Semaphorin 3A promotes osteogenic differentiation in human alveolar bone marrow mesenchymal stem cells

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Abstract. The aim of the present study was to investigate the role of Semaphorin 3A (Sema3A) in the osteogenic differentiation of human alveolar bone marrow mesenchymal stem cells (hABMMSCs). To investigate whether Sema3A affects hABMMSC proliferation and osteogenic differentiation, a stable Sema3A-overexpression cell line was generated by infection with the pAdCMV-SEMA3A-MCS-EGFP vector. Cell counting kit-8 and clone formation assays were performed to determine the proliferation ability of hABMMSCs, while cell osteogenic differentiation was assayed using Alizarin Red S staining. In addition, reverse transcription-quantitative polymerase chain reaction was employed to detect the mRNA expression level of osteogenesis-associated genes, Runt-related transcription factor 2 (Runx2), osteopontin (Opn) and osteocalcin (Ocn), during the osteogenic differentiation. The results revealed that, compared with the normal control group, the cell morphology of the infected cells was stable and no significant alterations were observed. Overexpression of Sema3A in hABMMSCs significantly increased the cell proliferation ability compared with the control group. Furthermore, the Alizarin Red S staining assay results indicated that the ossification process of hABMMSCs over-expressing Sema3A was evidently faster in comparison with that of the control group cells. Overexpression of Sema3A by pAdCMV-SEMA3A-MCS-EGFP infection also significantly increased the mRNA expression levels of the osteogenic marker genes Runx2, Opn and Ocn. In conclusion, Sema3A was observed to be a key positive regulator in hABMMSC osteogenic differentiation.

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Introduction

Mesenchymal stem cells (MSCs), which belong to the pluripotent stem cells, were initially identified in the bone marrow. Due to their various characteristics, including multidifferentiation potential, hematopoiesis support, stem cell implantation promotion, immune regulation and self-renewal (1,2), MSCs are currently a research focus. In addition, MSCs have become an attractive cell source for use in bone repair and tissue engineering due to their capacity for self-renewal and differentiation into osteoblasts (3).

Semaphorins (Semas) are a large family of conserved guidance proteins that regulate cellular shape and function (4). Semas were first identified as axon guidance factors during nervous system development, while they were found to be regulators of various developmental processes, including the heart, bone, kidney, lung and immune development, as well as angiogenesis (5-9). Previous studies have indicated that Semas serve important roles in osteoporosis, cardiovascular diseases, cancer and immune-mediated diseases (10-12). As a member of class 3 Semas, Sema3A serves a role in suppressing the progression of various types of cancer by inhibiting angiogenesis (13-16). More recently, Sema3A has been found to serve key roles in bone metabolism, at the same time, Sema3A could promote osteoblast differentiation and inhibit osteoblast activity, and is the hotspot in research of bone diseases (17,18). However, the role of Sema3A in the osteogenic differentiation of human alveolar bone marrow MSCs (hABMMSCs) remains unclear.

Therefore, in the present study, the fundamental functions of Sema3A in hABMMSC osteogenic differentiation were investigated and the underlying mechanism was analyzed.

Materials and methods

Materials. The α-minimum essential medium (MEM) culture medium, fetal bovine serum (FBS), streptomycin, penicillin and L-glutamine were supplied by Thermo Fisher Scientific, Inc. (Gibco; Waltham, MA, USA). Tryptase was obtained from Amresco, LLC (Solon, OH, USA). Dexamethasone,

β-glycerin sodium phosphate, ascorbic acid, dimethyl sulfoxide and Alizarin Red S were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The flow cytometer was purchased from Beckman Coulter, Inc. (Brea, CA, USA), while the enzyme-linked immune detector was supplied by BioTek Instruments, Inc. (Winooski, VT, USA). The ultraviolet spectrophotometer instrument (BioSpectrometer) and the polymerase chain reaction (PCR) instrument (Mastercycler nexus) were supplied by Eppendorf (Hamburg, Germany).

