

## Original Article

# The effects of Sema3A overexpression on the proliferation and differentiation of rat gingival mesenchymal stem cells in the LPS-induced inflammatory environment

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**Abstract:** Semaphorin3A has been identified as a potent osteoprotective factor that simultaneously inhibits bone resorption and promotes bone formation. The present study demonstrates the effect of the overexpression of Sema3A on the proliferation and differentiation of rat gingival mesenchymal stem cells (GMSCs) in the lipopolysaccharide (LPS)-induced inflammatory environment. rGMSCs were transfected with viral stocks of pLenO-GTP-Sema3A (Lv-Sema3A group) or pLenO-GTP (Lv-NC group), with rGMSCs as a control. The transfection efficiency was determined by flow cytometry. Cell proliferation was assessed using a Cell Counting Kit-8 assay. The expressions of alkaline phosphatase (ALP), osteocalcin (OCN), and runt-related transcription factor 2 (Runx2) were determined at 3, 7 and 14 days after the osteogenic induction culture with or without LPS using real-time PCR and Western blot. Alizarin Red staining was performed at 28 days. A pLenO-GTP-Sema3A-mediated transfection of rGMSC stably overexpressing Sema3A was built up. The overexpression of Sema3A promoted cell proliferation in the LPS-induced inflammatory environment. In addition, osteogenesis-related genes were upregulated in the Lv-Sema3A group compared with the control group. Also, after LPS administration, the overexpression of Sema3A enhanced the expression of the osteogenic genes in the LPS-induced inflammatory environment. Hence, Sema3A gene-modified rGMSCs show better osteogenic differentiation and proliferation capacities compared with rGMSCs in the LPS-induced inflammatory environment.

**Keywords:** Semaphorin3A, gingival mesenchymal stem cells, bone tissue engineering, transfection, lipopolysaccharide

## Introduction

Bone loss is the hallmark of periodontitis. Bone tissue engineering is an effective method for reconstructing alveolar bone and peri-dental structures [1]. However, in the complex micro-environment of periodontitis, alveolar bone regeneration is difficult due to relatively closed jaw regeneration and bone regeneration in the implant environment. Injectable growth factors and absorbable frames were developed for bone regeneration applications. Among them, bone growth factor, such as bone morphogenetic protein (BMP), is easily diluted by the tissue fluid or blood when it is directly injected

into the body [2]. Even if it achieves an effective therapeutic concentration, it is rapidly inactivated by proteolytic enzyme hydrolysis and cannot be used for long-term osteogenic induction in vivo. It is not feasible to directly mix growth factors with bone defects due to the intricate microenvironment. Therefore, genetically modified stem cells may be applied to bone tissue engineering in order to obtain alveolar bone regeneration in the complex microenvironment of periodontitis.

Gingival mesenchymal stem cells (GMSCs) derived from oral gingival tissues are easy to access, are homogenous, and have high prolifer-

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eration rates, which may be related to the faster healing of gingival damage in the oral cavity than in other areas and to less scar tissue being repaired [3-5]. Studies have demonstrated that GMSCs are superior to bone marrow mesenchymal stem cells (BMSCs) for cell therapy in regenerative medicine [6, 7]. Although GMSCs have been shown to have the potential for multi-directional differentiation and directional migration, a highly efficient differentiation protocol is yet to be reported.

Increasing evidence has proved that the nervous system may have the role of regulating bone remodeling. As we all know, the semaphorin family is a neuronal axon guiding factor with the same domain protein molecules, which plays a critical role in the development of neuronal development and injury, cardiovascular reconstruction, and tumorigenesis [8-11]. Semaphorin3A (Sema3A) is a member of the semaphorin family that plays multiple roles in neurosteering and tumorigenesis. As it has been described as having a certain correlation with the stability of the skeletal system, Sema3A might influence bone homeostasis by promoting osteoblast production and inhibiting bone regeneration of osteoclast activation, which is expected to be a new target for the treatment of bone metabolic diseases [12, 13]. However, due to its protein properties and high cost, the Sema3A protein is rarely used in bone tissue engineering.

In this study, an overexpressing Sema3A lentiviral vector, pLenO-GTP-Sema3A, was constructed and transfected into rGMSCs to get a high expression of Sema3A. The effects of overexpressing Sema3A on the proliferative activity and osteogenic differentiation of rGMSCs in the LPS-induced inflammatory environment were analyzed in vitro.

### Materials and methods

#### Cell culture

rGMSCs were isolated from four- to six-week-old Wistar rats using enzymolysis, for which the use of the animals was approved by the Animal Care and Use Committee of the School of Stomatology, Shandong University. Briefly, after anesthesia and euthanasia by an intraperitoneal injection of 10% chloralhydrate (0.4 g/kg body weight), the body was immersed in 75%

alcohol for 15 min. In the biosafety cabinet, mandibles were excised and healthy gingival tissue consisting of an approximately 1 mm fragment from around the molars was obtained and flushed with penicillin (100 U/ml; Solarbio, China) and streptomycin (100 mg/ml; Solarbio, China) in phosphate-buffered saline (PBS; Hyclone). Then, the tissue was digested with dispase II (3 mg/ml; Sigma-Aldrich) and collagenase I (3 mg/ml; Sigma-Aldrich) for 2 h at 37°C to separate single cells, which were cultured later in a culture dish. After subculture, the adherent cells were called GMSCs. The explant culture was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM/10% FBS; HyClone, USA; control medium [CM]). When the density of cells reached 80% confluence, they were passaged or seeded into 6-well plates at a density of  $10^5$  cells/cm<sup>2</sup>. Cells within 3-5 passages were used for the following experiments. All cells were cultured in a humidified incubator under 5% CO<sub>2</sub>/95% air at 37°C. The culture medium was changed every 2-3 days, and the cells were subcultured at 1:3.

