Tumor necrosis factor alpha suppresses osteogenic differentiation of MSCs by inhibiting semaphorin 3B via Wnt/β-catenin signaling in estrogen-deficiency induced osteoporosis

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ABSTRACT

The proinflammatory cytokines, especially tumor necrosis factor alpha (TNF-α), have been shown to inhibit osteogenic differentiation of mesenchymal stem cells (MSCs) and bone formation in estrogen-deficiency-induced osteoporosis, but the mechanisms of TNF-α impairs bone formation remain poorly understood. Semaphorins have been shown to regulate cell growth, cell migration, and cell differentiation in a variety of tissues, including bone tissue. Here, we identified a novel mechanism whereby TNF-α suppressing Semaphorin3B expression contributes to estrogen-deficiency-induced osteoporosis. In this study, we found that TNF-α could decrease Semaphorin3B expression in osteogenic differentiation of MSCs. Overexpression of Semaphorin3B in MSCs attenuated the inhibitory effects of TNF-α on MSCs proliferation and osteoblastic differentiation. Mechanistically, activation of the Wnt/β-catenin signaling markedly rescued TNF-α-inhibited Semaphorin3B expression, suggesting that Wnt/β-catenin signaling was involved in the regulation of Semaphorin3B expression by TNF-α. Taken together, our results revealed a novel function for Semaphorin3B and suggested that suppressed Semaphorin3B may contribute to impaired bone formation by elevated TNF-α in estrogen-deficiency-induced osteoporosis. This study may indicate a therapeutic target gene of Semaphorin3B for osteoporosis.

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1. Introduction

Osteoporosis is the most common metabolic bone disease, characterized by bone structural alterations including the reduction of bone mass and the destruction of bone microstructure [1]. It is well known that estrogen is an important protective factor for the maintenance of normal bone mass [2]. The reduction of estrogen level in postmenopausal women is associated with a rapid bone loss, eventually resulting in osteoporosis[3]. Previous studies showed that inflammatory cytokines, especially tumor necrosis factor alpha (TNF-α), were produced in excess in estrogen-deficiency-induced osteoporosis [4–6]. Bone loss caused by estrogen withdrawal was significantly alleviated by sequestration of TNF-α with soluble TNF-α receptors [7], suggesting that TNF-α is a critical player in the pathophysiology of postmenopausal osteoporosis.

Normal bone remodeling maintains constant bone mass by an orchestrated balance between the resorption of old bone by osteoclasts and the formation of new bone by osteoblasts. Recent studies have shown that increased levels of inflammatory cytokines not only promoted bone resorption but also impaired bone formation in postmenopausal osteoporosis and other inflammatory bone diseases such as arthritis and periodontitis [8,9]. Further studies confirmed that inhibition of mesenchymal stem cells (MSCs) osteoblastic differentiation played an important role in TNF-α-impaired bone formation[10,11]. Osteoblasts are derived from pleiotropic MSCs which are capable of differentiating into a variety of cell types, including osteoblasts, chondrocytes, myocytes, and adipocytes [12].
TNF-α suppresses osteogenic differentiation of MSCs by inhibiting the expression of the master osteogenic transcription factors Runx2 and Osterix, resulting in a reduction of osteoblast-specific differentiation markers[13]. In addition, TNF-α has been found to activate nuclear factor-kappa B (NF-κB), which is a negative regulator of bone formation [14]. Even though there have been a few studies regarding how TNF-α affects bone formation, the exact mechanisms of TNF-α-inhibited osteogenic differentiation of MSCs need to be further elucidated.

Semaphorins as secreted glycoprotein in cell surface is able to regulate cell growth, cell migration, and cell differentiation in a variety of tissues [15]. Semaphorin family proteins are divided into eight subclasses based on their C-terminal structure and similarity of amino acid, of which five (classes III–VII) are widely expressed in mammalian tissues [16,17]. There have been a few studies elucidating the role of the class III semaphorins in the skeleton. Togari et al. revealed that semaphorin-III was expressed in human osteoblasts and osteoclasts [18]. Gomez et al. found that Semaphorin3A (Sema3A) and its receptors played an important role in skeletal development[19]. Furthermore, Semaphorin3B (Sema3B) has been found to be a 1, 25-dihydroxyvitamin D3-induced gene in osteoblasts that promotes osteoclastogenesis and induces osteopenia in mice [20]. In addition, semaphorin-III family proteins have also emerged as critical factors for the regulation of various cell functions by TNF-α [21]. Taken together, we speculated that semaphorin-III family proteins may be involved in TNF-α-inhibited bone formation in estrogen-deficiency-induced osteoporosis.