Separation and purification of hABMMCs. The present study was approved by the Ethics Committee of the Stomatological Hospital of Jiangsu Province (Nanjing, China), and written informed consent was obtained from each patient. hABMMSCs were isolated and expanded as described by Zhang *et al* (19). Between January 2014 and December 2015, a total of 15 patients (male 8, female 7; aged 18-22 years; mean age: 20.5 years), were admitted at the Department of Oral and Maxillofacial Surgery in Stomatological Hospital of Jiangsu Province. All patients with systemic or metabolic disease were excluded from the present study and received orthognathic surgery due to malocclusion. All the bone collected from these patients were fresh and healthy. Briefly, healthy jaw cancellous bone was collected from patients during the orthognathic surgery under sterile conditions. Subsequently, the samples were washed and centrifuged with 1 mol/l PBS at 800 x g for three or four times for 5 min each time at 4˚C. The collected endothelial cells ($5x10^4$ cells per well) and bone fragments were then seeded into a 6-well plate and cultured in α-MEM medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine and incubated in a 5% CO_2 incubator at 37˚C. The culture medium was replaced every 3 days, and cells were passaged until 80% confluence was reached. Next, first generation log-phase cells were seeded into 6-well plates (300-450 cells per well) and cultured for 7-10 days prior to the observation of visible hABMMSC colonies. Subsequently, the cells were harvested with using 0.25% trypsin and then cultured in maintenance medium consisting of 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37˚C in an atmosphere containing 5% CO_2, and third to fifth generation cells were used in subsequent experiments.

Cell infection and morphology observation. The adenovirus expression vector pAdCMV-SEMA3A-MCS-EGFP, which overexpressed human Sema3A, and the control vector pCMV-MCS-EGFP were synthesized by Genechem (Shanghai Genechem Co., Ltd., Shanghai, China). The hABMMSCs were infected with the pAdCMV-SEMA3A-MCS-EGFP (Sema3A group) or pCMV-MCS-EGFP (control group) vector in 10 μg/ml hexadimethrine bromide and incubated for an additional 48 h at 37˚C in an atmosphere containing 5% CO_2. The efficiency of infection was observed under an inverted fluorescence microscope at 48 h after the infection, and the cell morphology was examined.

Clone formation assay. Log-phase hABMMSCs were harvested with trypsin, counted with a hemocytometer, and transferred to 75-cm^2 cell culture flasks ($3x10^4$ cells/cm^2; three replicates per sample). Subsequent to incubation for 10 days, the cells were carefully rinsed twice with PBS, followed by fixing with 4% paraformaldehyde for 20 min at room temperature and staining with 0.5% crystal violet for 20 min. Subsequently, the cells were washed with distilled water and dried naturally. The number of cell clones with >50 cells was counted under the microscope and the cloning efficiency was calculated according to the following formula: Cloning efficiency (%)=(number of clones/number of cells incubated) x100% (20).

Cell proliferation assay by cell counting kit-8 (CCK-8). Third generation log-phase hABMMSCs were infected with an empty vector (pCMV-MCS-EGFP) or pAdCMV-SEMA3A-MCS-EGFP, and then the cells were seeded into a 96-well plate with an initial density of $2x10^3$ cells per well (three replicates per sample) and cultured in the osteogenesis-inducing media containing 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mol/l dexamethasone, 0.01 mol/l β-glycerin sodium phosphate and 50 μg/ml ascorbic acid at 37˚C in an atmosphere containing 5% CO_2. The viability of these cells was detected on days 1, 2, 3, 4, 5 and 6 according to the protocol of the CCK-8 assay (Dojindo, Molecular Technologies, Inc., Kumamoto, Japan), and the results were statistically analyzed.

