

Original Article

Cell signaling negative regulatory factor 3 regulates differentiation of bone marrow mesenchymal stem cells in osteoporotic rats

Hai Xu¹, Lan Zheng², Huijie Wang³, Ying Li¹, Shan Hu¹, Shuping He¹, Haixia Chen¹, Qing Zou⁴, Yetong Tan³, Xiangdong Tian³

¹Huangjiahua Hospital of Hubei University of Chinese Medicine, Wuhan 430070, Hubei, China; ²The First Clinic College of Hubei University of Chinese Medicine, Wuhan 430065, Hubei, China; ³Beijing University of Chinese Medicine Third Affiliated Hospital, Beijing 100029, China; ⁴Huangshi Puren Hospital, Huangshi 435002, Hubei, China

Received October 20, 2018; Accepted May 7, 2019; Epub June 15, 2019; Published June 30, 2019

Abstract: The present study aimed to investigate the effects of cell signaling negative regulatory factor 3 (SOCS3) on differentiation of bone marrow mesenchymal stem cells (BMSCs) in osteoporotic rats, examining its application in osteoporosis. A rat model of osteoporosis was established. BMSCs were isolated, cultured, and transfected with SOCS3 small hair RNA (shRNA) using a lentivirus. Immunohistochemical staining was performed, detecting protein expression levels of SOCS3, signal transducer and activator of transcription 3 (STAT3), β -catenin, peroxisome proliferator-activated receptor- γ (PPAR- γ), and adiponectin. Expression levels of genes coding these proteins were detected using reverse transcription-polymerase chain reaction and Western blot analysis. Protein expression levels of osteoprotegerin (OPG) and receptor activators of nuclear factor kappa-B ligand (RANKL) were analyzed using enzyme-linked immunosorbent assays. When infected with SOCS3 shRNA lentivirus, expression of SOCS3 mRNA was inhibited ($P<0.05$), expression of STAT3 was increased ($P<0.05$), expression of PPAR- γ , RANKL, and adiponectin was reduced ($P<0.05$), expression of OPG was increased ($P<0.05$), and the RANKL/OPG ratio was reduced (all differences were $P<0.05$). Results suggest that SOCS3 may inhibit bone resorption and reduce bone loss. This provides a novel theoretical basis for the pathogenesis of osteoporosis, suggesting SOCS3 as a potential novel target for treatment of osteoporosis.

Keywords: Bone marrow mesenchymal stem cells, osteoporotic rats, cell signaling negative regulatory factor 3, activator of transcription 3, signal transducer

Introduction

Stem cells are progenitor cells in many tissues, playing a crucial role in maintaining normal metabolism and the steady state of tissues. Many studies have demonstrated that stem cell dysfunction is closely associated with occurrence of numerous diseases [1]. Bone marrow mesenchymal stem cells (BMSCs), a type of adult stem cells like hematopoietic stem cells, can differentiate into many cells, including osteoblasts, adipocytes, skeletal muscle, and hematopoiesis-supporting stromal cells. BMSCs have good plasticity. Thus, unique immunological properties do not pose any ethical controversy. They have become the primary

seed cells for tissue engineering and cell therapy, widely used in a variety of studies. In a normal physiological state, BMSCs differentiate into osteoblasts and adipocytes, maintaining a dynamic balance maintained between the two. However, when BMSC differentiation into adipocytes exceeds differentiation into osteoblasts, an imbalance between bone resorption and bone formation occurs. This results in diseases, such as osteoporosis [2].

Cell signaling negative regulatory factor 3 (SOCS3; a suppressor of cytokine signaling) affects Janus kinase (JAK) signal transducers and activators of the JAK/signal transducer and activator of transcription (STAT) transcription signal-

ing pathways. JAK/STAT signaling pathways are primarily involved in cell proliferation, differentiation, apoptosis, and immune regulation. Subtle changes in this signaling pathway may lead to changes in cytokine expression and pathology [3]. As a result, SOCS3 can affect cell growth, proliferation, apoptosis, and other basic biological behaviors. In addition, SOCS3 is closely associated with abnormal activation of signaling pathways involved in cell proliferation and movement. SOCS3 is regulated by the cytokine interleukin-6 (IL-6), negatively regulating JAK/STAT signaling pathways by inhibiting signal transduction via a feedback regulating mechanism. It activates transcription factors, such as STAT. Cao et al. [4] observed that the function of STAT-3 is more marked in cells lacking SOCS-3. In addition, Ilamathi et al. [5] observed that JAK-STAT signaling pathways and osteoporosis are closely related.

Many studies have demonstrated that bone metabolism and osteoporosis are closely associated with Wnt/ β -catenin pathways, peroxisome proliferator-activated receptor- γ (PPAR- γ), adiponectin, osteoprotegerin (OPG; an activating factor), and the nuclear factor κ B receptor activator of nuclear factor kappa-B ligand (RANKL) system. However, association levels between SOCS3 and incidence levels of osteoporosis have not yet been reported. Previously, it was demonstrated that osteopenia, caused by osteoporosis, is often accompanied by an increase in adipose tissue differentiated from BMSCs [6]. Investigating the mechanisms underlying this phenomenon, the present study established a rat model of osteoporosis. BMSCs were isolated and cultured *in vitro*, aiming to explore the regulation of BMSC differentiation by SOCS3, laying an experimental foundation for treatment of osteoporosis on a molecular level.

