluorescein isothiocyanate-labeled CD29, CD90, CD105, CD73, CD34, or CD45. All antibodies were purchased from eBioscience (SanDiego, CA, USA). Cells stained with non-specific immunoglobulin G (IgG) were used to set the parameters. Negative control for anti-CD34 and anti-CD45 was the same.

Overexpression of Sema3A and HIF1α fusion protein in iPSC-MSCs

The coding sequence (CDS) areas of *SEMA3A* and *HIF1A* genes were linked with a three (GGGGS; G, glycine; S, serine) linker and inserted into lentivirus-enhanced green fluorescent protein (LV-EGFP) shuttle plasmid (Addgene). The CDS areas of *SEMA3A* and *HIF1A* genes were also separately inserted into the shuttle plasmid. Then, the constructed shuttle plasmids together with other components of the lentivirus overexpression system were transfected into packaging cells to generate overexpression lentivirus (oeLenti)-Sema3A-HIF1α, oeLenti-Sema3A, and oeLenti-HIF1α particles.

Osteogenic differentiation

To stimulate the osteogenic differentiation, iPSC-MSCs were cultured in osteogenic medium which consisted of DMEM, 50 μmol/L ascorbic acid (Sigma, St. Louis, MO, USA), 100 nmol/L dexamethasone (Sigma), and 10 mmol/L sodium β-glycerophosphate (Sigma). The culture medium was refreshed every 2 days. Cells were harvested at 48, 96 h, 7 or 14 days post the differentiation induction.

The iPSC-MSCs incubated in osteogenic culture medium for 14 days were fixed with 4% paraformaldehyde, and stained with alizarin-red (Solarbio). Images were taken under a Motic microscope (Xiamen, China). The activities of alkaline phosphatase (ALP) of cells cultured for 7 days were determined with the A059-2 kit purchased from the Jiancheng Bioengineering Institute (Nanjing, China).

HA scaffolds

HA scaffolds were purchased from the Kunshan Chinese Technology New Materials Co., Ltd. (Suzhou, China), cut into small pieces to fit the culture well, and sterilized at 121°C. The scaffold piece was placed into each well for 16 h in complete medium, then iPSC-MSCs were seeded onto the scaffolds. Two days later, cells on the surface of HA scaffolds were scanned with a scanning electronic microscope (SEM) (Hitachi, Japan). Two weeks later, cells were harvested for following analysis.

Methyl thiazoleterazolium assay (MTT) assay

Forty-eight hours post the oeLenti infection, iPSC-MSCs were seeded into 96-well plates at a cell density of 5000 per well. Cell proliferation was determined with 0.5 g/L MTT following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Complementary DNAs (cDNAs) were processed with Super M-MLV reverse transcriptase (BioTeke, Beijing, China) using cellular RNAs as the templates according to the manufacturer's protocols. Primers are listed in Table 1. The mRNA expression levels of osteopontin (Opn) and osteocalcin (Ocn) were determined with qRT-PCR using SYBR Green (Solarbio) and Power Taq PCR MasterMix (BioTeke). The mRNA levels were normalized to housekeeping gene via $2^{-\Delta\Delta CT}$ method.

Western blotting analysis

The primary antibodies used were Sema3A antibody (1:1000; ABclonal, Wuhan, China), HIF1α antibody (1:10,000; Abcam, Cambridge, MA, USA), Opn antibody (1:2000; ProteinTech, Rosemont, IL, USA), and Ocn antibody (1:1000; Affinity, Cincinnati, OH, USA).

Protein fractions were prepared from iPSC-MSCs in standard radio-immunpresipitering assay lysis buffer (Solarbio), and the protein concentrations were determined with the bicinchoninic acid Kit (Solarbio). The same amounts of protein sample were loaded onto and separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transformed onto polyvinylidene fluoride membrane, and blocked in skim milk. These membranes were blotted with one of the above mentioned primary antibodies, and then with goat anti-rabbit or anti-mouse IgG. After visualized with enhanced chemiluminescence (ECL, Solarbio), the protein densities were analyzed with software gel-pro-analyzer.

Statistical analysis

All statistical data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The data conformed to the normal distribution and were presented as means ± standard deviation. Data between two groups were compared using a Student' *t* test, and from over three groups were analyzed with one-way analysis of variance followed by TUKEY's multiple comparison. Differences were considered statistically significant at $P < 0.05$.

Results

Identification of the surface markers of iPSC-MSCs

The surface markers of iPSC-MSCs of passage three were analyzed with flow cytometry assays. The results showed that cells positive for CD34 and CD45 were less than 3% [Figure 1]. Further, over 90% cells expressed characteristic MSC markers, such as CD29, CD90, CD105, and CD73. The multi-potential differentiation of iPSC-MSCs was further determined by culturing cells in appropriate induction media. We found that the iPSC-MSCs can be readily differentiated into osteocytes and adipocytes (data not shown).

Successful construction of lentiviruses overexpressing Sema3A-HIF1α fusion protein

The encoding fragments of Sema3A, HIF1α, and Sema3A-linker-HIF1α were inserted a lentivirus vector to promote the transcription in iPSC-MSCs. Three days post the infection, iPSC-MSCs were harvested, and subjected to Western blotting analysis using specific Sema3A and HIF1α antibodies. We found that oeLenti-Sema3A and oeLenti-HIF1α infection upregulated Sema3A and HIF1α protein expression in iPSC-MSCs, separately [Figure 2]. Furthermore, an approximate 180,000 Da protein was probed by both Sema3A and HIF1α antibodies in iPSC-MSCs infected with oeLenti-Sema3A-HIF1α, indicating that the fusion protein retained the immune reactivity for Sema3A and HIF1α antibodies [Figure 2].