Alizarin Red S staining. At 24 h after third generation log-phase hABMMSCs were infected with pCMV-MCS-EGFP or pAdCMV-SEMA3A-MCS-EGFP, the cells were seeded into 6-well plates ($5x10^4$ cells per well) and grown in osteogenesis-inducing media consisting of α-MEM medium supplemented with 10% FBS, 10 mmol/l β-glycerin liquid sodium phosphate, 0.3 mmol/l vitamin C and $1x10^{-5}$ mmol/l dexamethasone. Following incubation for 7, 14 and 21 days, Alizarin Red S staining was performed as described by Cai *et al* (21) with minor modification. Briefly, the cultured cells in the 6-well plates were rinsed twice with PBS and fixed with 4% paraformaldehyde for 20 min. The cells were then washed with PBS and exposed to Alizarin Red S (2% aqueous) for 5 min. Subsequently, they were washed again with PBS and observed under a microscope. Positive staining is represented as a red/purple color.

Reverse transcription-quantitative PCR (RT-qPCR). Following infection for 7 or 14 days, RT-qPCR was performed to detect the mRNA expression level of three osteogenesis-associated genes, namely Runt-related transcription factor 2 (Runx2), osteopontin (Opn) and osteocalcin (Ocn). Briefly, total RNA was extracted from the cell lines using TRIzol reagent (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. RNA concentration and quality were measured using a NanoDrop spectrophotometer (ND-1,000; NanoDrop Technologies, Wilmington, DE, USA). Next, cDNA was obtained from total RNA using a cDNA RT kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. qPCR was performed to analyze the synthesized cDNA using a PCR thermal cycler with the following amplification parameters: 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec, and 60˚C for 60 sec. All the primers used in qPCR were synthesized by Nanjing GenScript Co., Ltd. (Nanjing, China), and were as follows:

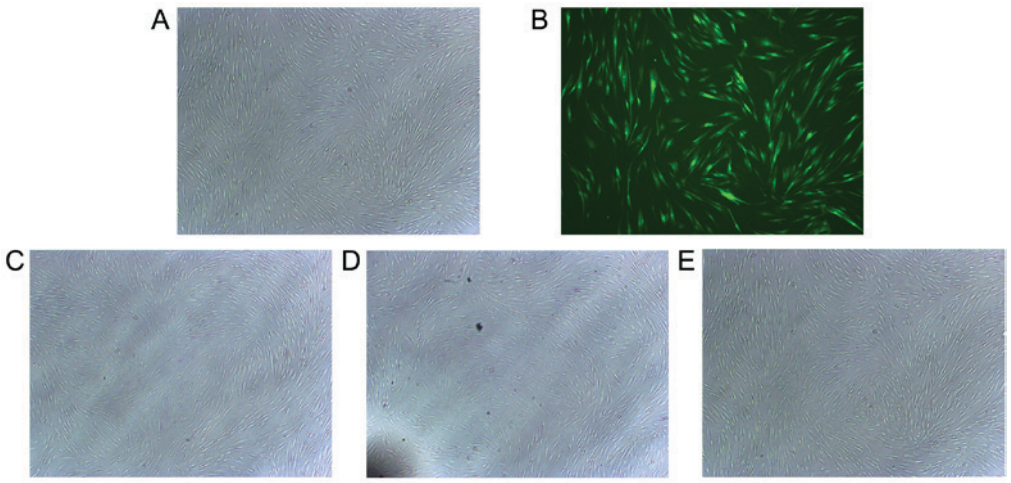

Figure 1. Overexpression of Sema3A is successfully achieved by vector infection and the cell morphology is examined. Representative overlay images of (A) phase contrast and (B) fluorescence microscopy demonstrated the transduction efficiency of Sema3A for green fluorescent protein using adenoviral transduction. The cell morphology of the (C) untreated control cells, (D) cells infected with pCMV-MCS-EGFP (control vector) and (E) cells infected with pAdCMV-SEMA3A-MCS-EGFP (Sema3A overexpressing vector) was examined under an electron microscope. Experiments were performed in triplicate. Sema3A, semaphorin 3A.

