Semaphorin 3A enhances osteogenesis of MG63 cells through interaction with Schwann cells *in vitro*

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Abstract. Bone remodeling is under the control of various signals and systems in the body, including the nervous system. Semaphorin (Sema) 3A is a chemorepellent protein which regulates bone mass. Schwann cells, having a pivotal role following nerve injury, interact with Sema3A under numerous circumstances. The present study established a co-culture system of MG63 and Schwann cells to investigate the role of the interaction between Sema3A and Schwann cells in osteogenesis. The results from the alkaline phosphatase assay, calcium nodule staining and the analysis of the osteogenic gene expression revealed that Sema3A inhibits osteogenic differentiation of MG63 cells in single-cell culture and promotes osteogenic differentiation of MG63 cells in co-culture with Schwann cells, in a concentration-dependent manner. These findings suggest that the presence of Schwann cells induces Sema3A-associated osteogenic differentiation in bone cells, and also reveals the pivotal role of Sema3A as a regulator in the skeletal and nervous systems, thus contributing to a better understanding of the interaction between these systems.

Introduction

Bone remodeling is strongly regulated by hormones, cytokines and other cellular interactions that affect communication between osteoblasts and osteoclasts. Any disruption in this network may result in abnormal bone mass, including osteoporosis (1).

It has previously been demonstrated that the nervous system is an important regulator of bone formation. Due to the thick innervation in bone tissue, chemical messengers are transduced to bone and periosteum through the sensory and sympathetic nerve fibers (2-4). In addition, catecholaminergic positive axons may be visualized near osteoblasts *in vivo* and damaged or missing peripheral nerve fibers may result in abnormal bone formation (5,6). Furthermore, the existence of β-adrenergic receptors in osteoblasts and osteoclasts verifies the role of the central nervous system in the regulation of bone formation (7). However, the molecular mechanisms through which neurons and nerve fibers reach their targets and function during osteogenesis are still poorly understood.

In the central nervous system, several protein families involved in wiring, location, and migration of axons, affecting the length and branching of dendrites were previously reported. One of the most prevalent neural signaling molecular groups are the Semaphorins, a verified set of neuro-immune molecules, which exhibit a predominant role in cardiac and skeletal development, epithelial morphogenesis, angiogenesis and tumor regression (8-12). Semaphorin (Sema) 3A, the first protein discovered in this large protein family, is a chemorepellent for the nervous system that serves to induce the retraction and collapse of the structure of axonal growth cone, and to affect fiber plasticity in adults (13-15). Sema3A completes its biological function via binding to a receptor complex encompassing ligand-binding component Neuropilin (Nrp) 1 and class A Plexins (Plx) a 1, 2, 3 and 4, the latter of which is necessary for proper signal transduction (16). Previously, Sema3A was reported to have a significant role in bone remodeling (17-19). In a previous study, Sema3A-deficient mice and neuro-specific Sema3A-deficient mice were generated and both exhibited low bone mass, low expression of sensory-nerve markers and their positive nerve fibers, and defective innervations. Conversely, osteoblast-specific Sema3A-deficient mice and wild-type mice did not exhibit any of these malfunctions or abnormalities (20). These results suggested that the absence of Sema3A in osteoblasts was not the only factor resulting in bone defects and that Sema3A modulated sensory nerve innervation during bone remodeling. However, the specific role of Sema3A through which bone formation is affected is still unclear.

Previous studies have exposed the key role of Schwann cells in repair and reconstruction following peripheral nerve injury. Injury-induced Schwann cells undergo a series of alterations including downregulation of myelin genes, upregulation of trophic factors and cytokines, activation of myelin autophagy and invasion of macrophages. Altogether, these alterations lead to increased regeneration of axons, remyelination of nerve fibers, avoidance of tissue loss and improved function (21-23).

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Furthermore, Schwann cell migration is significantly impaired following the loss of Nrp1 (24) and binding between Sema3A and Nrp1 has been described as a key interaction in bone cell differentiation (18,19). It was therefore hypothesized that during Sema3A-associated osteogenesis, neural cells, including Schwann cells, may exhibit a pivotal role by interacting with Sema3A and osteoblastic cells.

The present study investigated the role of neural cells in bone remodeling. Sema3A was added to a single cell culture of MG63, and to a co-culture of MG63 and Schwann cells. Osteogenic differentiation was assessed to determine if Sema3A osteogenic induction is influenced by the addition of Schwann cells.