Table 1: Primers for quantitative real-time polymerase chain reaction.

Gene ID	Name	Sequence	Amplified fragment (bp)
NM_001204201.1	OPN F	5′TTCACTCCAATCGTCCCTAC3′	161
	OPN R	5′TTAGACTCACCGCTCTTCAT3′	
NM_007541.3	OCN F	5′GAGGGCAATAAGGTAGTGAA3′	160
	OCN R	5′CATAGATGCGTTTGTAGGC3′	

Opn: Osteopontin; Ocn: Osteocalcin; F: Forward; R: Reverse.

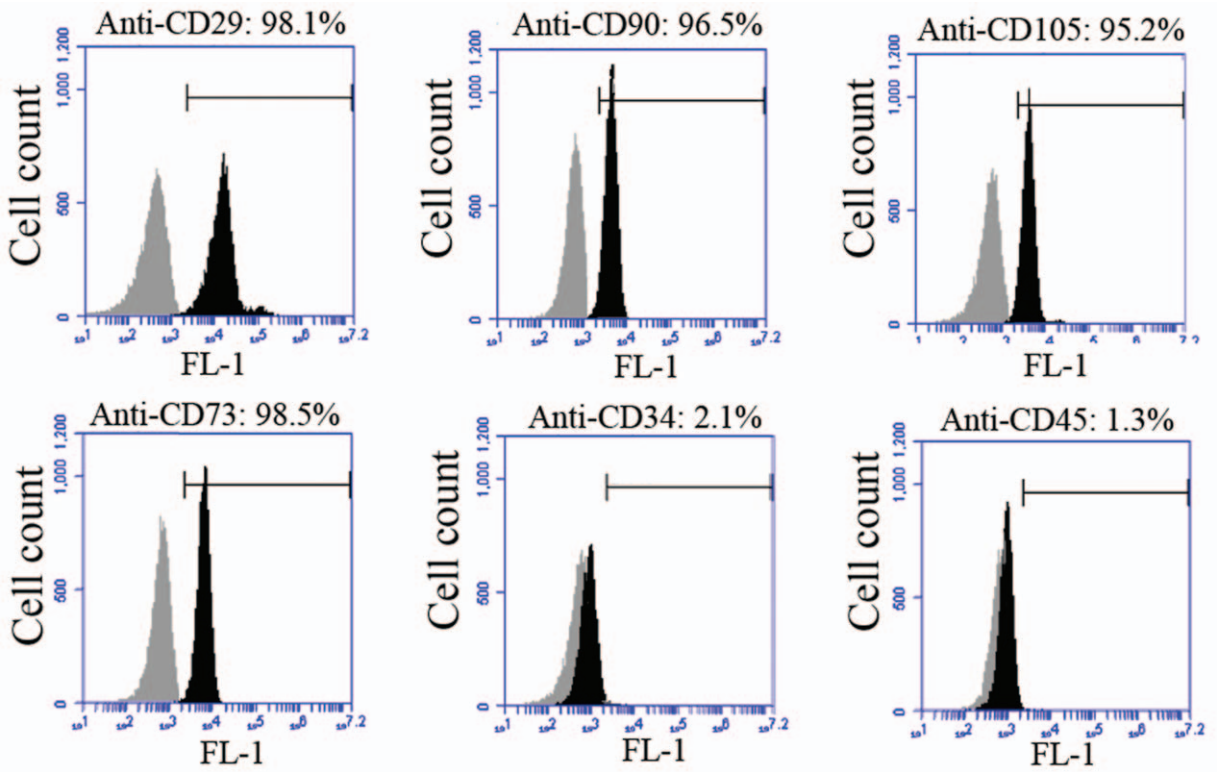

Figure 1: Identification of the surface markers of iPSC-MSCs. The iPSC-MSCs of passage three were analyzed with flow cytometry to determine their surface markers, including CD34 and CD45 (hematopoietic cell lineage-specific antigens), and CD29, CD90, CD105, and CD73 (MSC antigens). iPSCs: Induced pluripotent stem cells; MSCs: Mesenchymal stem or stromal cells.