In this study, Sema3B was downregulated in MSCs derived from ovariectomized (OVX) mice, and its role was confirmed in the promotion of the osteogenic differentiation of MSCs. In addition, TNF-α-impaired bone formation both in vivo and in vitro in association with suppressed Sema3B. Administration of anti-TNF-α in OVX mice increased bone formation by upregulating Sema3B. Furthermore, the overexpression of Sema3B in vitro partially rescued the osteogenic defect in MSCs treated with TNF-α. Mechanistically, TNF-α suppressed the expression of Sema3B by inhibiting Wnt/β-catenin signaling pathway. This study indicated that suppressing the expression and function of Sema3B may contribute to the TNF-α-induced inhibition of bone formation in estrogen-deficiency-induced osteoporosis.

2. Materials and methods

2.1. Animal models

Generation of OVX mice was performed as described in detail elsewhere [22]. Briefly, two-month-old female C57BL/6j mice were divided into two groups: SHAM group (the bilateral ovaries were exposed but left intact) and OVX group (the bilateral ovaries were removed). TNF-α neutralization in vivo were performed as previous studies [23]. Briefly, mice were injected with 100 mg/kg body weight of TNF-α neutralized antibody (anti-TNF-α) (R&D Systems, Minnesota, USA), and control OVX mice received a comparable volume of PBS (0.15 ml) via a tail vein injection two times per week. After 8 weeks, the femurs and tibias were collected from SHAM, OVX, and OVX + anti-TNF-α mice for micro-CT scans. In order to determine TNF-α levels in serum and the cultured medium, the Mouse TNF-α ELISA Max standard kit (Biologend, San Diego, CA) was used according to the manufacturer’s instructions. 1–25 μl of each sample was used in the ELISA.

2.2. Isolation of mouse bone marrow mesenchymal stem cells (BMSCs)

Isolation of BMSCs was accomplished as previous reports with a simple modification [24]. Briefly, the bone-marrow-derived all nucleated cells (ANCs) were isolated from femurs and tibias of mouse. The cells were seeded at a density of 1 × 10⁵ into 100 mm culture dishes (Corning, NY, USA) at 37 °C and 5% CO₂. Non-adherent cells were removed by replacing the medium after 1 day and attached cells were maintained in alpha minimum essential medium (α-MEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Equitech-bio, Kerrville, TX, USA), 2 mM L-glutamine, 55 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Paisley, UK) for further experiment. Osteogenic differentiation of MSC was carried out using osteoblastic induction medium (OIM) containing standard growth medium (α-MEM supplemented with 10% FBS and 100 μg/ml penicillin/streptomycin) supplemented with 10−7 M Dexamethasone (Dex), 50 μg/ml ascorbic acid, and 10 mM βglycerolphosphate (Sigma–Aldrich, St Louis, USA).

2.3. Analysis of micro-CT scans

Analysis of micro-CT scans was performed according to the method reported by Jia et al. [22]. Briefly, for the distal femurs, the whole secondary spongiosa at the left distal femur from SHAM, OVX, and OVX + anti-TNF-α mice were scanned ex vivo using a micro-CT system (GE eXplore Locus SP) with X-ray tube settings of 55 kV and 70 μA. The femur was placed in a microcentrifuge tube filled with PBS. 437 slices with a voxel size of 8 μm were scanned at the region of the distal femur beginning at the growth plate and extending proximally along the femur diaphysis. Ninety continuous slices beginning at 0.1 mm from the most proximal aspect of the growth plate in which both condyles were no longer visible were selected for analysis. All trabecular bone from each selected slice was segmented for three-dimensional reconstruction (sigma = 0.8, supports = 1, and threshold = 220) to calculate the following parameters: bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and bone mineral density (BMD).

2.4. Enzyme-linked immunosorbsent assay (ELISA) assay

In order to determine TNF-α levels in serum and the cultured medium, the Mouse TNF-α ELISA Max standard kit (Biologend, San Diego, CA) was used according to the manufacturer’s instructions. 1–25 μl of each sample was used in the ELISA. The values obtained were calculated back to the amount of TNF-α per ml of serum and the cultured medium for graphic depiction of the results.