#### Construction of pLenO-GTP-Sema3A

The pLenO-GTP-Sema3A was provided by Bio-Link Technology (Shanghai, China). The 2nd-3rd passage rGMSCs were seeded in 6-well plates at a density of  $10^5$  cells/well and transfected with pLenO-GTP-Sema3A or pLenO-GTP in the presence of polybrene (4 µg/mL) for 6 h. After that, the cells were washed twice with PBS and incubated in CM. After transfection for 72 h, fluorescence microscopy was employed to determine transfection efficiency. Numbers of green fluorescence positive cells were counted using a fluorescent microscope under 100-fold magnification in four fields of view, and the total number of cells was quantified under a lighted microscope.

#### Flow cytometry analysis

After transfection with pLenO-GTP-Sema3A or pLenO-GTP and screening by puromycin for 3 days, the transfection efficiency of rGMSCs was analyzed using flow cytometry (BD FACSCalibur, USA). Briefly, rGMSCs was transfected with pLenO-GTP-Sema3A or pLenO-GTP and puromycin (2.5 µg/ml) was added for screening.  $10^6$ /well rGMSCs from each group were

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**Table 1.** Primer sequences used for real-time PCR

Primer	Genbank	Sequence (5'-3')	Length (bp)
Sema3A	NM_017310.1	CTTATGACCCCAAACCTTCTGACTG GTTCTCCATCTATTGCATTTTCTCG	258
GAPDH	NM_017008.4	CCTGAAGTCTCAGCAGCAAGTCCT TCAGAGGTGGCAGTGTATCA	138
ALP	NM_013059.1	TGGTACTCGGACAATGAGATGC GCTCTTCCAAATGCTGATGAGGT	219
RUNX2	NM_001278483.1	CCTGAAGTCTCAGCAGCAAGTCCT TCAGAGGTGGCAGTGTATCA	237
OCN	XM_006232594.3	CCCTGACTGCATTCTGCCTCT CCAAGTCCATTGTTGAGGTAGCG	136

placed in 500 µl PBS for flow cytometry using rGMSCs as a control.

### Cell Counting Kit-8 assay

To evaluate the rGMSC proliferation after LPS treatment or normal conditions on 1, 3, 5, and 7 days, a Cell Counting Kit-8 assay (CCK-8, Dojindo, Japan) was used. Briefly, 10 µl CCK-8 reagents were gently mixed with 100 µl CM or CM with 5 µg/ml lipopolysaccharide (LPS) and added evenly into each well. After 2 h of incubation at 37°C, the absorbance was measured at the 450 nm wavelength using a microplate reader (SPECTROstar Nano; BMG Labtech, Germany), and a proliferation curve was generated on the basis of absorbance and time.

### Real-time PCR

The cells were cultured under an osteogenic differentiation medium [DM], CM with 50 mg/ml ascorbic acid (Sigma-Aldrich), 10 nM dexamethasone (Sigma-Aldrich), and 10 mM β-glycerophosphate (Sigma-Aldrich), or DM with 5 µg/ml LPS for 7 and 14 days, and the mRNA expressions of alkaline phosphatase (ALP), osteocalcin (OCN), and runt-related transcription factor 2 (Runx2) were determined using real-time PCR. The mRNA levels of Sema3A were examined after transfection for 3 days. Total RNA was extracted from the cells using Trizol reagent (Takara, Dalian, China) before being subjected to reverse transcription using the PrimeScript RT Reagent kit (Takara, Dalian, China) in accordance with the manufacturer's recommendations. The real-time PCR analysis was conducted with a SYBR Premix Ex Taq II (Takara, Dalian, China) using the LightCycler 96

Real-time PCR System (Roche, Switzerland) in a 10 µl reaction volume. The PCR conditions were as follows: incubation at 95°C for 15 s, 45 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 35 s. GAPDH was selected as the internal control. The primers used are listed in **Table 1**.