Materials and methods

Animals

A total of 40 female Sprague-Dawley rats of clean specific pathogen-free grades (age, 6 months; weight, 220 ± 6.32 g) were provided by the Center for Animal Experiment of Wuhan University (Wuhan, China). Treatment of the animals in the experiment complied with animal ethical requirements. Current research was conducted in accordance with the Declaration

of Helsinki and in accordance with Guidelines for Care and Use of Laboratory Animals, as adopted by the United States National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of the Fourth Military Medical University (Xi'an, China).

Reagents

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from PAN-Biotech GmbH Aidenbach, Germany). Bovine serum albumin (BSA) and phosphate-buffered saline (PBS) were purchased from Cyagen Biosciences (Santa Clara, CA, USA). Fetal bovine serum, indole Mischa Star, and isobutylmethylxanthine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Muse anti-rat CD34, CD44, CD90, and CD105 antibodies were purchased from Caltag Medsystems (Buckingham, UK). SOCS3 mouse monoclonal antibody was purchased from EMD Millipore (Billerica, MA, USA). β -catenin antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit anti-human STAT3 antibody was purchased from Beyotime Institute of Biotechnology (Haimen, China). PPAR- γ was purchased from Shanghai SAB (Shanghai, China). Moreover, shRNA sequences and polymerase chain reaction (PCR) primers were synthesized by Takara Biotechnology Co., Inc. (Dalian, China), which also supplied the PCR reverse transcription kit. Total RNA was extracted using TRIzol Reagent, purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). OPG and RANKL enzyme-linked immunosorbent assay (ELISA) kits were purchased from the Biomedica Group (Windham, NH, USA).

Establishment of the osteoporosis rat model

The rats were fed and acclimatized for 1 week prior to the operation. They were anesthetized with intraperitoneal (i.p.) injections of ketamine hydrochloride (100 mg/kg). A bilateral oophorectomy was performed under sterile conditions. The rats were placed in the supine position. An incision was made inferior to the abdomen and along the ventral acetabular line to cut open the abdominal cavity. Double ovaries were identified and then resected. Surrounding blood vessels and fallopian tubes were ligated. The wounds were stitched and disinfected with 75% ethanol. The rats were injected with peni-

cillin (i.p.) to prevent infections. They had access to food and water *ad libitum* for 3 months at room temperature in a ventilated environment. After this period, they were sacrificed following anesthetization. Tibias and the lumbar were removed, performing a histomorphometry and detecting biomechanical indicators [7]. Finally, 20 rats with osteoporosis were selected for subsequent experimentation.

BMSC isolation and in vitro culturing

Under sterile conditions, the femur was removed and soaked in 75% ethanol for 30 seconds. The epiphyseal ends were also cut. A total of 4 mL of PBS was injected into the bone marrow cavity from one end, flushing the bone marrow from the other end. The liquid was repeatedly blown with a dropper, then centrifuged to discard the supernatant. The cells were resuspended in 5 mL of DMEM containing 10% FBS to collect the bone marrow cell suspension. The cells were adjusted to a concentration of 1.0×10^8 cells/L, inoculated in culture bottles, and placed in an incubator at 37°C. The culture solution was discarded after 10–14 days. The cells were washed with PBS and digested with 2.5 g/L trypsin. Digestion was terminated using a fresh culture solution containing blood serum when the cells became round. Next, the cell suspension was collected and centrifuged. Finally, the cells were adjusted to a concentration of 2×10^8 cells/L following inoculation.

Cell membrane flow cytometry

Third-generation rat BMSCs were digested with 0.25% trypsin at 4°C. This was followed by centrifugation at 1,000 r/min for 5 minutes with a 4.9-cm centrifugal radius. The cells were washed three times with PBS/1% BSA and counted. Monoclonal antibodies CD34, CD44, CD90, and CD105 were added into the cells. A negative control sample was set for each tube. After incubation on ice for 30 minutes in the dark, the cells were washed with PBS 3 times. The solution was repeatedly blown to create a single-cell suspension. The samples were then analyzed using flow cytometry.

Establishment of the SOCS3 small hair RNA (shRNA) lentivirus

A lentivirus can efficiently infect dividing and non-dividing cells. It can be integrated into a

host cell genome for long-term expression. SOCS3 shRNA can effectively inhibit expression of SOCS3. AgeI and EcoRI were digested and gelled to recover the SOCS3 plasmid. When the fragments of the annealed shRNA sequences were connected, reverse transcription-PCR (RT-PCR) was performed. The empty vector PCR fragments were 185 base pairs (bp) in size and the PCR product fragment inserted into the shRNA sequence was 243 bp. The PCR product was purified using 2% agarose gel electrophoresis. Clones were identified by PCR and the sequence was detected, building a lentivirus.