2.5. Immunohistochemistry

Femurs harvested from SHAM, OVX, and OVX + anti-TNF-α mice were paraffin embedded. Ten micron longitudinal tissue sections were cut deparaffinized and stained in HE to observe the structure of bone trabecular. Furthermore, some of tissue sections were used for immunohistochemistry staining of Sema3B and TNF-α. Immunohistochemistry staining was accomplished using methods previously described [25]. Briefly, femur sections were incubated with 3% hydrogen peroxide for 10 min to reduce endogenous peroxides activity and blocked with 3% normal goat serum in Tris-buffered saline. Next, the sections were incubated with anti-Sema3B antibody (Santa Cruz, CA, USA) and anti-TNF-α antibody (Abcam, Cambridge, UK) for 2 h at room temperature. Subsequently, biotinylated secondary antibodies were added into the sections, followed by a peroxidase-labeled streptavidin–biotin staining technique (DAB kit, Invitrogen, Paisley, UK).

2.6. Cells transfections and treatments

Mouse MSCs (mMSCs) were seeded at a density of 2 × 10⁵ into a 6-well tissue culture plate in 3 ml of α-MEM supplemented with 10% FBS and 100 μg/ml penicillin/streptomycin per well. When MSCs were grown to 40–80% confluence, infected cells by adding the Sema3B lentiviral activation particles (Santa Cruz, CA, USA) or Sema3B shRNA lentiviral particles (Santa Cruz, CA, USA) to the culture for 24 h. Furthermore, negative control (NC) cells were transduced with control

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lentiviral activation particles (Santa Cruz, CA, USA) or control shRNA lentiviral activation particles (Santa Cruz, CA, USA). The Sema3B-overexpressing MSCs were treated with 10 ng/ml TNF-α (Sigma, St Louis, USA) for different time points as previous report [23]. To investigate whether TNF-α decreases Sema3B expression through Wnt/β-catenin signaling pathway, MSCs were respectively pre-incubated with Wnt/β-catenin signaling activator, 20 mM LiCl (Sigma, St Louis, USA) or 5 μM BIO (Sigma, St Louis, USA), for 6 h as previous reports [26,27], followed by adding TNF-α (10 ng/ml) for 24 h. Subsequently, the expression of Sema3B was measured by western blot.

2.7. Western blot analysis

Fifty micrograms of protein samples from MSCs were separated by SDS-PAGE (7.5–10% polyacrylamide gels). Proteins were electroblotted onto a polyvinylidene difluoride membrane (0.45 mm; Millipore, Bedford, MA, USA). Next, the membranes were blocked by 5% non-fat dry milk in Tris-buffered saline with Tween 20 for 1 h. The membranes were then incubated with anti-Sema3B antibody (1:400), anti-DKK-1 antibody (1:200), anti-P-tyrosine-GSK-3β antibody (1:500), anti-GSK-3β antibody (1:400) (All from Santa Cruz, CA, USA), at 4 °C for overnight, followed by the incubation of membranes with HRP conjugated secondary antibody (1:5000) (Santa Cruz, CA, USA) at room temperature for 1 h. Enhanced chemiluminescence assay (Thermo Scientific, Pierce, USA) was used to observe antigenantibody complexes.

2.8. Quantitative real-time PCR analysis (qRT-PCR)

Total RNA from MSCs was extracted using TRIzol reagent (Invitrogen, Paisley, UK) as previous report [25]. CDNA was synthesized using 1 μg of RNA and a RevertAid First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Gene expression was detected with real-time PCR with SYBR green qRT-PCR kit (TaKaRa, Dalian, China). The threshold number of cycles (Ct) was set within the exponential phase of the PCR reaction, and the ∆Ct value for each target gene was calculated by subtracting the Ct value of GAPDH (internal control) from the target gene. Relative gene expression levels were calculated by comparing the ∆Ct values between control and experimental conditions for each target PCR, and calculated using the following equation: Relative gene expression = 2^(-∆Ct sample - ∆Ct control). The primer pairs used to detect the mRNA levels of target genes are presented in Table 1.

2.9. ALP activity measurement and histochemical staining

MSCs were cultured in α-MEM medium, containing with 10% FBS, 10−7 M Dex, 10 mM glycerophosphate disodium, and 50 μg/ml ascorbic acid (All from Sigma, St Louis, USA), to induce osteogenic differentiation. After 7 days, the cell lysates were collected to measure ALP activity according to the instruction of ALP assay kit (Sigma, St Louis, MO). Briefly, osteoblasts were suspended in 0.3 ml lysis buffer (0.1% triton X-100, 50 mM NaF, 1% aprotinin, 1% pepstatin, and 1% phenylmethanesulfonyl fluoride). An aliquot of cell lysate was added to ALP substrate buffer containing 2 mg/ml p-nitrophenyl phosphate in 1.5 M alkaline buffer (Sigma, St Louis, MO), and the mixture was incubated at 37 °C for 50 min. The enzymatic reaction was stopped by the addition of 10 mM NaOH, and the absorbance was read at 405 nm. A protein assay was then performed using the BCA Protein Assay reagent (Pierce Biotechnology, Rockford, IL, USA) and ALP activity was normalized to protein concentration.