Materials and methods

Materials. Recombinant human Sema3A protein was purchased from Sino Biological Inc. (Beijing, China). The human osteoblast cell line MG63 and the human immortalized Schwann cell line sNF96.2 (SCs) were provided by the School of Stomatology of Jilin University (Changchun, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen; Thermo Fisher Scientific, Inc., (Waltham, MA, USA). Trypsin and Dulbecco's phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Cell Counting Kit-8 (CCK8) was purchased from 7Sea Pharmatech (Shanghai, China). In Situ Cell Apoptosis Detection kit (AP kit) was purchased from Boster Biological Technology (Pleasanton, CA, USA). Total RNA was extracted using the TRIzol® method (Invitrogen; Thermo Fisher Scientific, Inc.). All absorbance represented by optical density (OD) value was measured using Synergy HT spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Single and co-cell culture systems. The MG63 and SCs were cultured in DMEM supplemented with 10% FBS, 1% penicillin, and 1% streptomycin, according to previously reported culturing conditions (25,26). The medium was replaced every 3 days. Cells were trypsinized and centrifuged at 183 x g, 37˚C for 5 min when the confluence reached 80%. Following centrifugation, each cell was re-suspended. A total of 500 cells were seeded in each well of 96-well plates at various MG63 to SCs proportions (1:0, 1:1, 1:2, 2:1 and 0:1) to establish single and co-culture systems. Cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell proliferation and apoptosis assay. MG63 and SCs single-cell cultures and co-cultures were seeded onto 96-well plates and cultured for 5 days to test their level of proliferation. At days 1, 3 and 5, 10 μl of CCK8 reagent was added to each well. Cells were incubated at 37˚C for 40 min for interaction with the reagent. Then the plate was examined by spectrophotometer at a wavelength of 450 nm to assess OD. The same test was conducted on the two single-cell cultures and the co-culture system of MG63 to SCs at 1:1 (1:1 co-culture), with addition of Sema3A to investigate if Sema3A affected proliferation. Briefly, Sema3A (dissolved in PBS) was added to each well of the 96-well plates to reach final concentration of 25, 50 and 100 ng/ml. At days 1, 3 and 5, cell proliferation was measured following CCK8 treatment by spectrophotometer (450 nm). Controls (0 ng/ml Sema3A) for both single-cell cultures and 1:1 co-culture, were created using the same volume of PBS. The apoptosis assay was carried out to investigate apoptosis in the 1:1 co-culture treated with Sema3A using 6-well plates at days 1, 3 and 5 by AP kit according to the manufacturer's protocol. The principle of this kit is that cells in apoptosis produce DNA breakpoints with 3'-OH terminals. Terminal Deoxynucleotidyl Transferase (TdT) tags digoxigenin (Dig)-marked dUTP to 3'-OH terminals of broken DNAs. Then by reaction with the anti-Dig-biotin and streptavidin-biotin complex/alkaline phosphatase (SABC/AP), broken DNA will become colored following the addition of a chromogenic substrate. Briefly, cells were first fixed in 4% paraformaldehyde at room temperature for 30 min, then went through reactions with TdT/Dig-dUTP, anti-Dig-biotin and SABC/AP respectively, finally were stained by 20X 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium at room temperature for 20 min and washed by 0.01 M Tris buffered saline adequately to remove the excess dye. Under microscope, nuclei of apoptotic cells were stained dark blue. For each group, three individual visual fields of 500 cells each were observed and the total amount of dead cells were counted.

Cytotoxicity assay. To assess the cytotoxicity of Sema3A, Sema3A was added to the culture medium to reach the final concentrations of 25, 50 and 100 ng/ml. Medium with the same volume of PBS was used as the control group (0 ng/ml Sema3A). 1:1 co-culture was maintained in each medium for 6, 12 and 24 h and then tested by CCK8 at each timepoint. OD values were measured by spectrophotometer (450 nm) following incubation for 40 min.

Expression of Sema3A receptors. Gene expression of Sema3A receptors in MG63 and SCs was analyzed using reverse transcription-semi quantitative polymerase chain reaction (RT-sqPCR). Total RNA was extracted from each cell group using the TRIzol method according to manufacturer's protocol. Total RNA density was measured using NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, complementary DNA (cDNA) was synthesized from 1 μg total RNA using the PrimeScript™ RT kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. RT-sqPCR amplification was carried out on cDNA using the Sapphire Amp Fast PCR Master Mix (Takara Bio, Inc.). Synthesis and amplification were conducted in an Alpha Thermal Cycler (Bibby Scientific, Ltd., Staffordshire, UK). Primers for the house-keeping gene β-actin as well as Sema3A receptors (Nrp1, Nrp2, Plxa1, Plxa2, Plxa3, and Plxa4) were used for amplification. Sequences of these primers, as previously described (27), are listed in Table I. Each 50 μl PCR reaction system consisted of 25 μl SapphireAmp Fast PCR Master Mix, 1 μl 10 μM forward primer, 1 μl 10 μM reverse primer, 2 μl 50 ng/μl cDNA, and 21 μl dH_2O. The PCR cycle reaction was performed under the following conditions: Polymerase activation step at 94˚C for 1 min followed by 30 cycles (98˚C for 5 sec, 55˚C for 5 sec, 72˚C for 10 sec), and final extension step at 72˚C for 10 min. The PCR products were then loaded onto a 2% agarose gel (Invitrogen; Thermo

Table I. Primer sequences used for RT-sqPCR and RT-qPCR.

Gene	Forward sequence	Reverse sequence
β-actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
Nrp1	CTCCTGTTGTGTCTTCAGG	CCCGATGAGGATCGGATTC
Nrp2	CATGCACTATGACACCCCTG	ATGGGTTCCATGCAGTTCTC
Plxa1	GACTTCCTGCTGACCCTGAG	GACTTCAACCTGAAGCCAGC
Plxa2	GCTACAAGAGCTGGGTGGAG	CTCTCGGCTTGAAGAACCAC
Plxa3	CAGCAGATCGACTACAAGAC	GCCGTGTCAGGTAGATCTC
Plxa4	TGTCAGGGTGTCAACGAGAGC	ATACACCTGCTCCTTGGTGG
RUNX2	CACTGGCGCTGCAACAAGA	CATTCCGGAGCTCAGCAGAATAA
Osteocalcin (OCN)	CCCAGGCGCTACCTGTATCAA	GGTCAGCCAACTCGTCACAGTC

Nrp, Neuropilin; Plx, Plexin; RUNX2, Runt-related transcription factor 2; OCN, Osteocalcin; RT-sqPCR, reverse transcription-semi quantitative polymerase chain reaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Fisher Scientific, Inc.) containing 0.5 μg/ml ethidium bromide (Invitrogen; Thermo Fisher Scientific, Inc.). The size of the amplified bands was assessed against a 1,000 bp DNA ladder (Takara Bio, Inc.). Bands were visualized and captured using Molecular Imager Gel Doc XR+ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Image Lab (v4.0; Bio-Rad Laboratories, Inc.).