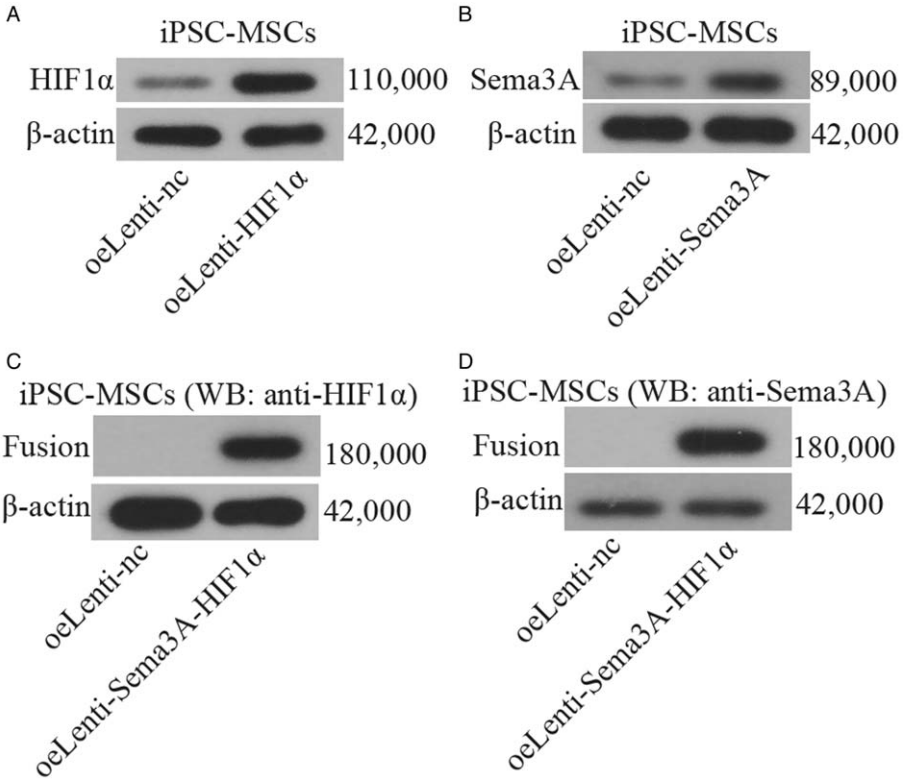

Figure 2: Successful construction of lentiviruses overexpressing Sema3A-HIF1α fusion protein. The CDS areas of *SEMA3A* and *HIF1A* genes were constructed into LV-EGFP shuttle plasmid to generate oeLenti-Sema3A and oeLenti-HIF1α viruses, separately. The two CDS areas were also linked together to generate oeLenti-Sema3A-HIF1α viruses. iPSC-MSCs were infected with the three types of lentiviruses, and 3 days later, cells were harvested for Western blotting using HIF1α antibody (A), Sema3A antibody (B), or both (C, D). CDS: Coding sequence; HIF1α: Hypoxia inducible factor 1 subunit alpha; iPSCs: Induced pluripotent stem cells; LV-EGFP: Lentivirus-enhanced green fluorescent protein; MSCs: Mesenchymal stem cells; nc: Negative control; oeLenti: Overexpression lentiviruses; Sema3A: Semaphorin 3A; WB: Western blotting.

Sema3A-HIF1α overexpression enhances the proliferation of iPSC-MSCs and promoted their differentiation in osteogenic induction media

We first analyzed the survival of iPSC-MSCs infected with three types of lentiviruses with MTT assay. As indicated in Figure 3A, the forced overexpression of Sema3A alone significantly hindered the proliferation of iPSC-MSCs. To the contrary, HIF1α overexpression promoted cell proliferation. Sema3A and HIF1α co-overexpression also promoted the growth of iPSC-MSCs, though to a less significant extent when compared to HIF1α overexpression alone.

Furthermore, the lentivirus infected cells were then incubated in osteogenic induction media for differentiation for 7 days, and then harvested to determine the ALP activity. We noted that the ALP activity was enhanced in response to Sema3A overexpression, either alone or in combination with HIF1α [Figure 3B], while HIF1α overexpression alone hardly affected ALP activity. The mineralization of iPSC-MSCs which were allowed to differentiate for 14 days was further determined with alizarin-red staining [Figure 3C]. The staining images confirmed the enhanced osteogenic differentiation of iPSC-MSCs overexpressing Sema3A and Sema3A-HIF1α fusion protein.

In addition, we also analyzed the expression levels of Opn and Ocn, two key osteogenic markers, in iPSC-MSCs. Results from qRT-PCR (48 h post-differentiation induction) and Western blotting (96 h post-differentiation induction) analyses uniformly showed that Sema3A and HIF1α co-expression enhanced Opn and Ocn expression, and the effects were similar to that of Sema3A alone [Figure 4]. HIF1α overexpression alone slightly reduced the expression of Opn and Ocn [Figure 4].

These data indicated that the forced overexpression of Sema3A or HIF1α alone either arrested cell proliferation or inhibited osteogenic differentiation of iPSC-MSCs. Co-expression of these two factors together may neutralize the adverse effects of each other.