As another index of osteogenesis in vitro, ALP staining was used to determine the osteogenic differentiation of MSCs. MSCs were cultured in osteogenic differentiation medium for 7 days, followed by ALP staining. The cells were washed twice with PBS, and then fixed in 2% formaldehyde for 10 min. Next the cells were stained with ALP reagent for 30 min at 37 °C. Each well cell was washed three times with distilled water and was photographed with digital camera. The differentiated osteoblasts from the MSCs were dyed with blue violet. ALP staining intensities in different treatment groups were used to estimate the ability of MSCs osteogenic differentiation.

2.10. Alizarin red S (ARS) staining

MSCs were cultured in osteogenic differentiation medium for 14 days, followed by ARS staining. The cells were washed twice with PBS and then fixed in 2% formaldehyde for 10 min. Next the cells were stained with 0.2% ARS solution for 30 min at 37 °C. Each well cell was washed three times with distilled water and was photographed. The red color obtained referred to calcium deposit. Stains were eluted with 100% DMSO to quantify the amount of Alizarin red staining and measured at 405 nm.

2.11. Cell counting kit (CCK-8) assay

MSCs were seeded at a density of 2 × 10³ per well into 96-well plates and cultured at 37 °C in 5% CO2. CCK-8 measurement was performed according to CCK-8 Kit (Dojindo Laboratories, Japan) instruction. Briefly, 10 μl WST-8 were added into each well at 37 °C in 5% CO2 for 1 h, the absorbance of each sample was measured at a wavelength of 450 nm. The result of cell viability measurement is expressed as the absorbance at OD450. Cell viability ratio = (OD day n experimental group−OD day n blank group)/(OD day 0 experimental group−OD day 0 blank group).

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2.12. 5-ethylidene-2'-deoxyuridine (EdU) incorporation assay

MSCs were seeded at a density of $1 \times 10^5$ per well into 24-well plates and cultured at 37°C in 5% CO$_2$, 50 μM of EdU (Sigma-Aldrich, St Louis, USA) was then added into each well for 2 h. Next cells were fixed with 4% formaldehyde for 15 min, followed by permeabilization with 0.5% Triton X-100 for 20 min at room temperature. After washing cells three times with PBS, each well cells were added 100 μl of 1× Apollo® reaction cocktail for 30 min at room temperature. Subsequently, the cells were stained with Hoechst 33258. The EdU incorporation rate was expressed as the ratio of EdU-positive cells (red cells) to total Hoechst 33342-positive cells (blue cells).

2.13. 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE) assay

MSCs were labeled with 2.5 μM CFSE (Invitrogen, Paisley, UK) at 37°C for 10 min, followed by washing cells twice with ice-cold PBS. CFSE-labeled MSCs were then seeded into 6-well plates and cultured in osteogenic differentiation medium. Cells were harvested on days 5 and fluorescence intensity was measured by flow cytometry. The results were analysed with CellQuest software.

2.14. Statistical analysis

Statistical analysis was performed using SPSS software. Two-tailed unpaired one-way ANOVA were used to compare between more than two groups. The results were expressed as the mean ± SD. Results were considered statistically significant if $P \leq 0.05$.

3. Results

3.1. The effect of TNF-α on expression of Class III Semaphorins in osteogenic differentiation of MSCs

Using a panel of qRT-PCR assays to measure all seven known members of the class III semaphorins, we examined the effects of 10 ng/ml TNF-α on their expression profile in osteogenic differentiation of MSCs. Two of the seven class III semaphorins, Sema3E and Sema3F, were not detectable. Five of the seven class III semaphorins were detected over a 14-day period, in which Sema3A and Sema3C remained unchanged (Fig. 1A, C). In addition, even though a significant decrease in expression of Sema3D was presented in MSCs differentiation into osteoblasts, inhibition of Sema3D expression was not affected by TNF-α (Fig. 1D). The expressions of Sema3B and Sema3E were significantly increased in a time-dependent manner during the osteogenesis of MSCs (Fig. 1B, E). However, TNF-α treatment significantly decreased the expression of Sema3B, but not Sema3E, revealing that Sema3B may be involved in TNF-α-inhibited MSC osteogenesis differentiation (Fig. 1B, E). Furthermore, in consistency with the results from qRT-PCR, western blot analysis also confirmed that the increased Sema3B expression levels were markedly reduced by TNF-α during the osteogenesis of MSCs (Fig. 1F).