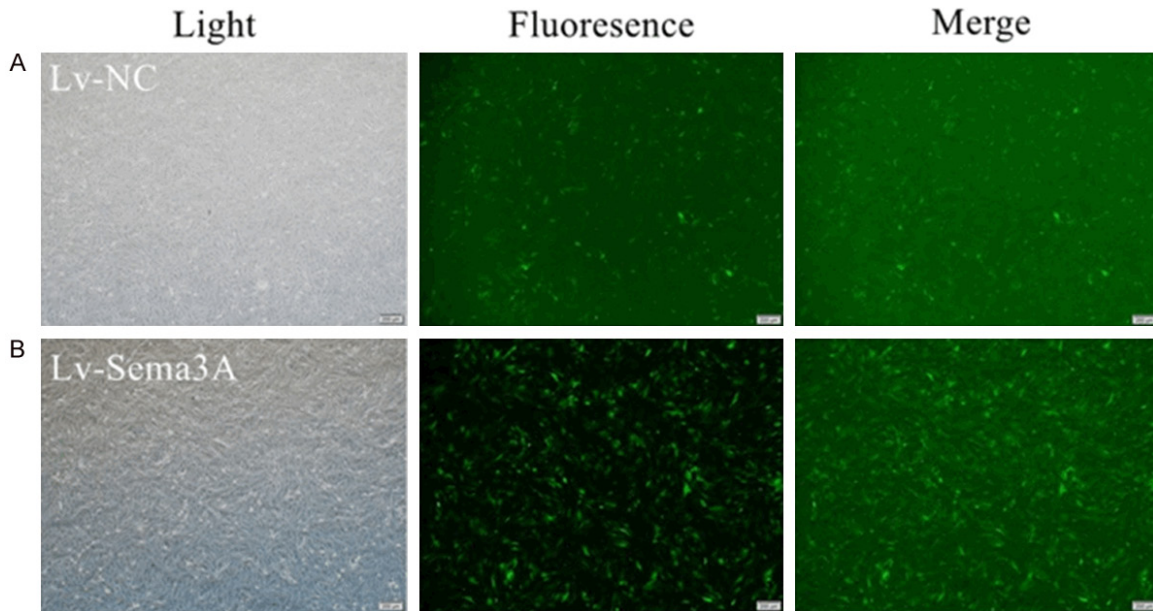
### Western blotting analysis

The cells were extracted with a RIPA buffer (Solarbio, China) and supplemented with a 1% phosphatase inhibitor (Boster, Wuhan, China) and 1% phenylmethanesulfonyl fluoride (Solarbio, China). The protein concentrations of the lysates were measured using a BCA Reagent kit (Beyotime, China). Then, 20 µg of the protein extracts was resolved by a 10% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis for 1 h and then transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% milk for 2 h. The blots were incubated with primary antibodies against Sema3A (diluted 1:1000; Abcam, UK), ALP (diluted 1:500; Abcam, UK), Runx2 (1:1000; Cell Signaling Technology, China), OCN (1:1000; Cell Signaling Technology, China), overnight at 4°C and then with a secondary antibody, horseradish peroxidase-linked goat-anti rabbit IgG, at room temperature for 1 h. The blots were exposed using an enhanced chemiluminescence system, and the integrated optical density values of each blot were analyzed using Gel Pro software version 4.0.

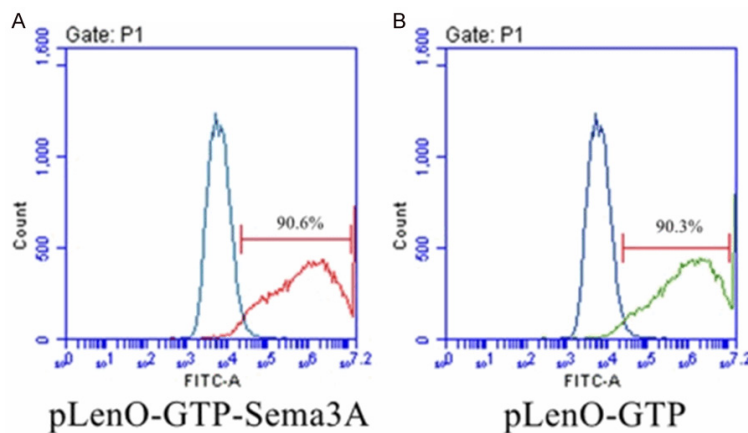
### Alizarin red staining

The rGMSCs from each group were seeded into 6-well plates at a density of 10<sup>5</sup> cells/well. When the cells extended over 80% of the bottom of the wells, the culture medium was replaced with DM or DM with 5 µg/ml LPS. After 28 days of incubation, the cells were rinsed thrice with PBS (pH 7.4) and fixed with 4% formalin on ice for 30 min, washed thrice with deionized water and then stained with 1% alizarin red (Sigma-Aldrich) (pH 4.2) for 15 min. Then, to estimate the relative content of calcium within the cell matrix, 10% cetylpyridinium chloride (Sigma-Aldrich) was added to the

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**Figure 1.** Transfection efficiency of rGMSCs under fluorescence microscopy. The transfection rate of the Lv-Sema3A group was 60% (B) and it was the same in the Lv-NC group (A) at 72 h after transfection.



**Figure 2.** Transfection efficiency of rGMSCs by flow cytometry. The transfection rate of the Lv-Sema3A group was 90.6% (A) and that of the Lv-NC group was 90.3% (B) after screening by puromycin for 3 days.

stained wells and the absorbance of the extracted dye was measured at the 562 nm wavelength.

### Statistical analysis

All data were presented as the mean  $\pm$  standard deviation (SD) from at least three independent experiments. The statistical analysis was determined using Student's *t* test of two groups or a one-way ANOVA of multiple groups, in which  $P < 0.05$  was considered statistically significant.

## Results

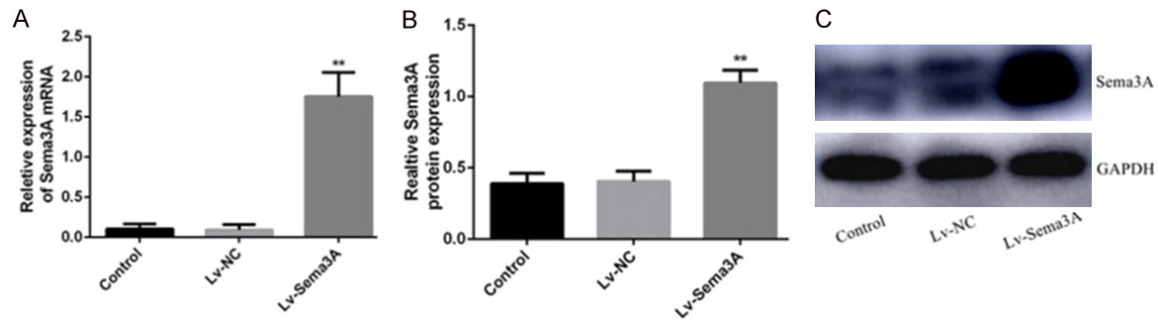
### Construction of pLenO-GTP-Sema3A transfected rGMSCs