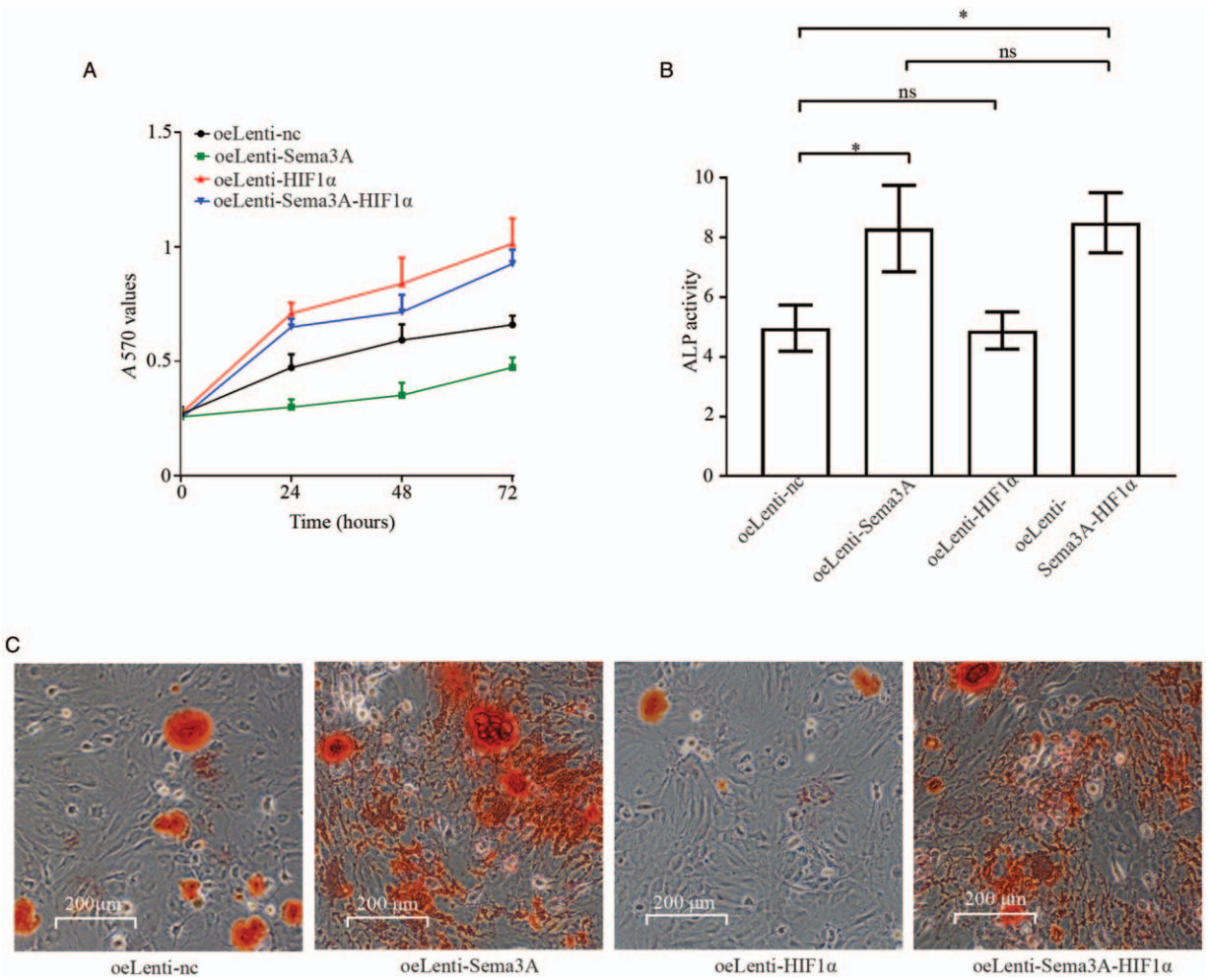

Figure 3: Sema3A and HIF1α co-expression enhanced the survival of iPSC-MSCs, and augmented their osteogenic differentiation. (A) The iPSC-MSCs infected with oeLenti-Sema3A, oeLenti-HIF1α, and oeLenti-Sema3A-HIF1α were subjected to MTT assay. iPSC-MSCs cultured in osteogenic induction media for 7 or 14 days were subjected for determining ALP activity (B) and mineralization via alizarin-red staining (C). $^{*}P < 0.05$. ALP: Alkaline phosphatase; HIF1α: Hypoxia inducible factor 1 subunit alpha; iPSCs: Induced pluripotent stem cells; MSCs: Mesenchymal stem cells; MTT: Methyl thiazoleterazolium; nc: Negative control; ns: Not significant; oeLenti: Overexpression lentiviruses; Sema3A: Semaphorin 3A.