3.2. TNF-α inhibited Sema3B expression in vivo

To further examine the inhibitory effect of TNF-α on Sema3B expression in vivo, we performed OVX or SHAM operation on 2-month-old mice. After 3 days, the OVX mice were treated with anti-TNF-α or PBS, and the SHAM mice were treated with PBS twice a week for 8 weeks. Micro-CT analysis of distal femurs metaphysics revealed that the BMD of OVX mice was significantly lower compared to SHAM control group. Similarly, BV/TV, Tb.Th, and Tb.N were also significantly lower in OVX mice compared to SHAM mice. However, the micro-CT analysis showed significant improvement in the trabecular bone structures in the anti-TNF-α-treated OVX mice compared with the untreated group (Fig. 2A, B). The results from micro-CT were consistent with the results of HE staining (Fig. 2C). Then, we performed immunohistochemistry to examine the expression of TNF-α in the femurs of SHAM mice, OVX mice, and OVX+ anti-TNF-α mice. Our results showed that the expression of Sema3B was upregulated in OVX mice compared with the SHAM control mice, whereas the expression level of TNF-α was decreased after anti-TNF-α treatment in OVX mice (Fig. 2D, E). In addition, the serum TNF-α level was dramatically elevated in OVX mice, which was markedly attenuated in OVX+ anti-TNF-α mice, suggesting that the animal models of this study were reliable (Fig. 2F).

Subsequently, immunohistochemistry analysis showed that Sema3B expression in osteoblasts was much higher than in the surrounding tissue and that Sema3B expression was markedly lower in OVX mice.
compared to SHAM control mice. However, anti-TNF-α treatment obviously increased Sema3B expression in OVX mice, suggesting that TNF-α could decrease Sema3B expression in vivo (Fig. 3A, B). To further examine the effect of TNF-α on Sema3B expression, the expression levels of Sema3B in MSCs obtained from SHAM mice (SHAM-MSCs), OVX mice (OVX-MSCs), and OVX + anti-TNF-α mice (OVX + anti-TNF-α-MSCs) were measured by qRT-PCR. The results showed that the expression levels of Sema3B were reduced in OVX-MSCs when compared with the SHAM-MSCs, whereas the expression of Sema3B was significantly higher in OVX + anti-TNF-α-MSCs compared to OVX-MSCs (Fig. 3C).

Furthermore, when BM-MSCs derived from different groups mice were induced by osteogenic medium for 7 and 14 days, Sema3B protein and mRNA expression were also greatly decreased in OVX-MSCs, whereas the expression levels of Sema3B were increased after anti-TNF-α treatment (Fig. 3D, E). To examine whether the alteration of Sema3B expression was due to the change of TNF-α concentration in culture medium, the levels of TNF-α in culture medium of SHAM-MSCs, OVX-MSCs, and OVX + anti-TNF-α-MSCs were measured by ELISA assay. We found that the level of TNF-α was significantly increased in culture medium of OVX-MSCs, which was markedly reduced in culture medium of OVX + anti-TNF-α-MSCs (Fig. 3F).

Taken together, these results strongly suggested that TNF-α could markedly decrease Sema3B expression in vivo. Furthermore, the expression levels of Sema3B receptors, such as neuropilins (NRP1 and NRP2), plexins A1 (PA1), plexins A2 (PA2), plexins A3 (PA3) and plexins A4 (PA4) in SHAM-MSCs, OVX-MSCs, and OVX + anti-TNF-α-MSCs were also measured by qRT-PCR. The results showed that the expressions of NRP1, PA1, and PA4 were markedly downregulated, whereas the expression of PA3 was significantly upregulated (Fig. 3G).