The pLenO-GTP-Sema3A was constructed (Figure S1) and rGMSCs were used for transfection (Figure S2). After 72 hours of transfection, the transfection rate was 60% in the Lv-Sema3A group and the same in the Lv-NC group (Figure 1). A flow cytometry analysis showed that after screening by puromycin for 3 days, the transfection rate was 90% in the Sema3A group, and

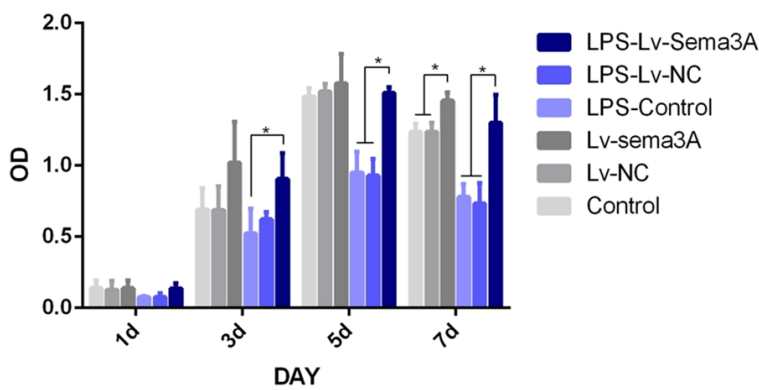
the rate was the same in the NC group (Figure 2).

Following transfection for 3 days, the cells were harvested, and the expression levels of Sema3A were analyzed using real-time PCR and Western blot analyses. The results revealed that the mRNA expression of Sema3A was higher in the Lv-Sema3A group compared with the control group (Figure 3A) ( $P < 0.01$ ). Sema3A protein expression was significantly increased in the Lv-Sema3A group (Figure 3B, 3C) ( $P <$

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**Figure 3.** The effect of the Sema3A gene transfection of rGMSC using real-time PCR and Western blot detection. The mRNA and protein levels of Sema3A were significantly higher in the Lv-Sema3A group compared with the control and Lv-NC groups (A-C) (\*\* $P < 0.01$ ).



**Figure 4.** Cell proliferation was measured using a Cell Counting Kit-8. There are statistical differences between the Lv-Sema3A group and the control groups at 7 days culture with DM and 3, 5, 7 days with DM+LPS (\* $P < 0.05$ ).

0.01). Transfection of mock lentivirus does not affect Sema3A gene expression in rGMSCs, and it was confirmed that the transfection of pLenO-GTP-Sema3A into rGMSCs increases Sema3A expression.

### Cell proliferation

The results showed that statistical differences between the Lv-Sema3A group and the control groups were significant on the 7th day, and the proliferative activity of the cells has been reduced by the effects of LPS. In the LPS-induced inflammatory environment, the statistical differences between the Lv-Sema3A group and the control groups were significant on the 3rd, 5th, and 7th days (**Figure 4**) ( $P < 0.05$ ).

### Osteogenic capacity detection

The expression levels of the osteoblast-related genes, including ALP, Runx2 and OCN, were measured using real-time PCR after they were

cultured with DM+LPS or DM for 5, 7, and 14 days. The results showed that the expression levels of ALP, Runx2, and OCN were significantly increased in the Lv-Sema3A group, compared with the control group. Nearly all these genes had lower expression levels in the inflammatory environment. LPS significantly inhibited the osteogenic activity of the cells. Under the action of LPS, compared with the control group, ALP, Runx2, and the OCN mRNA expression levels in the Lv-Sema3A group were

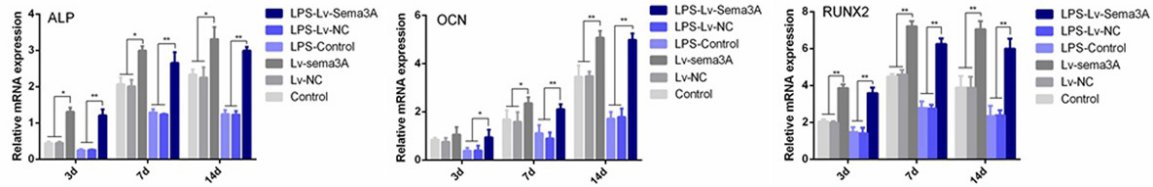
significantly increased ( $P < 0.05$ ) (**Figure 5**). Western blot showed that protein levels of ALP, Runx2, and OCN were significantly increased in the Lv-Sema3A group at 14 days in the LPS-induced inflammation, compared with the control group ( $P < 0.05$ ) (**Figure 6**). Moreover, as the culture time was prolonged, the overexpression of Sema3A attenuated the inhibitory effect of LPS on cell osteogenesis.

The mineral nodules stained with calcium-specific Alizarin Red revealed that the Lv-Sema3A group displayed larger areas covered by mineral nodules after 28 days of mineralization-induced culture. In the LPS-induced inflammatory environment, the mineral nodules in the Lv-Sema3A group were greater than those in the control groups (**Figure 7**).