Osteogenic mRNA expression. The expression of osteogenic genes was analyzed by RT-qPCR. MG63 and 1:1 co-culture were divided into four groups with the addition of Sema3A or PBS to the final concentration of 0, 25, 50, 100 ng/ml. The medium was replaced every 2 days. At days 3 and 7, total RNA of cells was obtained and cDNA was synthetized using the aforementioned procedure. Then, primers for Runt-Related Transcription Factor 2 (RUNX2) and Osteocalcin (OCN) were used for RT-qPCR amplification using SYBR Premix EX™ Taq II RT-PCR kit (Takara Bio, Inc.) in Stratagene Mx3005P (Agilent Technologies, Inc., Santa Clara, CA, USA). The primer sequences used for RUNX2 and OCN amplification were as previously described (26) and listed in Table I. Briefly, each 25 μl RT-qPCR mixture consisted of 12.5 μl SYBR Fast qPCR Mix, 1 μl 10 μM forward and reverse primers, 0.5 μl Rox Reference Dye (50x), 2 μl 10 ng/μl cDNA and 8 μl dH_2O. Each mixture was programmed to go through pre-denaturation at 95˚C for 30 sec, then 40 cycles of 5 sec at 95˚C followed by 30 sec at 60˚C. β-actin was used as the reference gene in RT-qPCR analysis and $2^{-\Delta\Delta Cq}$ method was applied to normalize and analyze the data as previously described (27).

Alkaline phosphatase (ALP) assay. The MG63 single culture and 1:1 co-culture under different concentrations of Sema3A were assessed by the ALP assay. At days 7 and 14, ALP activity was measured using an Alkaline phosphatase assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to manufacturer's protocol. Briefly, cells were washed with PBS three times and then lysed using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). Cells were then centrifuged at 183 x g, 37˚C for 5 min. For the ALP assay, 50 μl supernatant and 50 μl reaction reagent was added to each well of the 96-well plate. A total of 100 μl stop buffer was finally added to each well following a 10 min incubation at 37˚C. Then, OD value at a wavelength of 405 nm was measured.

Extracellular matrix (ECM) mineralization. The single culture MG63 and 1:1 co-culture was cultured 14 days for mineralization staining. Cells were washed twice with PBS and fixed in 95% ethanol for 15 min at room temperature. Then, the Alizarin Red solution (Sigma-Aldrich; Merck KGaA) was used to dye the calcium nodules at room temperature for 5 min. Cells were rinsed adequately with distilled water to remove excessive dye. Then, the dyed nodules were dissolved in 10% (w/v) cetylpyridinium chloride (Shanghai Yuan Ye Biotechnology Co., Ltd., Shanghai, China) solution and OD was measured at a wavelength of 620 nm.

Statistical analysis. For data analysis of cell proliferation, apoptosis, cytotoxicity assays, calcium nodule staining and alkaline phosphatase assay, one-way analysis of variance followed by post-hoc Tukey's test was applied using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard error of the mean. All statistical graphs were produced by graphing software OriginLab 8 (v8.0725; OriginLab, Northampton, MA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of cell culture systems. The three co-culture groups demonstrated different cell viabilities. The 1:1 co-culture was the group with the most increased cell proliferation at days 1, 3 and 5 (Fig. 1A). Other groups demonstrated inferior cell proliferation ($P<0.05$) compared with this group, at all time points examined. Cell proliferation of single MG63 and SCs were not increased compared with the 1:1 group. Hence, 1:1 of MG63 to SCs was the ratio selected for the follow-up experiments.

Effect of Sema3A on cell proliferation, apoptosis, and cytotoxicity. Cell proliferation, cell death, and cytotoxicity following Sema3A addition were assessed. There was no

difference in cell proliferation among cell groups treated with different concentrations of Sema3A (0, 25, 50 and 100 ng/ml) at days 1, 3 and 5 (Fig. 1B). Similarly, the addition of Sema3A did not result in a significant alteration in cell proliferation of MG63 single culture during the testing period (Fig. 1C). The proliferation of SCs was also not affected by the addition of Sema3A at days 1 and 3. However, at day 5, OD value in 100 ng/ml Sema3A group was significantly decreased compared with 0, 25 and 50 ng/ml groups ($P<0.05$; Fig. 1D). This trend of reduction in the proliferation rate at day 5 may be observed with increasing concentrations of Sema3A (though not reaching statistical significance in other groups). For the apoptosis assay, there was no difference in levels of apoptosis in 1:1 co-culture, MG63 and SCs under different Sema3A concentrations at days 1, 3 and 5 (Fig. 1E). Similarly, there was no difference in levels of cytotoxicity in the 1:1 co-culture under different Sema3A concentrations (Fig. 1F).

Expression of Sema3A receptors. RT-sqPCR was performed to examine the expression of Sema3A receptors. Nrp1 was only expressed in MG63. Nrp2 expression was observed in SCs and was visible however weak in MG63. Gene expression of Plxa1 and Plxa2 was only detected in MG63. Plxa3 was the only Plexin receptor expressed in SCs (Fig. 2).