The following primers were used: SOCS3 forward, 5'-TCACCCACAGCAAGTTTCC-3' and reverse, 5'-GGATGCGTAGGTTCTTGGTC-3' (218 bp); STAT3 forward, 5'-CCTCTGAGAAGAGGGGACAA-3' and reverse, 5'-ACCGCTTCCAGCAAAAGAGT-3' (317 bp); β -catenin forward, 5'-AAGCTCATC-ATACTGGCTAGT-3' and reverse, 5'-CTTGCAATC-CACCAGCTTCT-3' (160 bp); PPAR γ forward, 5'-TTGATTCTCCAGCATTTC-3' and reverse, 5'-TTCAATCGGATGGTTCTT-3' (231 bp); adiponectin forward, 5'-ATCTGATTACACAAAAGT-3' and reverse, 5'-TCAGTTGGTATCATGGTAG-3' (757 bp); β -actin forward, 5'-TCTACAATTGAGCTGCGTGTG-3' and reverse, 5'-GGTCAGGATCTTCAT GAGGT-3' (314 bp); shRNA forward, 5'-CCGGCCTGCGTTTGGATGATTTCTCGAGAAATCATCCACCAA-ACGCAGGTTTTTG-3' and reverse, 5'-AATTCAA-AAACCTGCGTTTGGTGGATGATTTCTCGAGAAATCATCCACCAAACG CAGG-3'.

BMSC infection with SOCS3 shRNA lentivirus

BMSC suspension was added into the SOCS3 shRNA lentivirus, cultured for 24 hours at 37°C with 5% CO₂, and replaced with fresh culture medium.

Morphological changes of BMSCs prior to and following lentivirus infection

Penicillin (100 U/mL), streptomycin (100 μ g/mL), insulin (5 μ g/mL), 10% fetal calf serum, dexamethasone (10^{-6} mol/L), indole MischaStar (6×10^{-7} mol/L), and isobutylmethylxanthine (10^{-4} mol/L) were added to the cell suspension. It was then cultured in a 5% CO₂ incubator at 37°C. On day 7 of the culturing period, an inverted phase-contrast microscope was used to observe morphological changes in cells prior to and following infection with the lentivirus.

Table 1. Quantitative reverse transcription-polymerase chain reaction results of genes in bone marrow mesenchymal stem cells prior to and following lentivirus infection (n=20)

Gene	Before infection	After infection
SOCS3	5.32±0.021	1.37±0.013 ^a
STAT3	5.60±0.022	10.05±0.013 ^b
β-Catenin	2.09±0.012	4.57±0.024 ^a
PPARγ	6.25±0.022	2.36±0.02 ^a
Adiponectin	1.37±0.013	0.72±0.022 ^a

^aP<0.05, ^bP<0.01 vs. before infection. SOCS3, signaling negative regulatory factor 3; STAT3, signal transducer and activator of transcription 3; PPARγ, peroxisome proliferator-activated receptor-γ.

SOCS3, STAT3, β-catenin, PPARγ, and adiponectin mRNA detection

Total RNA was extracted using TRIzol Reagent 24 hours after BMSC shRNA lentiviral infection. Detection of expression of SOCS3, STAT3, β-Catenin, PPARγ, and adiponectin mRNAs was performed using quantitative RT-PCR (qRT-PCR), prior to and following infection. The BMSC single-cell suspension was centrifuged. The cells were then fixed with cold ethanol and incubated overnight at -20°C. They were centrifuged to remove ethanol and washed with PBS. Next, 40 µg/mL propidium iodide and 100 µg/mL RNA enzymes were added. The cells were incubated at 4°C in the dark for 30 minutes. PCR products were detected using 2% agarose gel electrophoresis. Scion Image version 4.03 was used to analyze PCR product gray values. Immunohistochemistry was then performed, detecting protein expression levels of each gene.

OPG and RANKL protein detection

Freshly-diluted OPG and RANKL protease-labeled antibodies were added to the BMSC suspension, prior to and following infection. OD values were measured at 450 nm using an ELISA detector and concentrations were calculated using a standard curve.

Statistical analysis

Statistical analysis was performed using SPSS version 15.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean ± standard error. Differences between the groups were

compared using one-way analysis of variance. P<0.05 indicates statistically significant differences.

Results

Establishment and identification of the osteoporosis rat model

The distal end of the femur was removed from rats in the model group, performing histological examinations. Resorption was clear in the cancellous bone at the distal end of the femur. The number of bone trabecula in the model group was lower, compared with the normal rats. However, the growth plate at the epiphyseal end of the tibia was thinner than the normal standard.

BMSC growth characteristics

At 24 hours of BMSCs incubation in flasks, slender spindle cells were detected, with a cell refractive index. Cells significantly increased in size after 9 days. They became round and intermixed with surrounding blood cells. Cell fusion reached 80%-90% and the cells grew into shoal whorls, attached to few small round or refractive cells with large cell bodies. The third generation of BMSCs was fusiform and closely arranged.

Expression of BMSC surface antigens

Flow cytometry showed that adherent cultured cells exhibited characteristics of a BMSC and that the third-generation rat BMSCs expressed CD44, CD90, CD105, and other MSCs. Stem cell surfaces positively expressed antigens. Positive expression rates of CD44, CD90, and CD105 were 96.20, 97.23, and 97.91%, respectively. Expression of CD34 on the stem cell surface was negatively expressed, with a positive rate of 3.83%. Primer sequences for each gene are presented in **Table 1**.

BMSC morphological changes prior to and following infection

On day 7 of lentiviral infection, cell morphology was observed under an inverted phase-contrast microscope. Results showed that BMSC morphology markedly changed from the former oval shape into a fibrous long spindle shape (**Figure 1**).