3.3. Involvement of Sema3B in TNF-α-inhibited osteogenic differentiation of MSCs

To explore whether Sema3B was involved in MSC osteogenesis differentiation, we performed loss-of-function and gain-of-function experiments in which we decreased and increased the expressions of Sema3B with lenti-shRNA-Sema3B and lenti-Sema3B, respectively. Western blotting confirmed that overexpression and depletion of Sema3B with lenti-Sema3B and lenti-shRNA-Sema3B were effective (Fig. 4A). Then, the MSCs were induced to osteoblast differentiation after transfection with either lenti-Sema3B or lenti-shRNA-Sema3B. Uprregulated Sema3B significantly enhanced osteoblast differentiation, which was indicated by a 1.5-fold increase of ALP activity and twofold increase of the amount of ARS staining (Fig. 4B, C). QRT-PCR analysis revealed that the levels of the osteoblast-specific genes Runx2, Osterix, and Osteocalcin were higher in the lenti-Sema3B-transfected MSCs compared with the cells transected with the lenti-Sema3B negative control (Fig. 4D).
were reduced in the lenti-shRNA-Sema3B-treated MSCs. These results suggested that Sema3B was a critical regulator in osteogenic differentiation of MSCs (Fig. 4B–D).

To further investigate the effect of Sema3B on TNF-α-inhibited MSC osteogenesis differentiation in vitro, Sema3B was overexpressed in MSCs, which was then cultured in osteogenesis medium in the presence of TNF-α. TNF-α treatment significantly decreased the ALP activity and the mineralized bone matrix formation in MSCs, whereas overexpression of Sema3B could markedly alleviate the inhibitory effect of TNF-α on MSC osteogenesis differentiation (Fig. 4E, F). In addition, qRT-PCR analysis showed that TNF-α was able to decrease the mRNA levels of the osteogenic marker genes Runx2, Osterix, and Osteocalcin, which could be attenuated by Sema3B overexpression (Fig. 4G). Based on the above results, we believed that Sema3B was a critical factor for TNF-α-inhibited MSCs osteogenesis differentiation.

3.4. Involvement of Sema3B in TNF-α-inhibited cells proliferation during the osteogenesis of MSCs

To further examine the effect of Sema3B on TNF-α-inhibited MSCs proliferation in vitro, Sema3B was overexpressed in MSCs, then cells were cultured in osteogenesis medium with 10 ng/ml TNF-α for different time points. CCK-8 activity assay showed that TNF-α led to a decrease in cell number during the osteogenesis of MSCs. However, overexpression of Sema3B significantly alleviated the inhibition of MSCs proliferation by TNF-α (Fig. 5A). Furthermore, CFSE staining was applied to further examine cells proliferation. When cells divide, CFSE labeling is distributed equally between daughter cells, which are half as fluorescent as their parents. After MSCs were cultured in osteogenic medium for 5 days, higher CFSE fluorescence intensity was observed in the TNF-α group compared with the control group, suggesting that TNF-α could inhibit cells proliferation. However, overexpression of

Fig. 3. TNF-α-inhibited Sema3B expression in vivo. A: Sema3B expression and localization (red arrow) in the distal end of intact femurs of SHAM mice, OVX mice, and OVX+anti-TNF-α mice through immunohistochemistry. Scale bars are 20 μm. B: Quantitative analyses of positive staining. Summarized data showed that Sema3B expression was downregulated in the distal end of intact femurs from OVX mice, whereas anti-TNF-α treatment could markedly increase Sema3B expression in OVX mice. n = 4. **P < 0.01. C: Western blot analysis of Sema3B expression was performed in MSCs from SHAM mice, OVX mice, and OVX+anti-TNF-α mice. n = 4. D: Western blot analysis of Sema3B expression was performed in MSCs cultured in osteogenic medium for 7 and 14 days, which were separately derived from SHAM mice, OVX mice, and OVX+anti-TNF-α mice. n = 4. E: qRT-PCR analysis of Sema3B expression was performed in MSCs cultured in osteogenic medium for 7 and 14 days, which were separately derived from SHAM mice, OVX mice, and OVX+anti-TNF-α mice. n = 4. **P < 0.01. F: The cultured medium TNF-α levels were measured in MSCs cultured in osteogenic medium for 7 and 14 days, which were separately derived from SHAM mice, OVX mice, and OVX+anti-TNF-α mice. n = 3, **P < 0.01. G: qRT-PCR analyses of Sema3B receptors expression were performed in MSCs from SHAM mice, OVX mice, and OVX+anti-TNF-α mice. n = 3. **P < 0.01.
Sema3B in MSCs could alleviate TNF-α-decreased cells proliferation (Fig. 5B). In addition, Edu staining was also used to observe the effect of TNF-α on cells proliferation in MSCs osteogenesis differentiation. We found that the less amount of cells with Edu-positive nuclei were presented in cells treated with TNF-α compared to control group, but overexpression of Sema3B significantly increased the amount of cells with Edu-positive, indicating that Sema3B was involved in TNF-α-decreased cells proliferation during the osteogenesis of MSCs (Fig. 5C, D).