ALP assay and calcium nodule staining. An ALP assay was undertaken to assess the level of osteogenic differentiation. ALP activity in MG63 single culture indicated a consistent decline at days 7 and 14 (Fig. 3A). At each time point, only 50 and 100 ng/ml Sema3A groups suggested statistical difference from control group (0 ng/ml Sema3A; $P<0.05$). Furthermore, in MG63, ALP activity decreased gradually with increasing concentrations of Sema3A. Conversely, ALP activity in the 1:1 co-culture increased gradually as concentrations of Sema3A increased at days 7 and 14 (Fig. 3B). ALP assay results in 1:1 the co-culture revealed that all Sema3A groups (25, 50 and 100 ng/ml) were markedly increased compared with the control group (0 ng/ml;) at days 7 and 14, however, there was no difference among Sema3A treatment groups (Fig. 3B; $P>0.05$). The osteogenic differentiation was further investigated by calcium nodule staining. Differences in staining were not visually detectable (data not shown), however OD values indicated differences between groups. In MG63 single culture, OD value declined as concentration of Sema3A increased, and the lowest OD appeared at 100 ng/ml (Fig. 3C; $P<0.05$). No meaningful difference was observed between 25 and 50 ng/ml groups (Fig. 3C; $P>0.05$). In 1:1 co-culture, OD value demonstrated a steady increase as Sema3A concentration increased, with all groups statistically different compared with each other ($P<0.05$; Fig. 3C).

Expression of osteogenic genes. RT-qPCR was performed to detect alterations in expression levels of osteogenic genes under different concentrations of Sema3A. The pattern of alteration in mRNA expression of osteogenic genes was in accordance with the ALP activity and calcium nodule mineralization results. At day 3, RUNX2 and OCN expression in MG63 cells declined when Sema3A was added, with the lowest gene expression detected at 100 ng/ml Sema3A compared with control group (0 ng/ml). On the contrary, the gene expression levels of RUNX2 and OCN were increased in 1:1 co-culture with the highest expression detected at 100 ng/ml Sema3A (Fig. 4A and B). Similarly, at day 7, RUNX2 and OCN indicated the same pattern of expression as described at day 3 in both MG63 single culture and 1:1 co-culture. As concentration of Sema3A increased, MG63 demonstrated a decline in mRNA levels of both genes, whereas 1:1 co-culture demonstrated increased expression levels of the genes (Fig. 4C and D). All Sema3A treatment groups (25, 50 and 100 ng/ml) in MG63 and 1:1 co-culture were statistically different ($P<0.05$) from the control group (0 ng/ml) at every time point.

Discussion

The present study established a co-culture system of MG63 and Schwann cells to investigate the role of Sema3A and the mechanisms underlying bone formation. Although co-culture systems have been used in numerous studies, to the best of the author's knowledge, the present study was the first to use a co-culture system to dissect Sema3A function on osteogenesis. First, a co-culture system was generated and optimal cell proliferation determined at 1:1 of MG63:SCs. Cell proliferation in the 1:1 co-culture was consistently increased compared with MG63 and SCs single cultures. This is in accordance with a previous study, in which proliferation in a co-cultured system of Schwann cells and osteoblasts has been demonstrated to be consistently increased compared with single cell cultures (28). Then, the MG63 and SCs single cultures and 1:1 co-culture were treated with different concentrations of Sema3A (0, 25, 50 and 100 ng/ml) to observe any effect of Sema3A on cell proliferation. The results demonstrated that no statistical difference was present among groups in cell proliferation of MG63 and 1:1 co-culture, indicating no influence of Sema3A on cell growth. However, a decreased OD value in the 100 ng/ml Sema3A group of SCs was observed at day 5, suggesting that a high concentration of Sema3A inhibited proliferation of SCs. The apoptosis and cytotoxicity results for MG63 and SCs single culture and 1:1 co-culture also suggested that Sema3A at applied concentrations was non-cytotoxic and exerted no effect among treatment groups on apoptosis.

RT-sqPCR was then performed to investigate gene expression of Sema3A receptors in MG63 and SCs. Results verified that MG63 expressed Nrp1, 2 and Plxa1, a2 and a3. Nrp2 and Plxa3 expression was detected in SCs. Previous studies reported that Nrp1 is the primary binding ligand for Sema3A (29) and Nrp1-Sema3A binding is involved in regulation of the neural and immune system (24,30-32). However, Nrp2, sharing 45% similarity in protein sequence with Nrp1 (33), has previously been implicated in Sema3A signaling. Glioma cell migration is regulated by Sema3A via Nrp2 (34), and Nrp2 improves Sema3A-dependent axonal targeting of the vomeronasal nerves in Nrp1 knockout mice (35). This contradicts with the previous view that Nrp1 bound preferentially with Sema3A and suggests a potential role for Nrp2 in Sema3A signaling. The present study observed low expression levels of Nrp2 in MG63 and high gene expression of Nrp2 in SCs. It was therefore proposed that Nrp2 of SCs may act as a principle receptor in Sema3A-mediated osteogenesis in the co-culture system. However, this still requires further investigation.