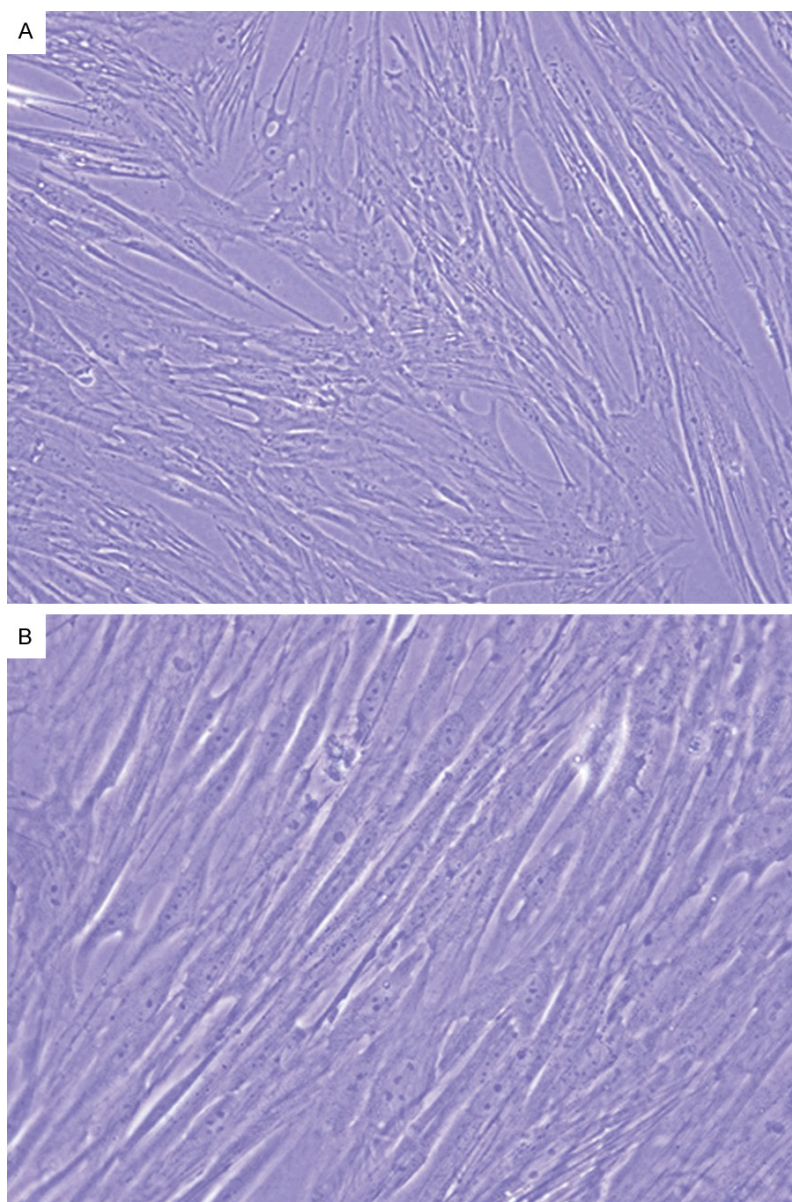


Figure 1. Observation of bone marrow mesenchymal stem cell morphology. (A) Prior to and (B) following infection (magnification $\times 100$).

MicroRNA protein expression in BMSCs prior to and following infection

When BMSCs were infected with the lentivirus, mRNA expression levels of SOCS3 ($P < 0.05$), PPAR γ , and adiponectin ($P < 0.01$) were significantly decreased, while expression levels of STAT3 and β -catenin were significantly increased ($P < 0.05$; **Figure 2**).

RT-qPCR analysis of BMSC genes prior to and following infection

Compared with results prior to infection, expression of SOCS3 ($P < 0.05$), PPAR γ , and adiponectin ($P < 0.01$) was significantly decreased, while expression of STAT3 and β -catenin was significantly increased ($P < 0.05$; **Table 1**).

SOCS3, STAT3, and adiponectin were primarily expressed in the cytoplasm of BMSCs, while β -catenin and PPAR γ were primarily expressed in the nucleus. A large quantity of SOCS3 and adiponectin positively-stained cells were detected in the cytoplasm of BMSCs prior to infection. SOCS3, adiponectin, and PPAR γ protein expression levels were significantly reduced following infection, compared with protein expression levels prior to infection. In addition, cytoplasmic STAT3 and β -Catenin protein expression levels significantly increased following infection, compared to those prior to infection (**Figure 3**).

BMSC immunohistochemistry

SOCS3, STAT3, and adiponectin were primarily expressed in the cytoplasm of BMSCs, while β -catenin and PPAR γ were primarily expressed in the nucleus. A large quantity of SOCS3 and adiponectin positively-stained cells were detected in the cytoplasm of BMSCs prior to infection. SOCS3, adiponectin, and PPAR γ protein expression levels were significantly reduced following infection, compared with protein expression levels prior to infection. In addition, cytoplasmic STAT3 and β -Catenin protein expression levels significantly increased following infection, compared to those prior to infection (**Figure 3**).

BMSC OPG and RANKL content and RANKL/OPG ratios, prior to and following infection

Following infection with the lentivirus, OPG content in BMSCs significantly increased, while RANKL content significantly decreased ($P < 0.05$). The RANKL/OPG ratio was significantly reduced following infection, compared with that prior to infection ($P < 0.01$; **Table 2**).