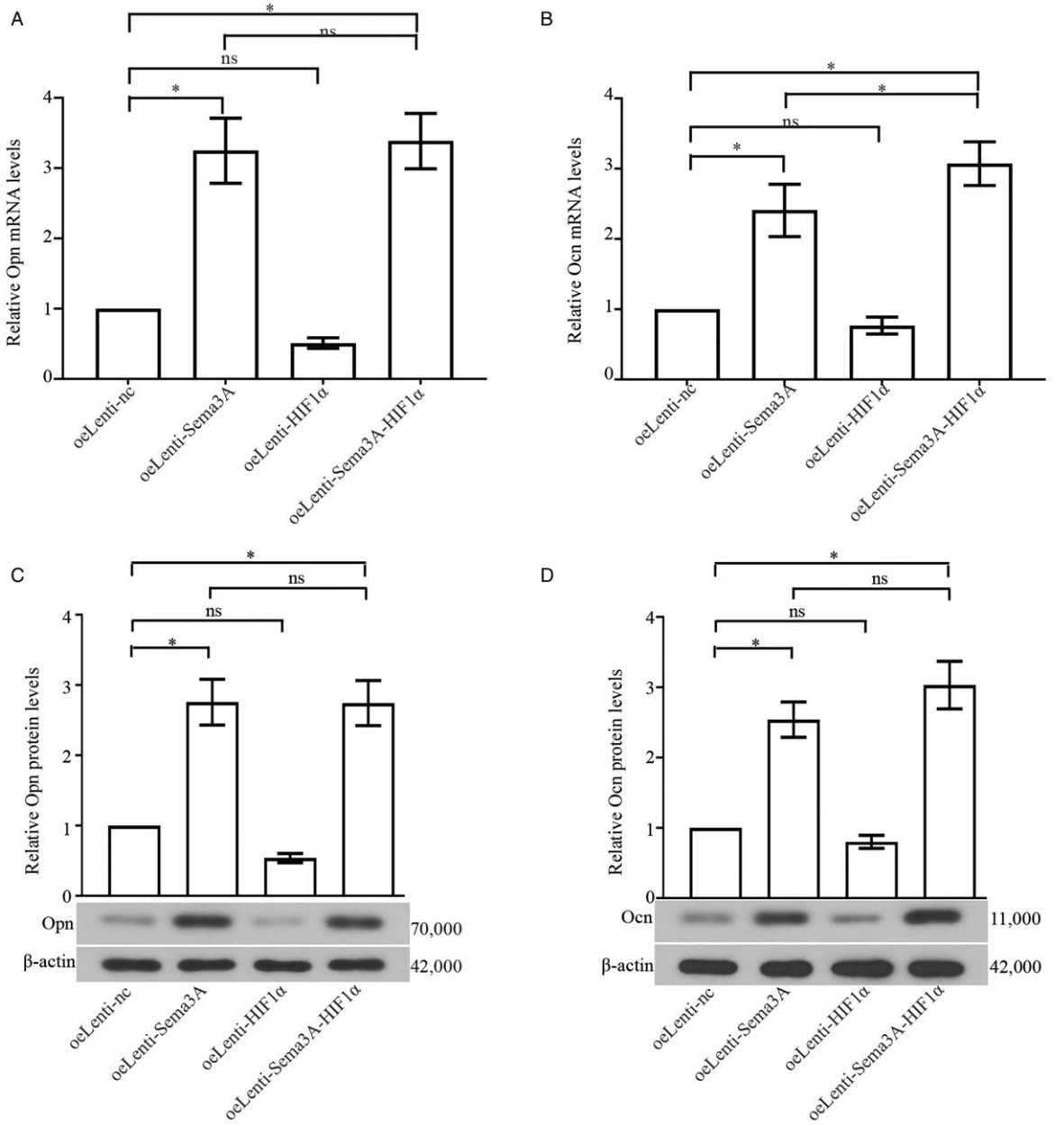

Figure 4: Sema3A and HIF1α co-expression upregulated the expression of osteogenic markers in iPSC-MSCs. The iPSC-MSCs were infected with oeLenti-Sema3A, oeLenti-HIF1α, and oeLenti-Sema3A-HIF1α, and then switched into osteogenic induction media. Cells were harvested 2 or 4 days following the induction, and their mRNAs and proteins were extracted for analyzing the mRNA (A, B) and protein levels of Opn and Ocn (C, D). $^{*}P < 0.05$. HIF1α: Hypoxia inducible factor 1 subunit alpha; iPSCs: Induced pluripotent stem cells; MSCs: Mesenchymal stem cells; nc: Negative control; ns: Not significant; Ocn: Osteocalcin; oeLenti: Overexpression lentiviruses; Opn: Osteopontin; Sema3A: Semaphorin 3A.

iPSC-MSCs modified by Sema3A-HIF1α overexpression grow better and prefer to osteogenic differentiation on HA scaffold

Finally, we seeded iPSC-MSCs modified by Sema3A-HIF1α overexpression on HA scaffolds, and compared their survival and differentiation with cells seeded in regular culture dishes. The iPSC-MSCs were seeded onto the HA scaffold, and 2 days later, the HA scaffold was scanned with a SEM. The SEM image showed that iPSC-MSCs properly distributed and attached to the interior and surface of the HA scaffold [Figure 5A]. To induce differentiation, these iPSC-MSCs were incubated in osteogenic induction media for 14 days. Thereafter, cell vitality was assessed with MTT, and cell differentiation was evaluated through determining ALP activity and osteogenic marker expression. We found that iPSC-MSCs overexpressing Sema3A-HIF1α grew better, and prefer to osteogenic differentiation on HA scaffold [Figure 5].

Discussion

Following the methods first reported by Takahashi and Yamanaka in 2006,[25] MEFs were reprogrammed into iPSCs by ectopic expression of Oct3/4, Sox2, c-Myc, and Klf4, and then differentiated into MSCs. Like the MSCs