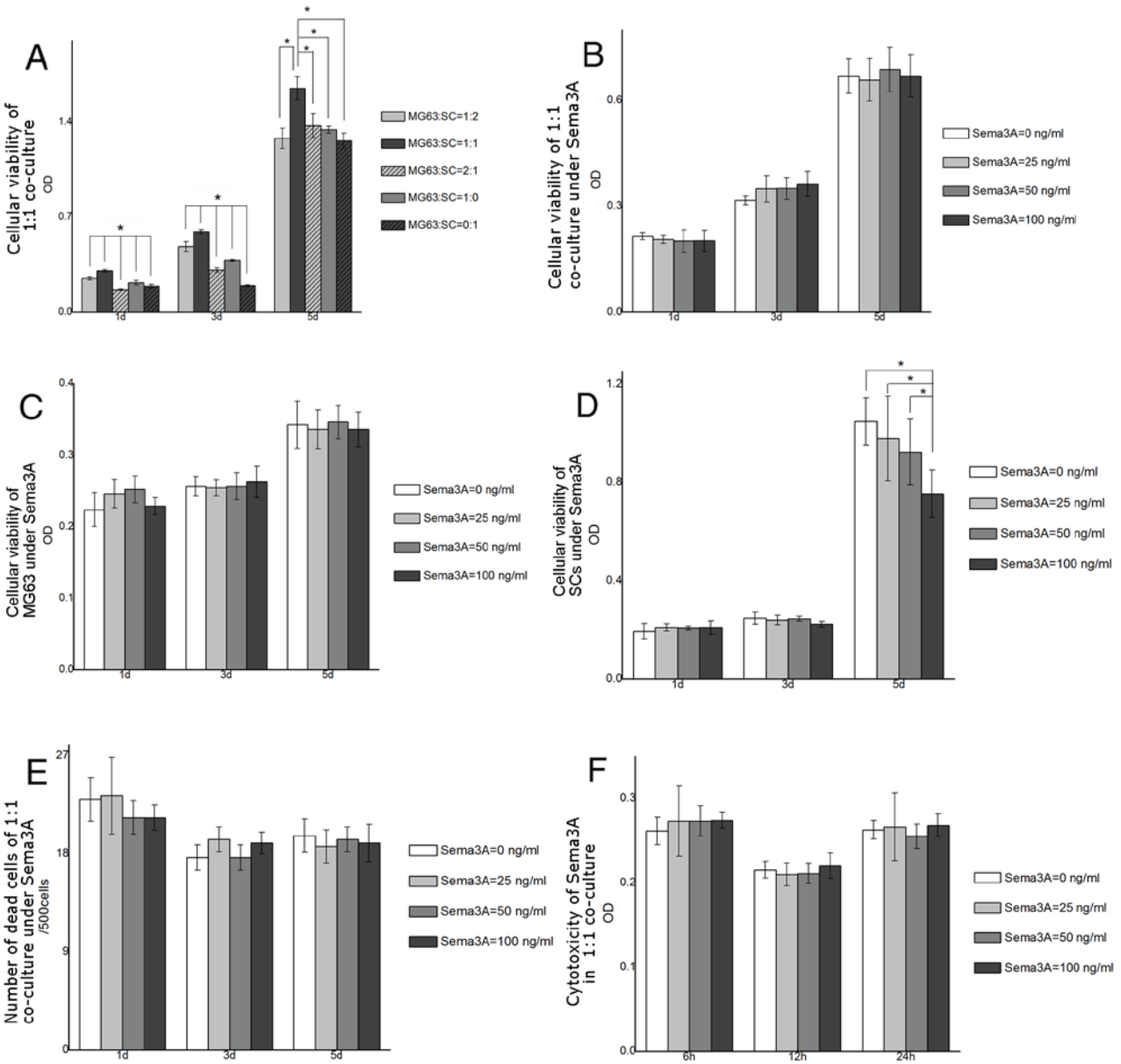

Figure 1. Cell proliferation, cytotoxicity and apoptosis. (A) Cell proliferation of MG63, SCs single cultures and of co-cultured cells. The 1:1 co-culture demonstrated a better vitality compared with other groups at all time points. (B) Cell proliferation of 1:1 co-culture under different concentrations of Sema3A. No difference in cell proliferation was observed among the four groups at any time point. (C) Cell proliferation of MG63 single culture under different concentrations of Sema3A. No difference in cell proliferation was observed among the four Sema3A treatment groups at any time. (D) Cell proliferation of SCs single cells under different concentrations of Sema3A. No difference in cell proliferation was observed among the four Sema3A treatment groups at days 1 and 3. At day 5, OD value of 100 ng/ml Sema3A treatment group was decreased compared with other groups. (E) Apoptosis conducted on 1:1 co-culture. The histogram illustrates dead cells counted in three fields of each group under a microscope. No statistical difference was observed among the four Sema3A treatment groups. (F) Cytotoxicity of 1:1 co-culture under different concentrations of Sema3A. No difference was observed among the four Sema3A treatment groups at any time point. All experiments were replicated three times to ensure the accuracy of the results. Data are presented as the mean ± standard error of the mean. *P<0.05. Sema3A, Semaphorin 3A; SC, Schwann cells; OD, optical density.

To dissect the role of Sema3A in osteogenesis and its interaction with neural cells, level of ALP, extracellular matrix mineralization and expression of osteogenic genes were assessed. Sema3A was first applied to MG63 single cell culture. However, contrary to previous findings on osteoblasts (36), ALP level, extracellular matrix mineralization and the expression of osteogenic genes suggested that Sema3A inhibited MG63 osteogenic differentiation compared with the control group (0 ng/ml Sema3A), with inhibitory levels rising with concentrations of Sema3A. Due to the lack of osteogenic medium, and low concentrations of Sema3A applied in the present study, 25 and 50 ng/ml Sema3A groups in calcium nodule staining and RUNX2 expression in RT-qPCR did not indicate a statistical difference. However, all Sema3A treatment groups, except the 25 ng/ml group of MG63 single culture in ALP assay, exhibited statistically significant differences compared with control group (0 ng/ml Sema3A) in all three assays. Furthermore, a trend of decline in osteogenic differentiation of MG63 was observed and verified in the present study. The authors present the following explanation to describe the decline in osteogenic expression in MG63 following Sema3A treatment. Firstly, MG63, alongside Saos-2