Discussion

The present study demonstrates that dysfunction of BMSCs is crucially involved in incidence of osteoporosis [8]. BMSCs are the only source of osteoblast precursors. Thus, the study of BMSC differentiation may provide novel targets for treatment of certain diseases, including bone aging and osteoporosis.

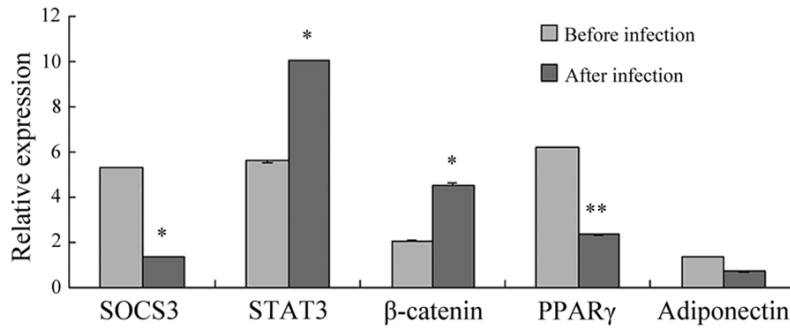


Figure 2. MicroRNA expression of SOCS3, STAT3, β -catenin, PPAR γ , and adiponectin in bone marrow mesenchymal stem cell mRNAs before and after infection; * $P < 0.05$ and ** $P < 0.01$ vs. before infection. SOCS3, signaling negative regulatory factor 3; STAT3, signal transducer and activator of transcription 3; PPAR γ , peroxisome proliferator-activated receptor- γ .

Numerous *in vitro* experiments have confirmed that promoting expression levels of fat-cell generation factors can inhibit osteoblast production. Osteogenic factors may inhibit adipogenesis [2]. PPAR- γ , a member of the nuclear hormone receptor superfamily, is a primary regulatory factor of adipogenesis. It is involved in the final step of regulating adipogenesis and associated signaling pathways [9]. When activated, PPAR- γ regulates expression of lipid metabolism genes, such as lipoprotein lipase and fatty acids synthase (FAS), leading to the mature phenotype of fat cells. In addition, PPAR- γ inhibits differentiation of BMSCs into osteoblasts, promoting differentiation into adipocytes [10].

Previous evidence has indicated that Wnt/ β -Catenin signaling pathways may play a crucial role in the self-renewal and directional regulation of BMSCs. This signaling pathway can promote BMSCs to differentiate into osteoblasts, inhibiting their differentiation into adipocytes [11]. Interruption of this signaling pathway will result in several skeletal or non-skeletal diseases [12]. Simultaneous activation of Wnt signaling pathways can prevent expression of PPAR γ and inhibit fat cell production, while PPAR γ induction may inhibit the signaling pathway [13].

Numerous studies have identified that incidence of osteoporosis in obese subjects is significantly reduced, compared with non-obese subjects, and that the leptin levels of obese subjects increase significantly. This suggests that leptin and bone metabolism are closely related [14]. Studies concerning bone metabolism have indicated that leptin may influence bone resorption and bone formation [15]. Ratra

et al. [16] determined that leptin acts on the neurons of hypothalamic arcuate nucleus, reduces the transcription of neuropeptide Y mRNA, and inhibits osteoblast differentiation via the neuropeptide Y receptor on osteogenic cells, affecting bone density. Inagaki et al. [17] and Pedrosa et al. [18] confirmed that SOCS3 is a negative feedback inhibitor of leptin signal transduction and that it is closely associated with fat metabolism. SOCS3 targets leptin

by inhibiting signal transduction pathways of leptin receptors by inhibiting the phosphorylation of JAK2 [19]. Therefore, it may be inferred that SOCS3 is closely associated with osteogenic and adipogenic differentiation of BMSCs. SOCS3 is a negative regulatory factor of STAT. Thus, it can be said that lowering expression levels of SOCS3 enhances expression of STAT3. This activates the conduction of Wnt/ β -catenin pathways and promotes differentiation of BMSCs into osteoblasts.

The lentivirus can efficiently infect dividing and non-dividing cells. It can be integrated into a host cell genome for long-term expression [20-22]. In the present study, a pre-fabricated lentiviral expression vector was used to inhibit expression of SOCS3 in BMSCs. Analyses suggested that expression of SOCS3 mRNA and protein was significantly inhibited following infection with lentivirus. Present results indicate that the lentivirus may effectively interfere with BMSC SOCS3 ($P < 0.05$). In addition, experimental results in the current study indicate that mRNA and protein expression levels of STAT3 and β -catenin are significantly increased ($P < 0.05$) following lentivirus infection, confirming that a reduction in expression of SOCS3 enhances expression of STAT3. STAT3 is an important factor downstream of Wnt/ β -catenin signaling pathways. Thus, enhanced expression activates the pathway and increases expression of β -catenin. The present study demonstrated that Wnt/ β -catenin signaling pathways prevent expression of PPAR γ and that the mRNA and protein expression levels of PPAR γ are significantly decreased following lentiviral infections ($P < 0.05$).