3.5. TNF-α inhibited Sema3B expression via canonical Wnt/β-catenin signaling

Previous evidences suggested that Wnt signaling pathway modulated differentiation, proliferation, and mineralization in bone formation. Furthermore, Wnt/β-catenin signaling activation could increase semaphorin-III family proteins expressions in many cells[28,29]. Thus, we hypothesized that TNF-α may suppress osteogenic differentiation of MSCs by inhibiting Sema3B expression via Wnt/β-catenin signaling. QRT-PCR analysis showed that TNF-α (10 ng/ml) significantly decreased the mRNA expressions of GSK3β and β-catenin, but significantly increased the mRNA expression of Dkk1 in MSCs with osteogenic medium for different time points (Fig. 6A–C). Furthermore, TNF-α could significantly decrease the expressions of Wnt/β-catenin signaling critical proteins, DKK-1, GSK3β, and β-catenin, in a time-dependent manner. In contrast, P38/ERK inhibitor was greatly inhibited by TNF-α (Fig. 6D), implying that TNF-α inhibited Wnt/β-catenin signaling during the osteogenesis of MSCs. Next, we investigated if TNF-α regulated Sema3B expression by inhibiting Wnt/β-catenin signaling in MSCs. Lici (20 mM) or BIO (5 μM) were used to activate Wnt/β-catenin signaling in MSCs, followed by treatment with TNF-α (10 ng/ml) for 24 h. We found that Wnt/β-catenin signaling activation was able to significantly attenuate the inhibitory effect of TNF-α on Sema3B expression, suggesting that TNF-α decreased the expression of Sema3B through inactivation of Wnt/β-catenin signaling (Fig. 6E, F).

4. Discussion

TNF-α has been found to be associated with impaired osteogenic differentiation potential of MSCs in estrogen-deficiency-induced osteoporosis, but the mechanism responsible remains poorly understood. In the present studies, we found that the expression of Sema3B was markedly downregulated by TNF-α during MSCs osteogenesis differentiation. Furthermore, inhibition of Sema3B expression was involved in TNF-α-inhibited proliferation and osteogenic differentiation of MSCs. Mechanistically, TNF-α decreased Sema3B expression by inhibiting Wnt/β-catenin signaling. This is the study linking a Sema3B with TNF-α, which provides a better understanding of the role of Sema3B in TNF-α-inhibited MSCs osteogenesis differentiation.

TNF-α has been shown to inhibit bone formation in estrogen-deficiency-induced osteoporosis. Huang et al. found that a low concentration of TNF-α (0.01 and 0.1 ng/ml) at short-term stimulation (<2 days) promoted the osteogenic differentiation of mMSCs. In contrast, TNF-α treatment at higher concentrations (10 and 100 ng/ml)
displayed inhibitory effect on osteogenic differentiation of MSCs. However, long-term treatment with TNF-α displayed dose-dependent inhibition of MSCs osteogenic differentiation [30]. Wang et al. confirmed that regardless of a short or long time, a high concentration of TNF-α (10 and 100 ng/ml) could inhibit the osteogenic differentiation of BMSCs [31]. In the present study, we found that TNF-α (10 ng/ml)
was able to significantly suppress osteogenic differentiation of MSCs, which were consistent with previous studies [30,31].

Some of semaphorins family proteins have emerged as critical regulators in bone homeostasis. Previous studies showed that Sema3A could promote osteoblasts differentiation and impair osteoclasts formation [19]. Hughes et al. found that Sema3E inhibited mouse osteoblast migration and reduced osteoclast formation in vitro[32]. Furthermore, transgenic mice expressing Sema3B from osteoblasts had a reduced bone mineral density and aberrant trabecular structure, demonstrating that Sema3B was involved in the process of bone metabolic. Osteoblasts from transgenic mice expressing Sema3B displayed enhanced differentiation and mineralization in vitro [20]. Even though these Semaphorins family proteins have been found to be associated with bone metabolic, only Sema3B was confirmed to involve in TNF-α-inhibited MSCs osteogenesis differentiation.

There have been a few studies elucidating the important roles of semaphorins family proteins in postmenopausal osteoporosis. Koh et al. found that Sema7A polymorphisms were associated with BMD and fracture risk in postmenopausal woman [33]. Negishi et al. showed that Sema4D-specific antibody treatment markedly prevented bone loss in a model of postmenopausal osteoporosis [34]. Furthermore, Hayashi et al. examined that Sema3A exerted an osteoprotective effect by both suppressing osteoclastic bone resorption and increasing osteoblastic bone formation in postmenopausal osteoporosis [35]. However, our studies were firstly to report that Sema3B was implicated in estrogen-deficiency-induced osteoporosis.