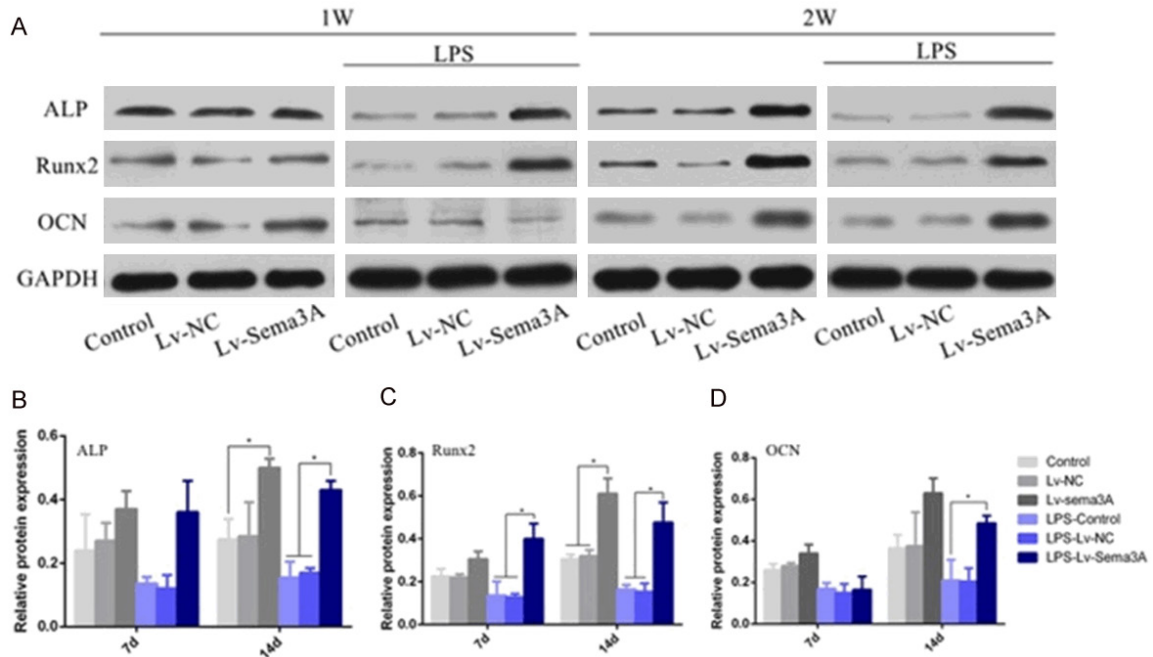
### Discussion

Periodontitis is a chronic progressive disease which involves a severe loss of alveolar bone

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**Figure 5.** The osteogenic gene expression of ALP, Runx2, and OCN were analyzed using Real-time PCR at 3, 7, and 14 days. The mRNA levels of ALP, Runx2, and OCN were up-regulated in the Lv-Sema3A group after being cultured with DM or DM+LPS (\*P < 0.05, \*\*P < 0.01).



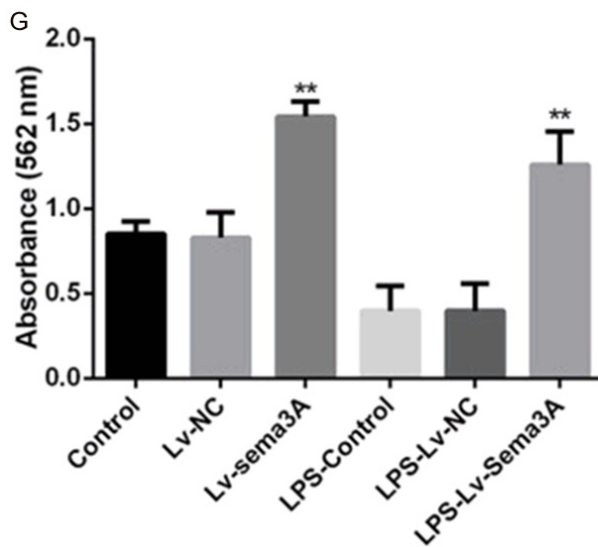
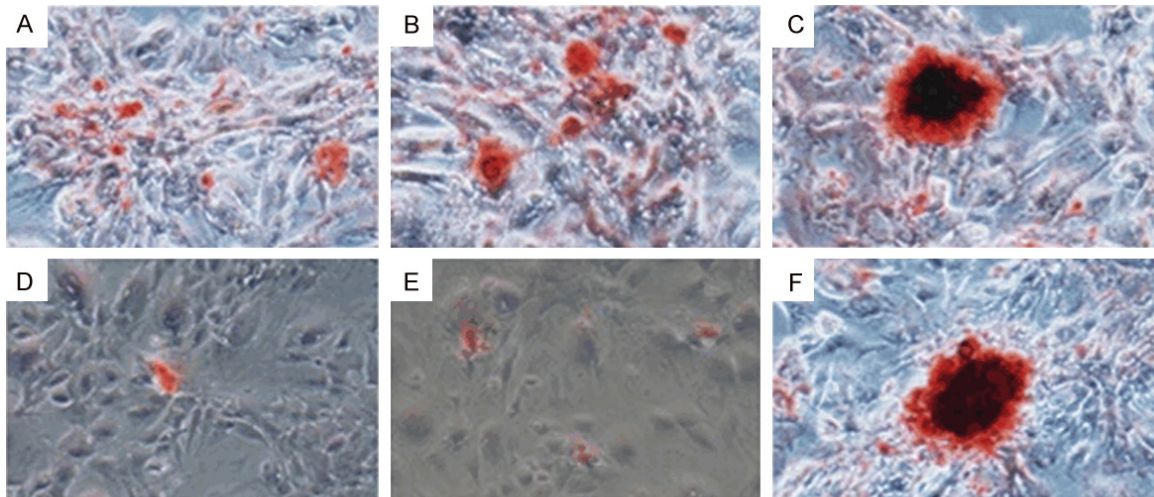
**Figure 6.** The protein expressions of ALP, Runx2, and OCN were detected by Western blot at 7 and 14 days. There are statistical differences between the Lv-Sema3A group and the LPS Lv-Sema3A groups cultured at 7 and 14 days with DM or DM+LPS (\*P < 0.05, \*\*P < 0.01).