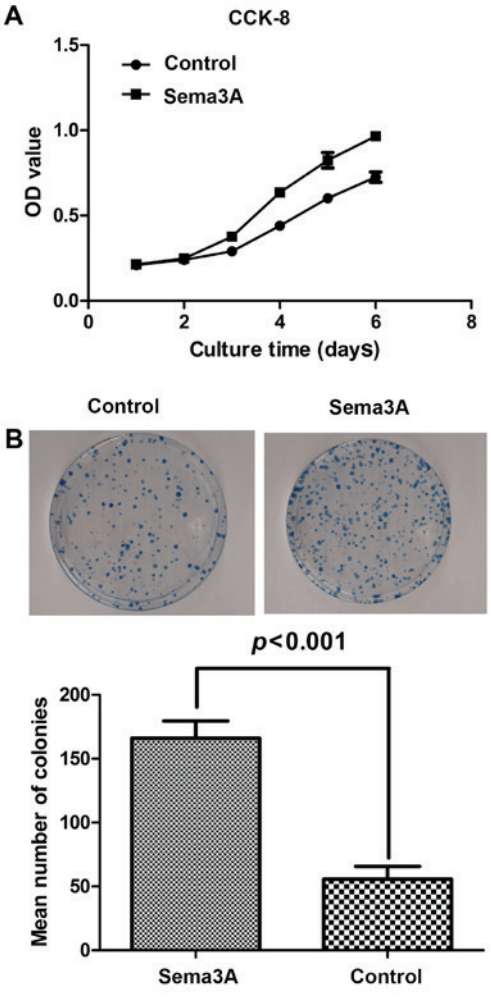

Figure 2. Sema3A promotes the cell proliferation ability of human alveolar bone marrow mesenchymal stem cells. (A) CCK-8 proliferation assay was conducted, and the OD was measured at 490 nm to determine the cell proliferation. (B) Clone formation assay results. The number of cell clones with >50 cells was counted under a microscope and the cloning efficiency was calculated. Tests were performed in triplicate, and data are represented as the mean ± standard deviation. Sema3A, semaphorin 3A; CCK-8, Cell Counting Kit-8; OD, optical density.

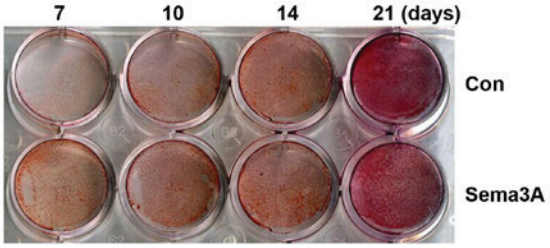

Figure 3. Alizarin Red S staining of hABMMSCs. At 24 h after third generation log-phase hABMMSCs were infected with the pCMV-MCS-EGFP (Con) or pAdCMV-SEMA3A-MCS-EGFP (Sema3A) vector, the cells were seeded in 6-well plates and grown in osteogenesis-inducing media. Following incubation for 7, 10, 14 and 21 days, Alizarin Red S staining was performed (n=3 per experiment). Sema3A, semaphorin 3A; Con, control; hABMMSCs, human alveolar bone marrow mesenchymal stem cells.

Runx-2, 5'-TGGCAGCACGCTATTAAATC-3' (forward) and 5'-TCTGCCGCTAGAATTCAAAA-3' (reverse); Opn, 5'-ACGCCGACCAAGGAAAACTC-3' (forward) and 5'-GTCCATAAACCACACTATCACCTCG-3' (reverse); Ocn, 5'-CAGACACCATGAGGACCATC-3' (forward) and 5'-GGACTGAGGCTCTGTGAGGT-3' (reverse); GAPDH, 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse). GAPDH served as an internal control.

Statistical analysis. All data are displayed as the mean ± standard deviation. Statistical comparisons between two groups were conducted with the Student's t-test. Statistical analysis was performed using the SPSS version 18.0 statistical software package (SPSS, Inc., Chicago, IL, USA). Values of $P<0.05$ were considered to indicate a difference that was statistically significant.