The Wnt/β-catenin signaling pathway plays an important role in MSCs growth and differentiation [36]. In the absence of Wnt ligands, cytoplasmic β-catenin protein is constantly degraded by the Axin complex comprised Axin, adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and GSK3β. Conversely, binding of a Wnt ligand to the cell surface receptors inactivates the Axin complex, leading to β-catenin accumulates in the cytoplasm. Then β-catenin translocates to the nucleus and interacts with members of the DNA-binding T cell factor/lymphoid enhancer factor [T/CELF] family to activate Wnt target genes [37]. Previous studies have shown that TNF-α inhibited osteogenic differentiation of BMMSCs mainly through blocking canonical Wnt pathway [38]. Consistent with previous reports, we also confirmed that TNF-α was able to inhibit Wnt/β-catenin signaling during the osteogenesis differentiation of MSCs. Furthermore, Chang et al. found that TNF-α could inhibit the osteogenic differentiation of MSCs by activating IKK-NF-κB, then IKK-NF-κB signaling promoted β-catenin ubiquitination and degradation through induction of Smurf1 and Smurf2 [39]. In our studies, the exact mechanisms of TNF-α-inactivated Wnt/β-catenin signaling were not fully elucidated. Therefore, further studies will be needed to explore how TNF-α inhibited Wnt/β-catenin signaling to mediate Sema3B expression during the osteogenesis differentiation of MSCs.

In this study, we confirmed that inhibition of Wnt/β-catenin signaling could decrease Sema3B expression in MSCs. Indeed, previous studies have elucidated the effects of Wnt/β-catenin signaling on semaphorins expression in various cells. Kettunen et al. found that Sema3A was positively regulated by Wnt4 in the dental mesenchyme [28], although it was unclear if this is via canonical or noncanonical Wnt signaling. Furthermore, Sema3B has been suggested to be downstream of Wnt signaling via TCF in zebrafish neural crest cells, indicating a possible role of canonical Wnt signaling in the positive regulation of Sema3B [29]. In addition, Hughes et al. found that Sema3A and -3E expression were decreased through activation of the canonical Wnt signaling pathway, but Sema3D expression was not affected in mouse calvarial osteoblasts [32]. Taken together, it is possible that there might be differential regulation of class III semaphorins downstream of canonical or noncanonical Wnt signaling and/or cell- or tissue-type differences. Our results demonstrated for the first time that Sema3B was downstream of Wnt signaling in mMSCs, but the exact mechanisms of Wnt signaling-mediated Sema3B expression need to be further elucidated.

In this study, we confirmed that TNF-α suppressed osteogenic differentiation of MSCs by inhibiting Sema 3B expression. Overexpression of Sema3B could promote osteogenic differentiation of MSCs, whereas depletion of Sema3B was able to impair MSCs osteoblastic differentiation. Thus, we speculated that Sema3B might be a novel target for the therapy of skeletal disorders. However, in the present studies, we only observed the effect of Sema 3B on osteogenic differentiation of MSCs in vitro. To further investigate the therapeutic potential of Sema3B on osteoporosis induced by estrogen deficiency, future studies should be performed to observe the effect of Sema3B on bone formation of OVX mice in vivo. Although further studies in vivo are needed, Sema3B may represent a novel target for treatment of bone metabolic disorders such as osteoporosis.

5. Conclusions

In conclusion, our results demonstrate for the first time that Sema3B expression is downregulated by TNF-α through inactivation of Wnt/β-catenin signaling during the osteogenesis of MSCs and that Sema3B is involved in TNF-α-inhibited proliferation and osteogenic differentiation of MSCs. This study is an effort to establish a molecular mechanism of TNF-α-inhibited bone formation in estrogen-deficiency-induced osteoporosis and to provide insights into the potential contribution of Sema3B in the regulation of osteogenic differentiation from MSCs.

Author contributions

L. G. and X. C. C. were involved in the conception and hypothesis delineation and wrote the article; C. L. S. and Q. Z. designed the experiments and conducted the western blot, quantitative real-time PCR experiments, cells proliferation assay; P. H., F. J. C., H. K., and P. S. W. performed ALP staining, ALP activity assay, and AR staining; Y. X. Z. designed and conducted the animal studies; J. X. performed ELISA assay.

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