[14]. Bacteria can invade periodontal tissues directly as well as through the indirect destruction of the immune response [15]. The tight balance of the activities of bone resorption and bone formation are destroyed. At present, due to the complex periodontal microenvironment, the practice of conventional stem cells being used as seed cells in bone tissue engineering may not have achieved the desired biological effects. The use of exogenous genetically modified stem cells in tissue engineering may lead to new advances in the reconstruction and regeneration of periodontal bone tissue.

The gingival covering the alveolar bone and surrounding the necks of teeth is a special part of the oral tissue and an important component of

the biochemical barrier and oral mucosal immunity[16].Gingivaldamagehealsquicklywithout scarring. Previously, bone marrow and periodontal ligaments were considered as an MSC source of periodontal tissue regeneration; however, there are some limitations and shortcomings in the use of these MSCs for periodontal tissue engineering and cell therapy [17]. In accordance with present studies, GMSCs have several advantages over periodontal ligament stem cells (PDLSCs) and bone marrow stem cells (BMSCs) [6]. GMSCs exhibit stable morphology and sustain MSC characteristics at higher passages. Also, GMSCs are not tumorigenic and maintain karyotype and telomerase activity in long-term cultures. Recent studies pointed out that not only can transplanted

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**Figure 7.** Mineralized nodules by Alizarin Red staining (original magnification 200 $\times$ , scale bar = 100  $\mu$ m) (A-F). The relative amount of calcium was calculated by absorbance at 562 nm (G). The mineralized nodules in the Lv-Sema3A group were higher in quantity than those in the control group in the LPS-induced inflammation (\*\* $P < 0.01$ ).

GMSCs be home to bone defects but they can also can promote bone regeneration [18]. However, the studies demonstrated that gingival-derived cells satisfy the minimum standards that can be defined as MSCs proposed by the International Cell Therapy Association [19]. Zhang et al. demonstrated that the transplantation of GMSCs mixed with HA/TCP in immunocompromised mice can form connective tissue without osteogenic differentiation [20]. Gene-modified GMSCs are expected to become a potential biological therapy [21]. The application of HIV-based viral vectors in gene therapy in vivo overcomes the short half-life of exogenous cytokines, high cost, possible toxic effects, repeated administration, and rapid diffusion in the body or decomposition by proteases and difficulty in maintaining an effective concentration.

Semaphorin 3A (Sema3A) as a diffusible axonal chemorepellent was initially described as an axon guidance molecule that plays a crucial role in the generation of the nervous system [22, 23]. Recent studies have demonstrated that Sema3A also exerts such diverse functions as regulating bone metabolism, participating in the immune response, controlling vascular endothelial cell movement, and invading various cancer cells. The Sema3A protein directly placed of into the defective area or the bone microenvironment, which is easily diluted by blood or tissue fluid or inactivated by proteolytic enzymes, cannot reach an effective concentration and exert its effects. In theory, the Sema3A protein can not only affect the target cells themselves, but it also affects the corresponding cells of the host site to further promote bone regeneration in vitro. Therefore, we think that a combination of an overexpression

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of the *Sema3A* lentiviral vector and an ideal target cell that has osteogenic and immunomodulatory effects may be more advantageous for the better repair of alveolar bone defects in the special microenvironment of periodontitis.

In the present study, a lentiviral vector carrying the gene *Sema3A* and green fluorescent protein (GFP) were constructed *in vitro*. GFP was derived from jellyfish, which emitted fluorescence under blue light irradiation [24]. After transfection and puromycin screening, the transfection efficiency was determined to be more than 90% using flow cytometry. The flow cytometry results showed that the inserted *Sema3A* gene initiates stable expression. There were no significant morphological changes in the rGMSCs overexpressing *Sema3A* compared with the rGMSCs. However, the expression of the *Sema3A* gene and protein in rGMSCs was significantly increased after lentivirus transfection according to the real-time PCR and Western blot results.

Whether gene-modified stem cells can maintain their original proliferative activity and multi-directional differentiation potential is important. There were no obvious changes in cell proliferation in the cultures with the DM. Also, the proliferation of the overexpression *Sema3A* cells was slightly increased. However, in addition to the overexpression of the *Sema3A* cells, the proliferation of all cells is significantly reduced in the process of DM treatment with LPS. In our study, the overexpression the *Sema3A* gene promoted the proliferative activity of GMSCs during osteogenic differentiation and reversed the inhibitory effect of LPS stimulation on the proliferation of GMSCs. Osteoblasts play a crucial part in bone formation and bone remodeling. On the one hand, osteoblasts participate in bone formation, synthesis, and the secretion of various osteogenic proteins. On the other hand, osteoblasts secrete a variety of cytokines or regulate osteoclast activity through intercellular contact. The present study revealed that mRNA expression level of ALP, *Runx2*, and *OCN* had gradually increased with the scheduled observation time points in the Lv-*Sema3A* group compared with the control group. However, LPS-induced inflammation significantly reduced the proliferation and osteogenic differentiation of the GMSCs. Also, the mRNA expression levels of ALP, *Runx2*, and *OCN* were significantly increased in the Lv-

*Sema3A* group compared with the control group in the LPS-induced inflammatory environment. This indicates that the *Sema3A* gene-modified rGMSCs reveal more powerful osteogenic activity compared with the rGMSCs in the LPS-induced inflammatory *in vitro*. Our study provides a novel idea for further study *in vivo*.