Results

Cell morphology. hABMMCs were separated and purified as described previously (22). The majority of the hABMMCs

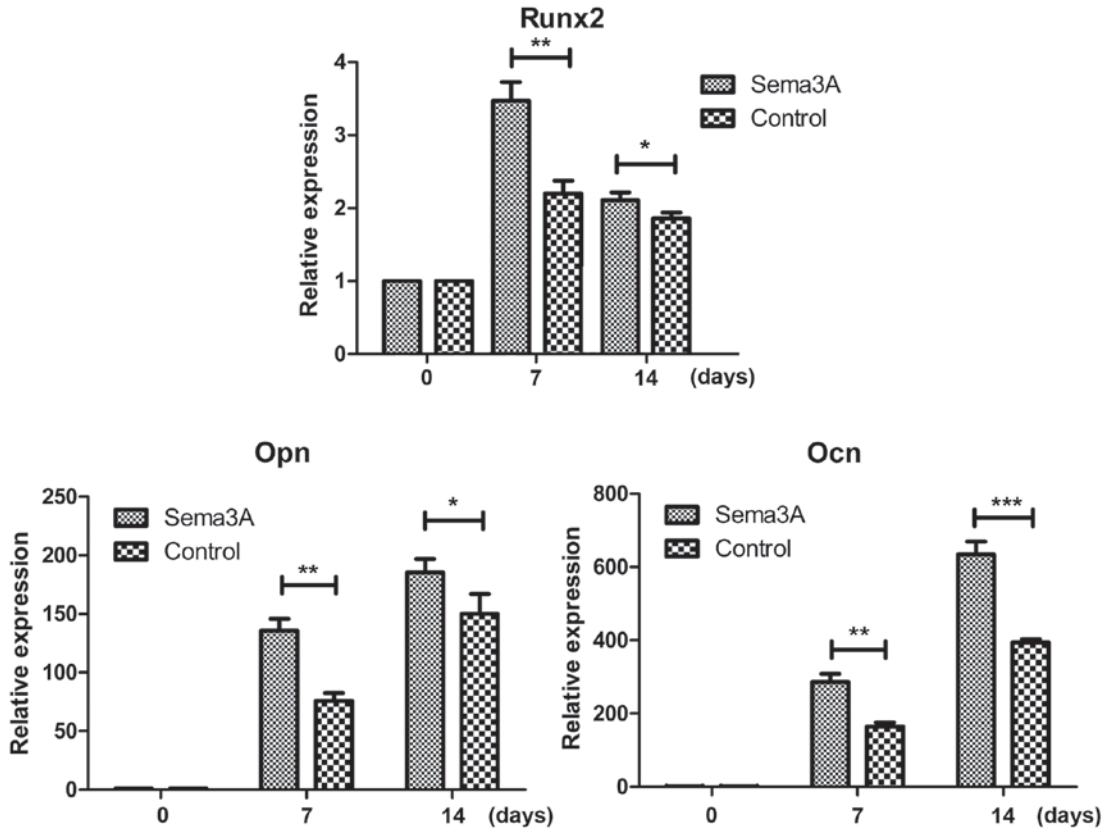

Figure 4. Relative expression levels of the osteogenesis-associated genes. After infection for 7 and 14 days, reverse transcription-quantitative polymerase chain reaction was performed to detect the mRNA expression levels of osteogenesis-associated genes (Runx2, Opn and Ocn). GAPDH served as an internal control. Experiments were performed in triplicate, and data are represented as the mean ± standard deviation. ***P<0.001, **P<0.01, *P<0.05 vs. control as indicated. Sema3A, semaphorin 3A; Runx2, runt-related transcription factor 2; Opn, osteopontin; Ocn, osteocalcin.

were spindle shaped, there was an abundant cytoplasm and only a few hABMMCs were oval-shaped. The efficiency of infection and cell morphology were examined under an inverted fluorescence microscope at 48 h after the infection. The results revealed that the cell transfection efficiency was >60%, while the cell morphology of the infected cells was stable and exhibited no significant alterations when compared with the normal control group (Fig. 1).