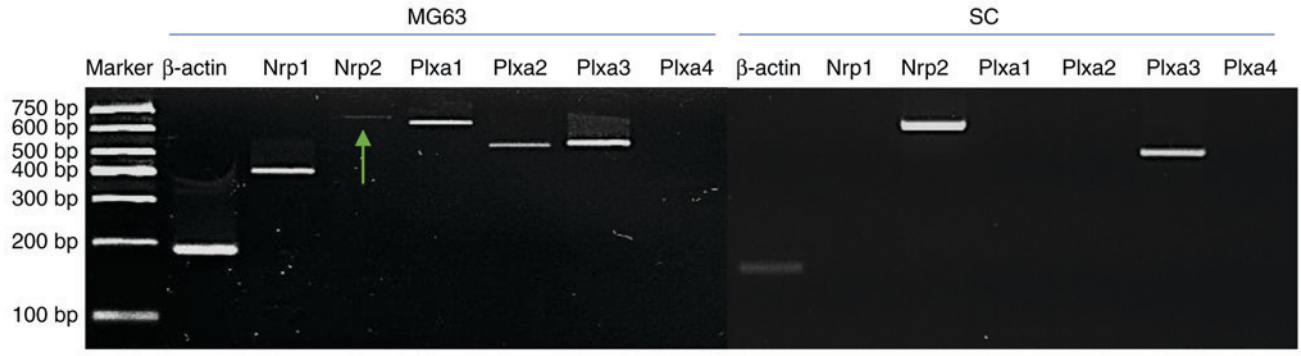

Figure 2. RT-sqPCR to assess gene expression of Sema3A receptors. Representative image captured following agarose electrophoresis of the RT-sqPCR products. DL 1000 DNA ladder was loaded onto the gel to assess the size of the PCR bands. MG63 cells abundantly expressed Nrp1, Plxa1, Plxa2, and Plxa3; weak expression of Nrp2 was be observed (green arrow). SCs abundantly expressed Nrp2 and Plxa3. PCR was replicated three times to verify the results. Sema3A, Semaphorin 3A; SC, Schwann cells; RT-sqPCR, reverse transcription-semi quantitative polymerase chain reaction; Nrp, neuropilin; Plx, plexins.