The jaw is the major supportive structure for the oral and maxillofacial region. The treatment of bone that is damaged by periodontal disease creates a challenge for both researchers and clinicians. These jaw defects may not be completely cured even in the course of a person's life. However, there are still opportunities to deliver gene-modified stem cells to the defective part to promote bone healing. Our results indicate that not only do *Sema3A* gene-modified rGMSCs have the potential to promote the osteogenic differentiation of GMSCs, but they can also weaken the induction of LPS. To confirm this, it is necessary to exploit the application of the *Sema3A* gene-modified GMSCs in animal models of periodontitis in the future.

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### Disclosure of conflict of interest

None.

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## Construction of the overexpressing lentiviral vectors of rSema3A

The Sema3A rat gene was amplified by PCR, and then the gene was inserted into the digested plasmid pLenO-GTP. The pLenO-GTP-Sema3A plasmids were produced by recombination. After sequencing identification, the recombinant vectors were used to transfect 293T cells to obtain virus pools.

## rGMSCs properties

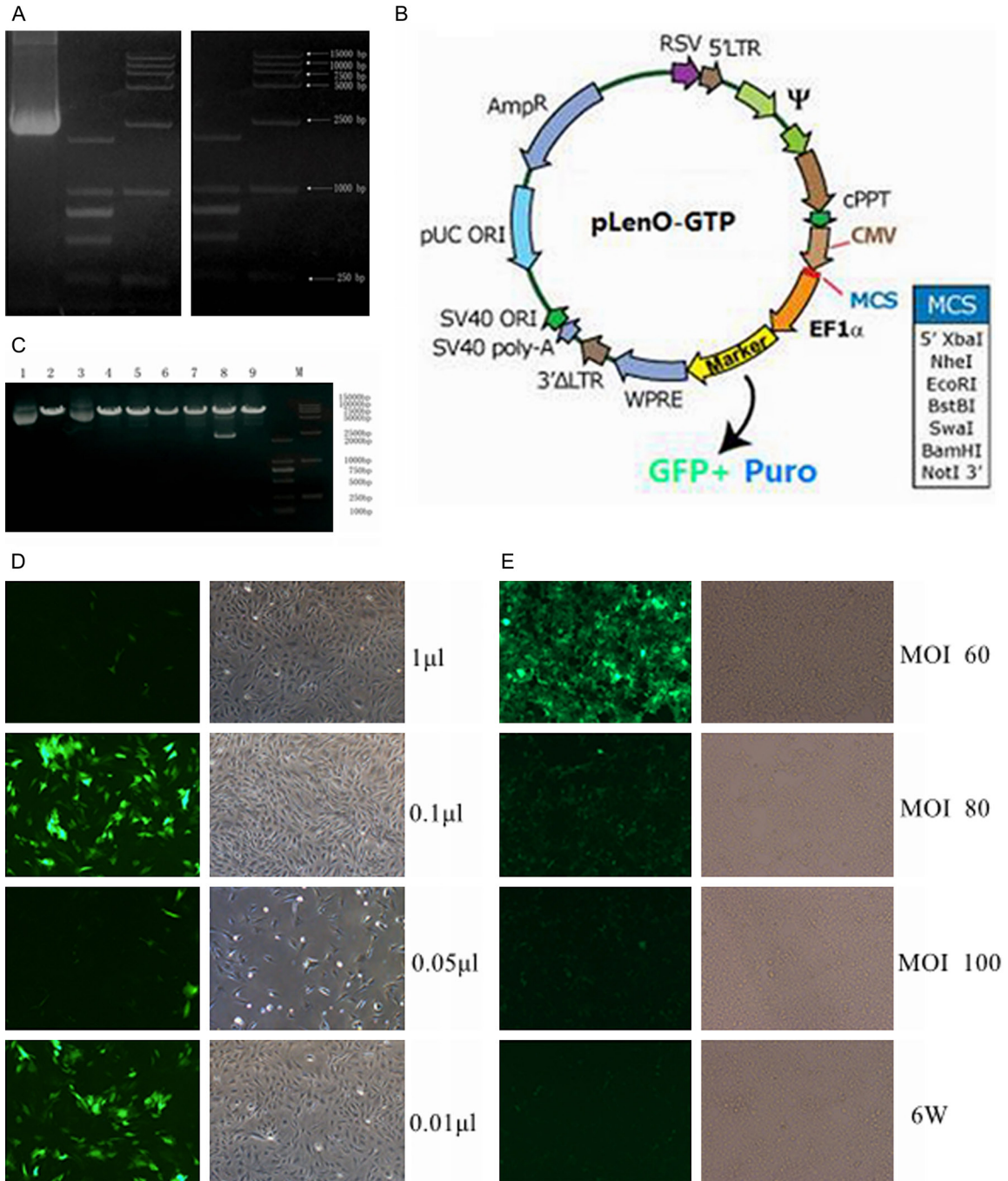
### *Phenotypic characteristics by flow cytometry*

The cells ( $10^6/100 \mu\text{l}$ ) were fixed and incubated for 20 minutes in the 12 EP tubes after the cell digestion and a PBS rinse,  $100 \mu\text{l}$  per tube, one of which is a blank control. The cells were then labeled with the following antibodies: PE conjugated anti-CD29, FITC-conjugated anti-CD45, PE conjugated anti-CD44, PE conjugated anti-CD11b, PE conjugated anti-CD90. The cells were washed and analyzed using a BD FACS Calibur machine and BD CSampler Plus software (BD Biosciences).