Effect of Sema3A on hABMMSC cell proliferation activity. To investigate the effect of Sema3A on the hABMMSC cell proliferation activity, clone formation assay and cell proliferation (CCK-8) assays were performed. As shown in Fig. 2A, compared with the control group, infection of hABMMSCs with the pAdCMV-SEMA3A-MCS-EGFP vector significantly affected the cell proliferation, while the cell viability was significantly enhanced. As shown in Fig. 2B, the results of the clone formation assay suggested that the clone formation ability of pAdCMV-SEMA3A-MCS-EGFP-infected cells was significantly increased as compared with that of the pCMV-MCS-EGFP-infected cells. All these data indicated that Sema3A overexpression significantly increased the hABMMSC proliferation.

Sema3A facilitates hABMMSC osteogenic differentiation. In order to investigate whether Sema3A exhibited an effect on osteogenic differentiation, pAdCMV-SEMA3A-MCS-EGFP or pCMV-MCS-EGFP vector was transfected into hABMMSCs. Alizarin Red S staining was then performed after 7, 10, 14 and 21 days of culturing in the osteogenesis-inducing media. hABMMSCs transfected with pAdCMV-SEMA3A-MCS-EGFP demonstrated matrix mineralization with more intense Alizarin Red S staining when compared with the pCMV-MCS-EGFP-transfected hABMMSCs (Fig. 3). Notably, the staining was more intense at earlier time points in Sema3A overexpressed hABMMSCs compared with the control. The mRNA expression levels of osteogenesis-associated genes (Runx2, Opn and Ocn) was also detected on days 0, 7 and 14 during the osteogenic differentiation using RT-qPCR. The results demonstrated that the mRNA expression levels of Runx2, Opn and Ocn were all significantly increased in the Sema3A overexpression hABMMSCs compared with the control hABMMSCs on days 7 and 14 (Fig. 4). These findings indicated that Sema3A serves an important role in promoting hABMMSC osteogenic differentiation.

Discussion

Sema3A has been reported to serve various important roles in the peripheral nerve, blood vessel and skeletal tissue development (23-25). In addition, a previous study has indicated that Sema3A-loaded chitosan intensely improved the osteogenic differentiation of osteoblasts and may be applied onto the Ti implant surface (26). In the present study, the aim was to investigate the role of Sema3A in hABMMSC osteogenic differentiation.

The process of osteogenic differentiation can be divided into three parts, including the proliferation, extracellular matrix (ECM) maturation and mineralization (27). To investigate whether Sema3A affects hABMMSC proliferation and osteogenic differentiation, hABMMSCs were initially isolated and expanded, and then a stable Sema3A-overexpression cell line was generated by infection with a pAdCMV-SEMA3A-MCS-EGFP vector, while cells infected with a control vector (pCMV-MCS-EGFP) were used as the negative control. The cell morphology of the infected cells was observed under a microscope, and no significant differences were detected between the Sema3A-overexpression and normal control groups. Subsequently, the cell proliferation ability of hABMMSCs was investigated using CCK-8 and clone formation assays, and the data suggested that Sema3A overexpression was able to significantly promote the proliferation ability of the hABMMSCs. Furthermore, Alizarin Red S staining was performed to analyze the cell osteogenic differentiation. As compared with the control group cells, the ossification process of hABMMSCs overexpressing Sema3A was evidently accelerated.

The current study also attempted to evaluate the expression levels of three osteogenic markers, Runx 2, Opn and Ocn. As an important transcription factor, Runx2 is essential for the initiation of osteoblast differentiation and bone formation (28). In the present study results, the relative expression level of Runx2 in hABMMSCs overexpressing Sema3A was markedly increased compared with that in the control cells. In addition, the osteoblast-associated proteins Ocn, which binds to calcium and promotes bone matrix calcification (29), and Opn, which is associated with cell attachment (30), were also investigated. These proteins are the main osteogenic genes that support proliferation, matrix formation and mineralization. The data of the current study revealed that these two proteins were increased in hABMMSCs overexpressing Sema3A when compared with the control cells. This observation confirmed the osteogenetic capacity of hABMMSCs demonstrated by the highest total protein content and the increased mRNA expression levels of osteogenic markers.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time that Sema3A is a key positive regulator in hABMMSC osteogenic differentiation. These findings suggested that Sema3A may be a potentially novel therapeutic agent in bone diseases.

Acknowledgements

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