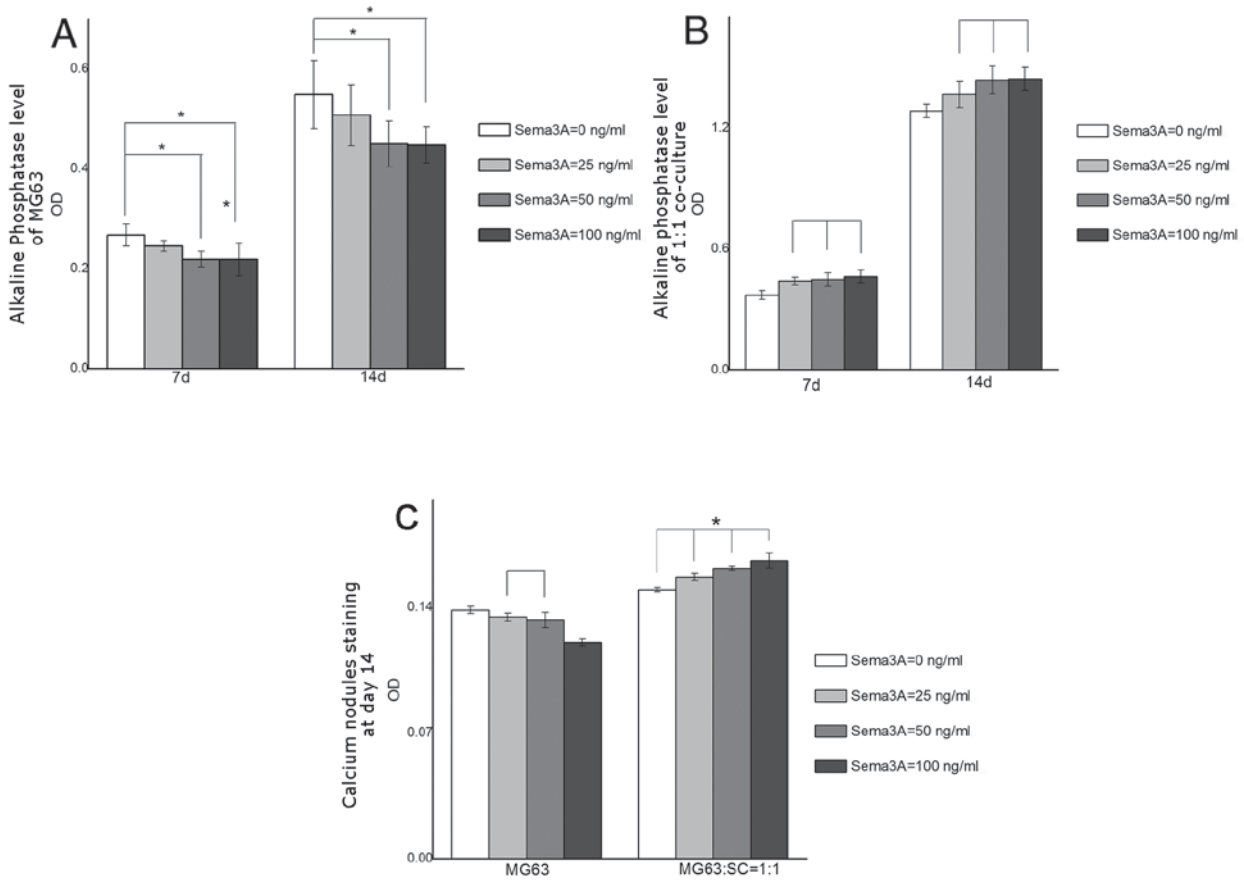

Figure 3. Alkaline phosphatase and extracellular matrix mineralization assay. (A) ALP activity of MG63 single culture at days 7 and 14. There were no statistical differences between the 0 and 25 ng/ml Sema3A treatment groups. (B) ALP activity of 1:1 co-culture at days 7 and 14. At all time points, 25, 50 and 100 ng/ml Sema3A treatment groups had increased ALP activity compared with the 0 ng/ml Sema3A control group. However, no statistical difference was observed among Sema3A treatment groups. (C) Absorbance values (620 nm) of calcium modules stained by Alizarin Red. All groups were statistically different from each other, with the exception being the 25 and 50 ng/ml Sema3A groups in MG63. In both alkaline phosphatase and extracellular matrix mineralization assays, three Sema3A treatment groups (25, 50, and 100 ng/ml) and the control group (0 ng/ml) were compared with each other within every time point for statistical analysis. Data are presented as the mean ± standard error of the mean. Lines above the bars without an asterisk in the histogram indicate groups with no statistical difference ($P>0.05$); $^{*}P<0.05$. Sema3A, Semaphorin 3A; SC, Schwann cells; OD, optical density.

and U-2 OS cell lines, is a malignant bone tumor cell line acquired from osteosarcoma. Although several osteoblastic features are shared between these cell lines (37), cellular and molecular functions including intercellular communication may vary due to chromosome alterations (38). For instance, MG63 expresses collagen-II and -IX whereas no expression of these collagens is observed in human osteoblasts (39). The expression level of ALP in human osteoblasts is decreased compared with osteosarcoma cells and is dependent on the rate of confluence, which is different from MG63 (40). In

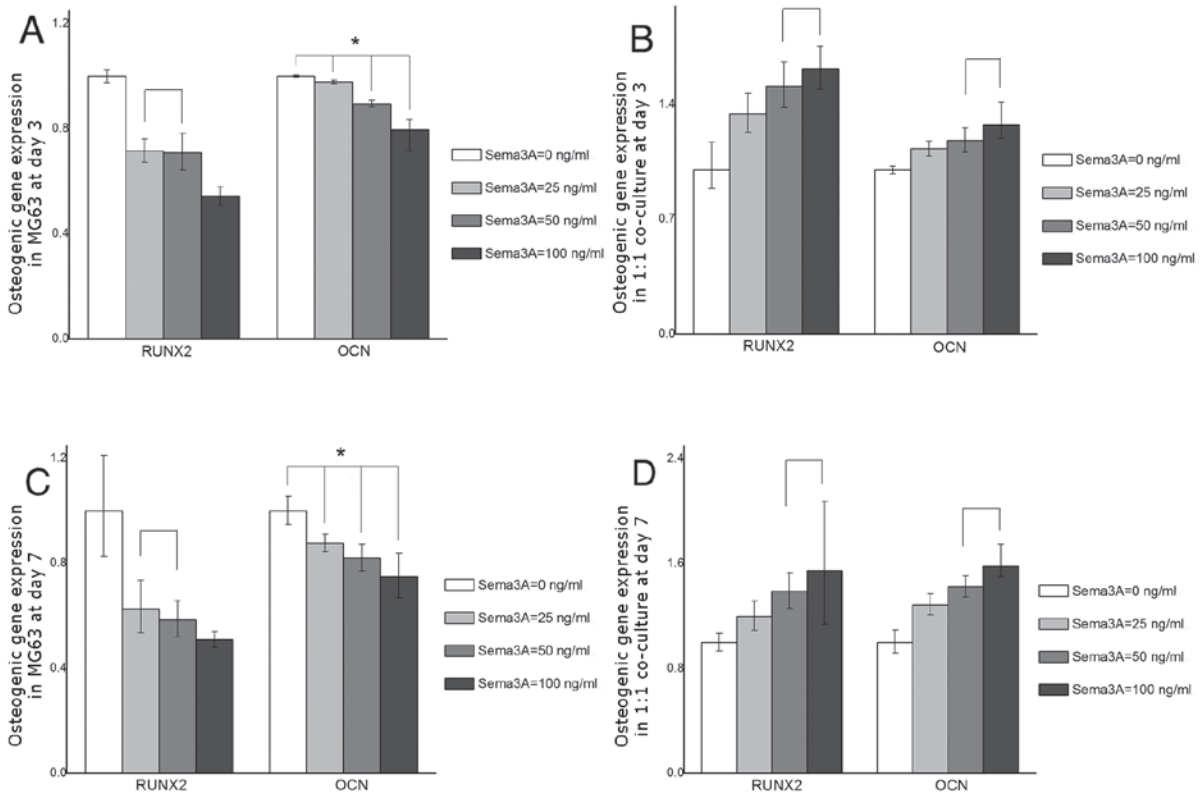

Figure 4. Osteogenic gene expression. (A) Expression of osteogenic genes in MG63 single culture at day 3. (B) Expression of osteogenic genes in 1:1 co-culture at day 3. (C) Expression of osteogenic genes in MG63 at day 7. (D) Expression of osteogenic genes in 1:1 co-culture at day 7. Osteogenic gene expression was normalized using the house-keeping gene. In both RUNX2 and OCN gene expression tests, three Sema3A treatment groups (25, 50, and 100 ng/ml) and the control group (0 ng/ml) were compared with each other within every time point for statistical analysis. Data are presented as the mean ± standard error of the mean. Lines above the bars without an asterisk in the histogram indicate groups with no statistical difference ($P>0.05$); $^{*}P<0.05$. Sema3A, Semaphorin 3A; SC, Schwann cells; RUNX2, Runt-related transcription factor 2; OCN, Osteocalcin.