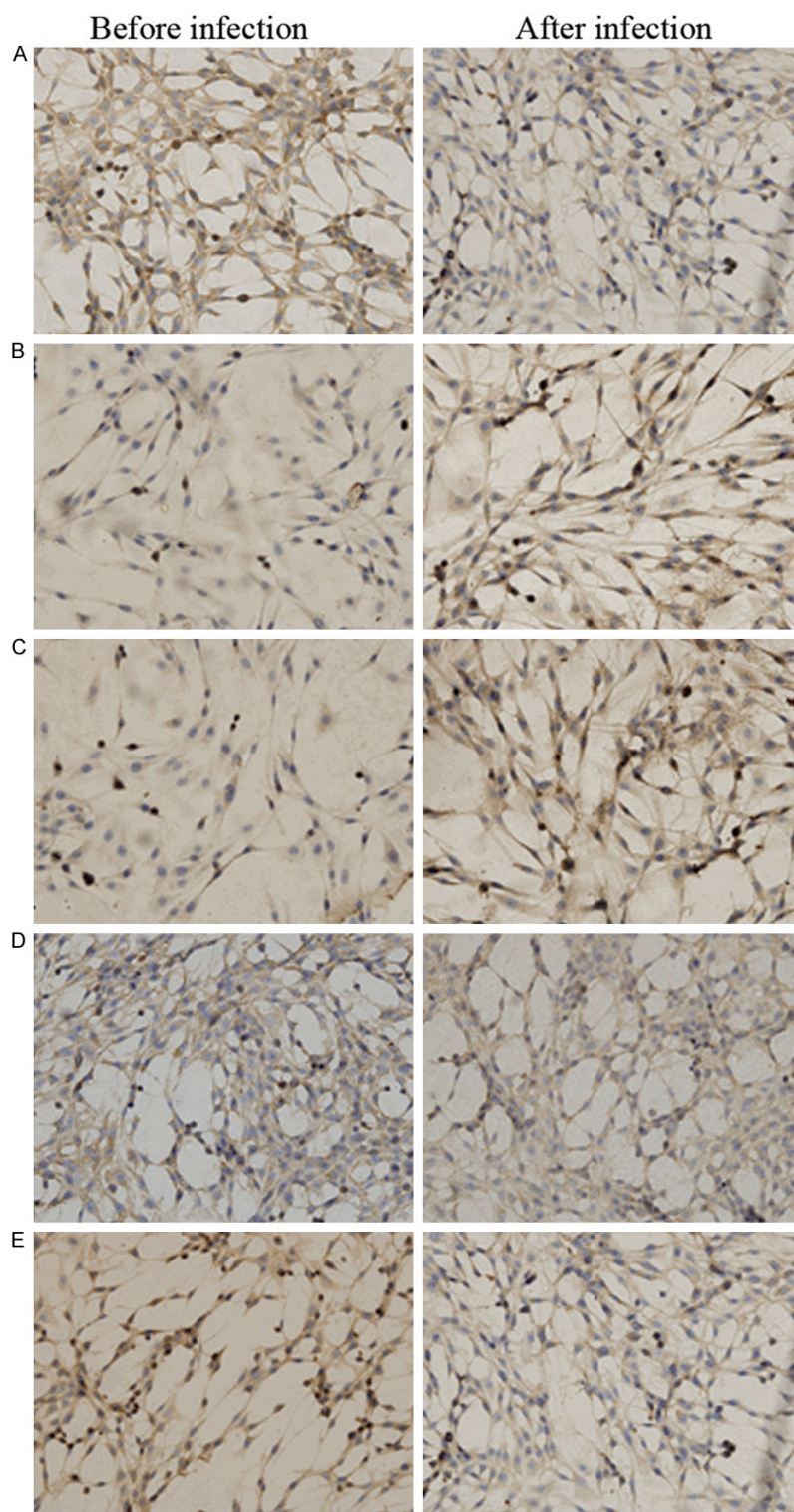


Figure 3. Immunohistochemical detection of (A) Signaling negative regulatory factor 3, (B) Signal transducer and activator of transcription 3, (C) β -catenin, (D) Peroxisome proliferator-activated receptor- γ and (E) Adiponectin in bone marrow mesenchymal stem cells before and after infection (magnification, $\times 400$).

PPAR γ regulates gene expression of key enzymes involved in the process of lipid metabolism

and secretion of the fat-cell proteins, such as leptin, TNF- α , pancreatic resistin (resistance and increased resistin secretion), and adiponectin [23]. Adiponectin is an adipocytokine, a type of internally-derived bioactive peptide or protein that can be specifically highly-expressed by fat cells. It has been suggested that adiponectin is a regulatory factor of bone metabolism or a mediator of adipose tissue and bone metabolism, negatively correlated with bone mineral density [24]. In the current study, it was demonstrated that expression levels of adiponectin mRNA and protein significantly decreased following infection ($P < 0.05$). Therefore, results suggest that the observed reduction in PPAR γ expression after infection decreased the secretion of adiponectin, resulting in a decline of adiponectin expression levels. The negative correlation between adiponectin and bone mineral density suggests that by downregulating expression of PPAR γ , expression of adiponectin can be reduced, bone density can be increased, and osteoporosis can be successfully treated.

Using the gene-deficient mouse model, researchers have found that SOCS3 can resist the inhibition of macrophage differentiation into osteoclasts from IL-6 and promote the formation of osteoclasts by blocking the conduction of IFN- γ , IL-6, and other cytokines. This accelerates the destruction of inflammatory bone [25].