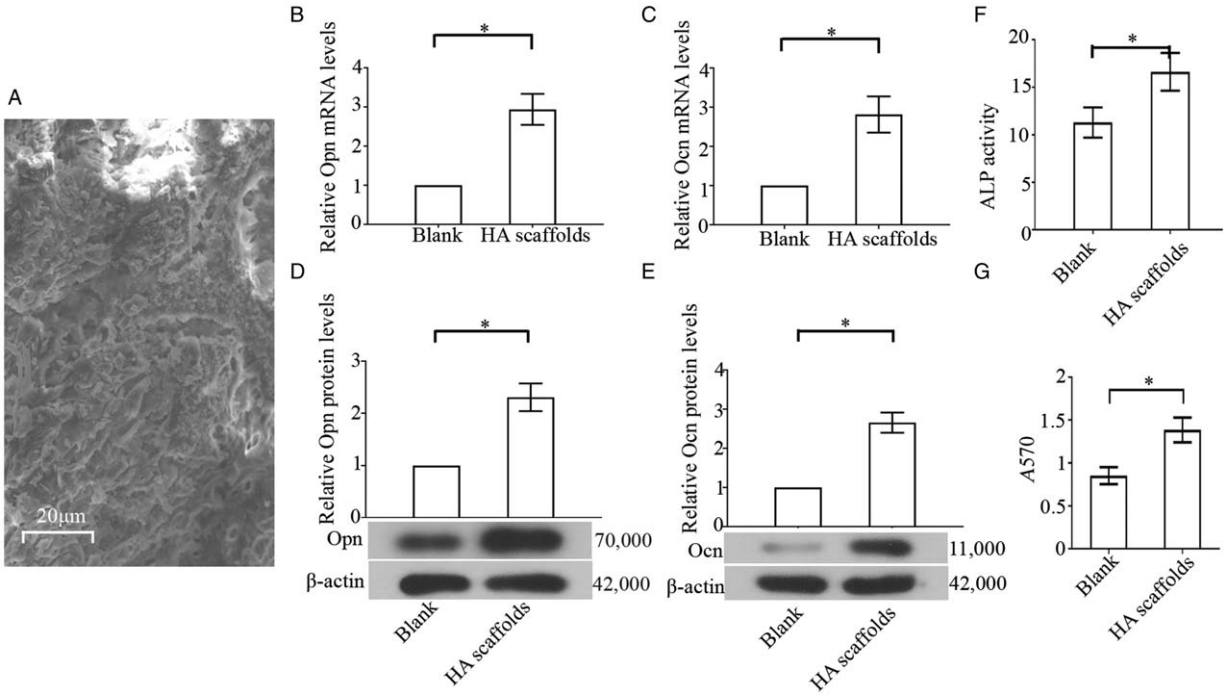

Figure 5: iPSC-MSCs modified by Sema3A-HIF1α overexpression grew better and preferred to osteogenic differentiation on HA scaffolds. (A) Two days post the seed of iPSC-MSCs onto HA scaffolds under a SEM (Bar = 20 μm). The iPSC-MSCs were cultured with or without (Blank) HA scaffold for 14 days, and then harvested for qRT-PCR (B, C), Western blotting (D, E), ALP activity (F), and MTT assays (G). $^{*}P < 0.05$. ALP: Alkaline phosphatase; HA: Hydroxyapatite; HIF1α: Hypoxia inducible factor 1 subunit alpha; iPSCs: Induced pluripotent stem cells; MSCs: Mesenchymal stem cells; MTT: Methyl thiazoleterazolium; qRT-PCR: Quantitative real-time polymerase chain reaction; Ocn: Osteocalcin; oeLenti: Overexpression lentiviruses; Opn: Osteopontin; Sema3A: Semaphorin 3A; SEM: Scanning electron microscope.

directly derived from the bone marrow,[5,26] iPSC-MSCs obtained in the present work were found to be positive for CD29, CD90, CD105, and CD73, and negative for hematopoietic cell lineage-specific antigens, CD34 and CD45. We further demonstrated that co-expression of Sema3A and HIF1α into iPSC-MSCs promoted their survival and osteogenic differentiation.

Lepelletier *et al*[27] have identified MSCs from bone marrow as Sema3A positive cells. Sema3A expression was also detectable, though not abundant, in iPSC-MSCs in our study. These findings suggest that Sema3A expresses in MSCs regardless of the different sources. A report from Liu *et al*[15] showed that Sema3A overexpression shifted the adipose MSCs towards osteogenic phenotype, and inhibited the adipogenic differentiation. In agreement with this study, we also found that Sema3A overexpression alone or together with HIF1α upregulated the osteoblast markers, ALP, Opn, and Ocn,[28] in iPSC-MSCs. We noted that, in Liu *et al*'s study,[15] the proliferation of adipose MSCs was barely affected by Sema3A overexpression, which is inconsistent with our findings. We found that Sema3A overexpression alone significantly suppressed the proliferation of iPSC-MSCs. Although the fact that MSCs used in Liu *et al*'s work and ours are derived from different sources may explain this inconsistent phenomena, most previous studies showing the apoptotic role of Sema3A supported our present findings.[10,16]

To counteract the pro-apoptotic effects induced by Sema3A in iPSC-MSCs, HIF1α was then infused with Sema3A via a 3(GGGGS) linker, a structure that is commonly used to combine two proteins together,[29] and then co-infected the iPSC-MSCs. Whether the two encoding fragments combined by this linker can produce functional proteins is the key for the co-expression experiment. Western blotting analysis was first performed using total protein extraction from cells infected with Sema3A-HIF1α viruses. We found that an approximate 180,000 Da blot was probed by both Sema3A and HIF1α antibodies, suggesting that the fusion protein retained the Sema3A and HIF1α immunoreactivity. Immune reactivity of a protein does not equal to its activity or function. Nonetheless, the immune reactivity of cells infected with SemaA-HIF1α to both Sema3A and HIF1α antibodies at least indicated that the antigenic structure of SemaA-HIF1α protein was not impaired.

HIF1α overexpression alone significantly promoted the proliferation of iPSC-MSCs, which confirmed its pro-survival role stated by previous studies.[21] Interestingly, the Sema3A-HIF1α fusion products enhanced the proliferation of iPSC-MSCs, though were less significant than HIF1α overexpression alone. The stronger proliferative ability of iPSC-MSCs induced by Sema3A-HIF1α viruses also indicated that the fusion products were functional.

Nanocrystalline HA materials are widely used as the scaffold in bone repair. HA is composed of $Ca10(PO_4)_6(OH)_2$, the major inorganic components of natural bone tissue.[30] HA scaffolds provide a three-dimensional microenvironment that allows cells to attach onto, grow

and differentiate.[31] Implantation of MSCs onto three-dimensional scaffolds shows a great promise in bone repair.[32] We next implanted iPSC-MSCs expressing Sema3A-HIF1α onto HA scaffolds, and evaluate their growth and differentiation on this scaffold. We found that the modified iPSC-MSCs could attach successfully onto the HA scaffold, suggesting that the adhesion was not affected by Sema3A-HIF1α forced overexpression. Additionally, the growth and osteogenic differentiation of iPSC-MSCs cultured on the HA scaffolds were also better than those cultured in two-dimensional dishes.

The timely formation of blood vessels in the fracture callus is a key component to accelerate bone regeneration.[33] Besides the anti-survival role of Sema3A, its anti-angiogenesis role shall be taken into serious consideration.[34] Of note, the pro-angiogenesis role of HIF1α has been widely reported.[35,36] Hypoxia-mimicking agents, such as dimethyloxalylglycine, can protect HIF1α from degradation, and promote the secretion of vascular endothelial growth factor-A *in vivo*.[37] Sole overexpression of Sema3A in adipose MSCs promoted the bone regeneration mediated by poly (lactic-co-glycolic acid) scaffolds.[15] As the growth and osteogenic differentiation of iPSC-MSCs overexpressing Sema3A-HIF1α were better, it is anticipated that these cells may have a superior effect to those only overexpressing Sema3A in promoting bone formation *in vivo*. This hypothesis shall be validated in animals with bone defect injury. Whether the angiogenesis is involved in Sema3A-HIF1α overexpression-mediated bone repair *in vivo* shall be evaluated.