addition, unlike osteoblasts, bone marrow mesenchymal stem cells do not express Nrp1 (41), whereas MG63 was verified to strongly express Nrp1 in the present study. Therefore, an altered chromosome expression of MG63 from human osteoblasts may exist, which may contribute to the deviated results of Sema3A function on osteogenesis from previous studies. Secondly, Sema3A has a complex role in cancer. Sema3A is downregulated in several cancers (42) and serves as an endogenous factor to inhibit tumor migration and progression (43). In addition, its binding with Nrp1 may inhibit cancer cell proliferation (44,45). In the present study, MG63 cells did not demonstrate any regression in proliferation under Sema3A. This may be due to the low concentrations applied. Sema3A may still exhibit an inhibitory role on the osteogenic differentiation of osteosarcoma-derived MG63, however to the best of the author's knowledge, no report has been discovered investigating the effect of Sema3A on osteosarcoma, and thus, its specific role in these cells requires further clarification. Furthermore, a previous study demonstrated that a low concentration of Sema3A may prevent Plexin receptors from performing signal transduction effectively due to insufficient binding with Sema3A, whereas other receptors including PlexinA3 may function properly at such concentrations (46). Therefore, instead of classic receptors including Nrp1, other ligands including Nrp2 and Plxa3, expression levels of which were verified in MG63 in the present study, may be involved in osteogenesis along with Sema3A under low concentrations. Sema3A may have engendered its osteogenic features on a dose-dependent basis due to receptor preference. However, the present study was rather limited in its ability to further illustrate the exact role of Sema3A in MG63 differentiation.

To explore the potential interaction between Sema3A and SCs in bone formation, the present study then added Sema3A at different concentrations to the 1:1 co-culture to test possible alterations in osteogenesis. The results indicated that ALP level, extracellular matrix mineralization, and osteogenic gene expression of 1:1 co-culture were gradually increased following addition of Sema3A, with the highest level observed at 100 ng/ml in all three tests, suggesting a dose-dependent pattern in skeletal function of Sema3A. However, 50 and 100 ng/ml groups in RT-qPCR did not reveal any statistical difference, indicating a more complicated role of concentration in Sema3A-induced osteogenesis. However, results revealed a positive role for SCs in bone formation. Schwann cells are of vital importance in nerve repair. Following peripheral nerve injury, class 3 Semaphorins including Sema3A are secreted by Schwann cells, and Nrp1/Nrp2 expression is upregulated in Schwann cells (47,48). This suggests the prominent involvement that Sema3A and SCs share in nerve regeneration. In the present study, the function of Sema3A in osteogenic expression in the 1:1 co-culture was completely reversed to that observed in the MG63 single culture. Firstly, according to a previous study (49), SCs excrete various numbers of neurotrophins including the brain-derived neurotrophic factor and the nerve

growth factor (NGF) following injury. These neurotrophins, particularly NGF, have already been reported to have a role in osteoblastic survival (50), and they bind receptors on multiple non-neural cells including osteoblasts to inflict trophic features (51). Therefore, the addition of injury-associated Sema3A into the co-culture system may have promoted secretion of trophic factors in SCs, which then promoted MG63 differentiation. Secondly, since SCs were demonstrated to express Nrp2 and Plxa3 in the present study, the link between osteogenesis and Nrp2/Plxa3 was investigated. Verlinden *et al* (52) demonstrated that Nrp2 knock out mice have an insufficient number of osteoblasts, an increased level of osteoclasts, and low bone mass. Notably, it has previously been suggested that Nrp2, along with Plxa3, are receptors of Sema3A that only partially and subordinately participate in the signaling pathway in comparison to Nrp1 or Plxa1/2 (32,53). However, Nrp2 is required for SCs normal assembly into functional units for repair activities (54), and Plxa3 is necessary for nerve fibers to be precisely aimed at their targets (46). Therefore, whether this promotion in osteogenesis is in part or entirely due to the binding of Sema3A to Nrp2/Plxa3 or to other receptors, and semaphorins expressed and secreted by SCs to form functional complexes, including the bone-associated Semaphorin 6D-Plxa1[17] or the nerve-associated Semaphorin 3F-Nrp2 (52,55), remains unclear.

Following the loss of a tooth, a lot of periodontal exteroceptors and nerve fibers are lost. It is now well established that oral implants gain stability and function through osseointegration. However, numerous patients have reported having felt a sensory difference following implant restoration compared with their natural tooth, which may sometimes lead to occlusal overload. Klineberg *et al* (56) suggested the term 'osseoperception' in 2005 to describe these alterations in sensation. Neurophysiological and psychological explanations have been proposed recently, however how oral sensations may be re-transduced, or nerves be regenerated, is still unclear. In the present study, Sema3A promoted osteogenesis of MG63 via interaction with SCs, suggesting the potential of Sema3A in both the bone and nervous system, which may be applied in periodontal rehabilitation, helping to restore not only masticatory however also sensory, thus completing oral function.

In conclusion, the present study established a co-culture system to explore the potential role of Sema3A in osteogenesis. Results revealed that *in vitro*, low concentrations of Sema3A inhibited MG63 osteogenic expression. Conversely, by interaction with Schwann cells, osteogenic expression was elevated with increasing concentrations of Sema3A. The molecular mechanisms underlying the function of Sema3A in osteogenesis have not yet been fully elucidated, however assumptions have been put forward to sustain the hypothesis that Sema3A mediates bone cells via function of neural cells. Further investigations will help to better understand the exact role that the nervous system exhibit bone formation.

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