Cunningham et al. [26] demonstrated that endogenous particulates may contribute to bone

Table 2. OPG and RANKL contents in bone marrow mesenchymal stem cells and the RANKL/OPG ratio prior to and following infection (n=20)

Parameter	Before infection	After infection
OPG (µg/ml)	0.35±0.013	0.47±0.013 ^a
RANKL (µg/ml)	9.53±0.018	2.05±0.014 ^a
RANKL/OPG	27.22	4.36 ^b

^aP<0.05, ^bP<0.01 vs. after infection. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand.

erosion via inhibition of anti-osteoclastogenic cytokine signaling, potential targets in overcoming these effects. In addition, studies have demonstrated that OPG and RANKL participate in the maintenance of the dynamic balance between osteoblasts and osteoclasts and that the OPG/RANKL ratio plays an important regulatory role in the formation and function of osteoclasts, bone formation, and bone resorption [27]. When RANK is activated, osteoclast differentiation is promoted and the number of osteoclasts is increased [28]. During the process of BMSC differentiation into osteoblasts, the RANKL/OPG ratio continually changes. It gradually decreases as osteoblasts differentiate and mature, thus losing its function of promoting osteoclast differentiation and activation. This results in the balance between bone resorption and bone formation [29, 30].

Miller [31] reported that subcutaneous injections of exogenous OPG drugs significantly reduce the levels of bone turnover markers in postmenopausal women and increase bone density. Results from the present study demonstrate that, when BMSCs are infected with a lentivirus, the OPG content significantly increases, RANKL content and RANKL/OPG ratios significantly decrease, and bone resorption is suppressed. This results in a decrease in bone loss. Present results suggest that SOCS3 may regulate bone metabolism by affecting dynamic changes between the OPG/RANKL system, osteoblasts, osteoclasts, bone formation, and resorption.

In conclusion, results of the current study suggest that SOCS3 regulates the differentiation of BMSCs via Wnt/β-catenin signaling pathways. Results also suggest that SOCS3 can decrease expression levels of adiponectin and increase bone density. In addition, SOCS3 inhibits bone

resorption by regulating the OPG/RANKL system, thereby reducing bone loss. This offers a theoretical basis for the pathogenesis of osteoporosis. The current study aimed to provide a novel target for treatment of this disease.

Acknowledgements

The present study was supported by the National Basic Research Program of China (973 Program; grant no. 2011CB964700-G), China Post-Doctoral Science Fund (2013M542498), Hubei Natural Science Foundation (2018CFC-808) and Hubei Provincial health Commission (WJ2017M168).

Address correspondence to: Dr. Yetong Tan and Xiangdong Tian, Beijing University of Chinese Medicine Third Affiliated Hospital, Beijing 100029, China. E-mail: xuhai1113@163.com (YTT); gxy19-82519@126.com (XDT)

References

- [1] Carvalho E, Verma P, Hourigan K, Banerjee R. Myocardial infarction: stem cell transplantation for cardiac regeneration. *Regen Med* 2015; 10: 1025-43.
- [2] Zhou D, Chen YX, Yin JH, Tao SC, Guo SC, Wei ZY, Feng Y, Zhang CQ. Valproic acid prevents glucocorticoid-induced osteonecrosis of the femoral head of rats. *Int J Mol Med* 2018; 41: 3433-3447.
- [3] Kim HJ, Baek J, Lee JR, Roh JY, Jung Y. Optimization of cytokine milieu to reproduce atopic dermatitis-related gene expression in HaCaT keratinocyte cell line. *Immune Netw* 2018; 18: e9.
- [4] Cao F, Hata R, Zhu P, Ma YJ, Tanaka J, Hanakawa Y, Hashimoto K, Niinobe M, Yoshikawa K, Sakanaka M. Overexpression of SOCS3 inhibits astrogliogenesis and promotes maintenance of neural stem cells. *J Neurochem* 2006; 98: 459-70.
- [5] Ilamathi M, Prabu PC, Ayyappa KA, Sivaramakrishnan V. Artesunate obliterates experimental hepatocellular carcinoma in rats through suppression of IL-6-JAK-STAT signalling. *Bio-med Pharmacother* 2016; 82: 72-9.
- [6] Li B. MicroRNA regulation in osteogenic and adipogenic differentiation of bone mesenchymal stem cells and its application in bone regeneration. *Curr Stem Cell Res Ther* 2018; 13: 26-30.
- [7] Mohamad NV, Ima-Nirwana S, Chin KY. The effects of gonadotropin-releasing hormone agonist (buserelin) and orchidectomy on bone turnover markers and histomorphometry in rats. *Aging Male* 2018; 1-8.