### *The induction of adipogenic and osteogenic differentiation*

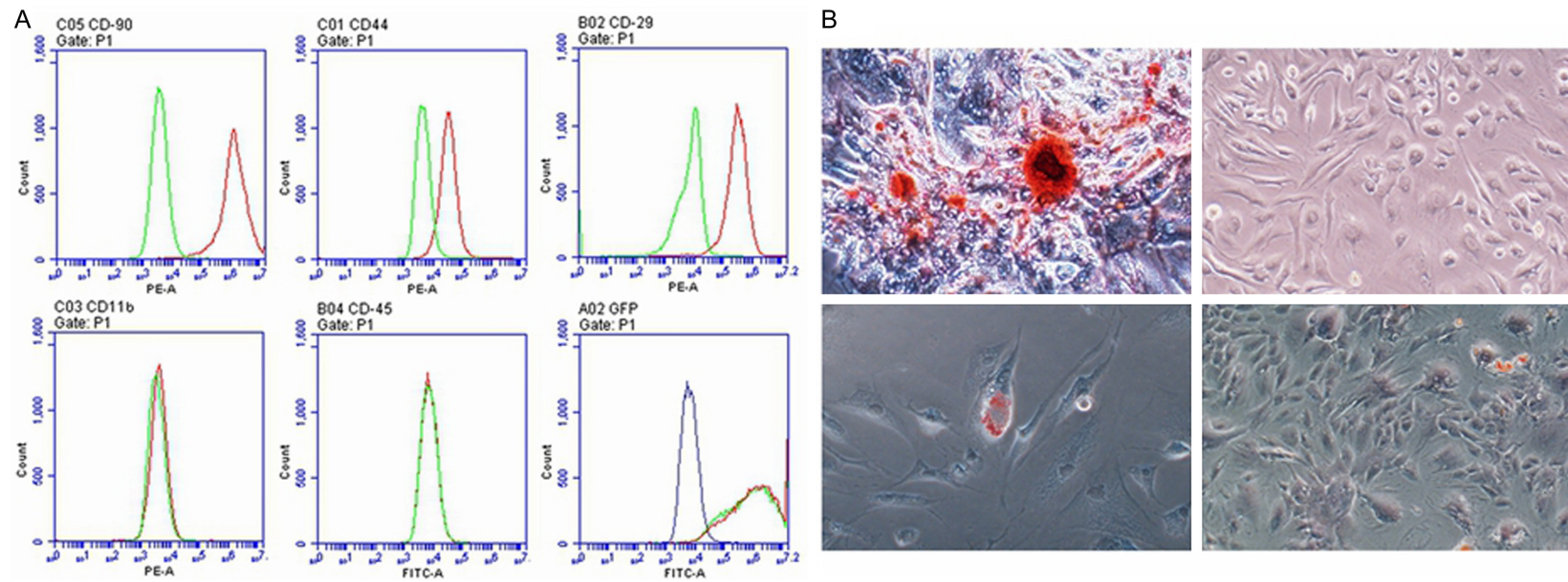
The osteogenic differentiation was induced in an osteogenic differentiation medium [CM with 50 mg/ml ascorbic acid (Sigma-Aldrich), 10 nM dexamethasone (Sigma-Aldrich), and 10 mM  $\beta$ -glycerophosphate (Sigma-Aldrich)] with  $5 \times 10^4$  cells/ml. After 28 days, Alizarin Red staining was performed to determine the calcium accumulation. To assess the adipogenic differentiation,  $5 \times 10^4$  cells/ml were cultured in an adipogenic differentiation medium (CM with 100 nM dexamethasone, 20  $\mu\text{M}$  indomethacin, 50  $\mu\text{M}$  3-Isobutyl-1-methylxanthine and 10  $\mu\text{g}/\text{ml}$  insulin). After 21 days, as the accumulation of lipid-rich vacuoles was detected within the cells, Oil Red O staining was done. The slides were fixed with 4% paraformaldehyde for 30 min and washed with PBS three times.

The effects of Sema3A overexpression on proliferation and differentiation



**Figure S1.** PCR amplification of gene Sema3A and identification by agarose gel electrophoresis (A). Vector map (B) and the identification of gene Sema3A positive clone by agarose gel electrophoresis (C). Different concentrations of lentivirus infected 293T cells to determine the virus titer (D). Determination of the MOI value of the lentivirus-infected cells and the stable fluorescence expression of the cells after 6 weeks of infection. Flow cytometric analysis of lentivirus transfection efficiency (E).

## The effects of Sema3A overexpression on proliferation and differentiation



**Figure S2.** Flow cytometric analysis of the surface-marker of rat gingival mesenchymal stem cells and GFP positive expression (A). Differentiation of rGMSCs into adipocytes, osteocytes (B).