In summary, the present work shows that Sema3A-HIF1α co-overexpression augments the survival of iPSC-MSCs, and promotes their osteogenic differentiation. Modifying iPSC-MSCs with pro-survival and pro-osteogenic factors may represent a promising strategy to optimize tissue engineering-based strategy in bone repair.

Funding

This work was supported by a grant from the National Natural Science Foundation of China (No. 81601726).

Conflicts of interest

None.

References

1. Zhou Q, Li SL, Ma YJ, de Tal V, Li W, Zhao YH. Detection of repair of the zone of calcified cartilage with osteoarthritis through mesenchymal stem cells by ultrashort echo time magnetic resonance imaging. Chin Med J 2018;131:1092–1098. doi: 10.4103/0366-6999.224725.
2. Fuchs E, Segre JA. Stem cells: a new lease on life. Cell 2000;100:143–155. doi: 10.1016/s0092-8674(00)81691-8.
3. Viti F, Landini M, Mezzelani A, Petecchia L, Milanesi L, Scaglione S. Osteogenic differentiation of MSC through calcium signaling activation: transcriptomics and functional analysis. PLoS One 2016;11:e0148173. doi: 10.1371/journal.pone.0148173.
4. Viswanathan S, Keating A, Deans R, Hematti P, Prockop D, Stroncek DF, *et al*. Soliciting strategies for developing cell-based reference materials to advance mesenchymal stromal cell research and clinical translation. Stem Cells Dev 2014;23:1157–1167. doi: 10.1089/scd.2013.0591.
5. Zhao Q, Gregory CA, Lee RH, Reger RL, Qin L, Hai B, *et al*. MSCs derived from iPSCs with a modified protocol are tumor-tropic but have much less potential to promote tumors than bone marrow MSCs. Proc Natl Acad Sci U S A 2015;112:530–535. doi: 10.1073/pnas.1423008112.
6. Zou L, Luo Y, Chen M, Wang G, Ding M, Petersen CC, *et al*. A simple method for deriving functional MSCs and applied for osteogenesis in 3D scaffolds. Sci Rep 2013;3:2243. doi: 10.1038/srep02243.
7. Wang F, Kong J, Cui YY, Liu P, Wen JY. Is human-induced pluripotent stem cell the best optimal? Chin Med J 2018;131:852–856. doi: 10.4103/0366-6999.228231.
8. Xie J, Peng C, Zhao Q, Wang X, Yuan H, Yang L, *et al*. Osteogenic differentiation and bone regeneration of iPSC-MSCs supported by a biomimetic nanofibrous scaffold. Acta Biomater 2016;29:365–379. doi: 10.1016/j.actbio.2015.10.007.
9. Inagaki S, Furuyama T, Iwahashi Y. Identification of a member of mouse semaphorin family. FEBS Lett 1995;370:269–272. doi: 10.1016/0014-5793(95)00850-9.
10. Moretti S, Procopio A, Lazzarini R, Rippo MR, Testa R, Marra M, *et al*. Semaphorin3A signaling controls Fas (CD95)-mediated apoptosis by promoting Fas translocation into lipid rafts. Blood 2008;111:2290–2299. doi: 10.1111/brv.12286.
11. Groppa E, Brkic S, Bovo E, Reginato S, Sacchi V, Di Maggio N, *et al*. VEGF dose regulates vascular stabilization through Semaphorin3A and the Neuropilin-1+ monocyte/TGF-beta1 paracrine axis. EMBO Mol Med 2015;7:1366–1384. doi: 10.15252/emmm.201405003.
12. Anderson JE, Do MQ, Daneshvar N, Suzuki T, Dort J, Mizunoya W, *et al*. The role of semaphorin3A in myogenic regeneration and the formation of functional neuromuscular junctions on new fibres. Biol Rev Camb Philos Soc 2017;92:1389–1405. doi: 10.1111/brv.12286.
13. Eastwood SL, Law AJ, Everall IP, Harrison PJ. The axonal chemorepellant semaphorin 3A is increased in the cerebellum in schizophrenia and may contribute to its synaptic pathology. Mol Psychiatry 2003;8:148–155. doi: 10.1038/sj.mp.4001233.
14. Hayashi M, Nakashima T, Yoshimura N, Okamoto K, Tanaka S, Takayanagi H. Autoregulation of osteocyte Sema3A orchestrates estrogen action and counteracts bone aging. Cell Metab 2019;29:627–637.e5. doi: 10.1016/j.cmet.2018.12.021.
15. Liu X, Tan N, Zhou Y, Zhou X, Chen H, Wei H, *et al*. Semaphorin 3A shifts adipose mesenchymal stem cells towards osteogenic phenotype and promotes bone regeneration in vivo. Stem Cells Int 2016;2016:2545214. doi: 10.1155/2016/2545214.
16. Sun J, Wei X, Wang Z, Liu Y, Lu J, Lu Y, *et al*. Inflammatory milieu cultivated Sema3A signaling promotes chondrocyte apoptosis in knee osteoarthritis. J Cell Biochem 2018;119:2891–2899. doi: 10.1002/jcb.26470.
17. Takubo K, Suda T. Opening the door for HIF1α tuning. Blood 2014;123:151–152. doi: 10.1182/blood-2013-11-536995.
18. Gonzalez FJ, Xie C, Jiang C. The role of hypoxia-inducible factors in metabolic diseases. Nat Rev Endocrinol 2018;15:21–32. doi: 10.1038/s41574-018-0096-z.
19. Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 2007;26:225–239. doi: 10.1007/s10555-007-9055-1.
20. Rohwer N, Jumpertz S, Erdem M, Egners A, Warzecha KT, Fragoulis A, *et al*. Non-canonical HIF-1 stabilization contributes to intestinal tumorigenesis. Oncogene 2019;38:5670–5685. doi: 10.1038/s41388-019-0816-4.
21. Calvani M, Rapisarda A, Uranchimeg B, Shoemaker RH, Melillo G. Hypoxic induction of an HIF-1alpha-dependent bFGF autocrine loop drives angiogenesis in human endothelial cells. Blood 2006;107:2705–2712. doi: 10.1083/jcb.201708105.
22. Ciria M, Garcia NA, Ontoria-Oviedo I, Gonzalez-King H, Carrero R, De La Pompa JL, *et al*. Mesenchymal stem cell migration and proliferation are mediated by hypoxia-inducible factor-1alpha upstream of notch and SUMO pathways. Stem Cells Dev 2017;26:973–985. doi: 10.1089/scd.2016.0331.
23. Hached K, Goguet P, Charrasse S, Vigneron S, Sacristan MP, Lorca T, *et al*. ENSA and ARPP19 differentially control cell cycle progression and development. J Cell Biol 2019;218:541–558. doi: 10.1083/jcb.201708105.
24. Hu GW, Li Q, Niu X, Hu B, Liu J, Zhou SM, *et al*. Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells attenuate limb ischemia by promoting angiogenesis in mice. Stem Cell Res Ther 2015;6:10. doi: 10.1186/scrt546.

25. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–676. doi: 10.1016/j.cell.2006.07.024.
26. Soleimani M, Nadri S. A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. Nat Protoc 2009;4:102–106. doi: 10.1038/nprot.2008.221.
27. Lepelletier Y, Lecourt S, Renand A, Arnulf B, Vanneaux V, Fermand JP, *et al*. Galectin-1 and semaphorin-3A are two soluble factors conferring T-cell immunosuppression to bone marrow mesenchymal stem cell. Stem Cells Dev 2010;19:1075–1079. doi: 10.1089/scd.2009.0212.
28. Marie PJ. Targeting integrins to promote bone formation and repair. Nat Rev Endocrinol 2013;9:288–295. doi: 10.1038/nrendo.2013.4.
29. Yuan W, Dong N, Zhang L, Liu J, Lin S, Xiang Z, *et al*. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine expressing a fusion protein of Ag85B-Esat6-HspX in mice. Vaccine 2012;30:2490–2497. doi: 10.1016/j.vaccine.2011.06.029.
30. Damien CJ, Parsons JR. Bone graft and bone graft substitutes: a review of current technology and applications. J Appl Biomater 1991;2:187–208. doi: 10.1002/jab.770020307.
31. Rufaihah AJ, Cheyyatraivendran S, Mazlan MDM, Lim K, Chong MSK, Mattar CNZ, *et al*. The effect of scaffold modulus on the morphology and remodeling of fetal mesenchymal stem cells. Front Physiol 2018;9:1555. doi: 10.3389/fphys.2018.01555.
32. Narayan R, Agarwal T, Mishra D, Maji S, Mohanty S, Mukhopadhyay A, *et al*. Ectopic vascularized bone formation by human mesenchymal stem cell microtissues in a biocomposite scaffold. Colloids Surf B Biointerfaces 2017;160:661–670. doi: 10.1016/j.colsurfb.2017.10.004.
33. Stegen S, van Gastel N, Carmeliet G. Bringing new life to damaged bone: the importance of angiogenesis in bone repair and regeneration. Bone 2015;70:19–27. doi: 10.1016/j.bone.2014.09.017.
34. Ochsenbein AM, Karaman S, Proulx ST, Berchtold M, Jurisic G, Stoeckli ET, *et al*. Endothelial cell-derived semaphorin 3A inhibits filopodia formation by blood vascular tip cells. Development 2016;143:589–594. doi: 10.1242/dev.127670.
35. Jeon M, Shin Y, Jung J, Jung UW, Lee JH, Moon JS, *et al*. HIF1A overexpression using cell-penetrating DNA-binding protein induces angiogenesis in vitro and in vivo. Mol Cell Biochem 2018;437:99–107. doi: 10.1007/s11010-017-3098-6.
36. Fu C, Tyagi R, Chin AC, Rojas T, Li RJ, Guha P, *et al*. Inositol polyphosphate multikinase inhibits angiogenesis via inositol pentakisphosphate-induced HIF-1alpha degradation. Circ Res 2018;122:457–472. doi: 10.1161/CIRCRESAHA.117.311983.
37. Peng J, Lai ZG, Fang ZL, Xing S, Hui K, Hao C, *et al*. Dimethyloxalylglycine prevents bone loss in ovariectomized C57BL/6J mice through enhanced angiogenesis and osteogenesis. PLoS One 2014;9:e112744. doi: 10.1371/journal.pone.0112744.

How to cite this article: Li JY, Wang TT, Li C, Wang ZF, Li S, Ma L, Zheng LL. Semaphorin 3A and hypoxia inducible factor 1 subunit alpha co-overexpression enhances the osteogenic differentiation of induced pluripotent stem cells and mesenchymal stem cells *in vitro*. Chin Med J 2020;133:301–309. doi: 10.1097/CM9.0000000000000612