- [8] Wang L, Zhang HY, Gao B, Shi J, Huang Q, Han YH, Hu YQ, Lu WG, Zhao ZJ, Liu BH, Jie Q, Yang L, Luo ZJ. Tetramethylpyrazine protects against glucocorticoid-induced apoptosis by promoting autophagy in mesenchymal stem cells and improves bone mass in glucocorticoid-induced osteoporosis rats. *Stem Cells Dev* 2017; 26: 419-430.
- [9] Vallée A, Vallée JN, Guillevin R, Lecarpentier Y. Interactions between the canonical WNT/beta-catenin pathway and PPAR gamma on neuroinflammation, demyelination, and remyelination in multiple sclerosis. *Cell Mol Neurobiol* 2018; 38: 783-795.
- [10] Cao J, Ou G, Yang N, Ding K, Kream BE, Hamrick MW, Isales CM, Shi XM. Impact of targeted PPAR γ disruption on bone remodeling. *Mol Cell Endocrinol* 2015; 410: 27-34.
- [11] Jing H, Su X, Gao B, Shuai Y, Chen J, Deng Z, Liao L, Jin Y. Epigenetic inhibition of Wnt pathway suppresses osteogenic differentiation of BMSCs during osteoporosis. *Cell Death Dis* 2018; 9: 176.
- [12] Liao L, Yu Y, Shao B, Su X, Wang H, Kuang H, Jing H, Shuai Y, Yang D, Jin Y. Redundant let-7a suppresses the immunomodulatory properties of BMSCs by inhibiting the Fas/FasL system in osteoporosis. *FASEB J* 2018; 32: 1982-1992.
- [13] Yuan Z, Li Q, Luo S, Liu Z, Luo D, Zhang B, Zhang D, Rao P, Xiao J. PPAR γ and Wnt signaling in adipogenic and osteogenic differentiation of mesenchymal stem cells. *Curr Stem Cell Res Ther* 2016; 11: 216-25.
- [14] Upadhyay J, Farr OM, Mantzoros CS. The role of leptin in regulating bone metabolism. *Metabolism* 2015; 64: 105-13.
- [15] Yan X, Kononenko NL, Brüel A, Thomsen JS, Poy MN. Neuronal cell adhesion molecule 1 regulates leptin sensitivity and bone mass. *Calcif Tissue Int* 2018; 102: 329-336.
- [16] Ratra DV, Elias CF. Chemical identity of hypothalamic neurons engaged by leptin in reproductive control. *J Chem Neuroanat* 2014; 61-62: 233-8.
- [17] Inagaki-Ohara K, Mayuzumi H, Kato S, Minokoshi Y, Otsubo T, Kawamura YI, Dohi T, Matsuzaki G, Yoshimura A. Enhancement of leptin receptor signaling by SOCS3 deficiency induces development of gastric tumors in mice. *Oncogene* 2014; 33: 74-84.
- [18] Pedrosa JA, Buonfiglio DC, Cardinali LI, Furigo IC, Ramos-Lobo AM, Tirapegui J, Elias CF, Donato J Jr. Inactivation of SOCS3 in leptin receptor-expressing cells protects mice from diet-induced insulin resistance but does not prevent obesity. *Mol Metab* 2014; 3: 608-18.
- [19] Liu Z, Gan L, Zhou Z, Jin W, Sun C. SOCS3 promotes inflammation and apoptosis via inhibiting JAK2/STAT3 signaling pathway in 3T3-L1 adipocyte. *Immunobiology* 2015; 220: 947-53.
- [20] Hron T, Fábryová H, Pačes J, Elleder D. Endogenous lentivirus in Malayan colugo (*Galeopoterus variegatus*), a close relative of primates. *Retrovirology* 2014; 11:84.
- [21] Robertson DS. Origin and activities of human lentivirus particles. *Biomed Pharmacother* 2016; 83: 1311-1314.
- [22] Lee JY, Lee HH. A new chemical complex can rapidly concentrate lentivirus and significantly enhance gene transduction. *Cytotechnology* 2018; 70: 193-201.
- [23] Paruthi J, Gill N, Mantzoros CS. Adipokines in the HIV/HAART-associated lipodystrophy syndrome. *Metabolism* 2013; 62: 1199-205.
- [24] Kanazawa I. Adiponectin in metabolic bone disease. *Curr Med Chem* 2012; 19: 5481-92.
- [25] Ohishi M, Matsumura Y, Aki D, Mashima R, Taniguchi K, Kobayashi T, Kukita T, Iwamoto Y, Yoshimura A. Suppressors of cytokine signaling-1 and -3 regulate osteoclastogenesis in the presence of inflammatory cytokines. *J Immunol* 2005; 174: 3024-31.
- [26] Cunningham CC, Corr EM, McCarthy GM, Dunne A. Intra-articular basic calcium phosphate and monosodium urate crystals inhibit anti-osteoclastogenic cytokine signalling. *Osteoarthritis Cartilage* 2016; 24: 2141-2152.
- [27] Park K, Ju WC, Yeo JH, Kim JY, Seo HS, Uchida Y, Cho Y. Increased OPG/RANKL ratio in the conditioned medium of soybean-treated osteoblasts suppresses RANKL-induced osteoclast differentiation. *Int J Mol Med* 2014; 33: 178-84.
- [28] Warren JT, Zou W, Decker CE, Rohatgi N, Nelson CA, Fremont DH, Teitelbaum SL. Correlating RANK ligand/RANK binding kinetics with osteoclast formation and function. *J Cell Biochem* 2015; 116: 2476-83.
- [29] Nardone V, D'Asta F, Brandi ML. Pharmacological management of osteogenesis. *Clinics (Sao Paulo)* 2014; 69: 438-46.
- [30] Sapir-Koren R, Livshits G. Osteocyte control of bone remodeling: is sclerostin a key molecular coordinator of the balanced bone resorption-formation cycles? *Osteoporos Int* 2014; 25: 2685-700.
- [31] Miller PD. Denosumab: anti-RANKL antibody. *Curr Osteoporos Rep* 2009; 7: 18-22.