Role of Osteoprotegerin (OPG) in Bone Marrow Adipogenesis

Lili Zhang\(^a\) Mengmeng Liu\(^b\) Xiaokang Zhou\(^a\) Yi Liu\(^b\) Bo Jing\(^c\) Xiaogang Wang\(^d\) Qi Zhang\(^a\) Yao Sun\(^a\)

\(^a\)Department of Implantology, School and hospital of Stomatolog, Tongji University, Shanghai Engineering Research Center of Tooth Restoration and Regeneration, \(^b\)Department of Endodontics, School of Stomatolog, Tongji University, \(^c\)Center of stem cell, Medical School, Tongji University, Shanghai, \(^d\)Department of Cell Biology & Institute of Biomedicine, College of Life Science and Technology, Jinan University, Guangzhou, China

Key Words
Adipogenesis • Osteoprotegerin • Bone aging • Peroxisome proliferator-activated receptor γ (PPAR-γ) • Bone marrow mesenchymal stem cells (BMMSCs)

Abstract
Background/Aims: Bone marrow adipogenesis is one of the major characteristics of aged bone. Bone marrow mesenchymal stem cells (BMMSCs) prefer to differentiate into adipocytes instead of osteoblasts in the bone marrow cavity in aged hosts. The mechanism of formation and function of adipocytes in aged bone marrow needs further investigation. Osteoprotegerin (OPG) is a member of the tumor necrosis factor receptor (TNFR) super family, and it can inhibit the activities of osteoclasts. We found that adipocyte numbers increased in the bone marrow of \(^b\)Opg\(^\) knockout mice. In this study, we investigated the role of OPG in the differentiation of BMMSCs and bone marrow adipogenesis. Methods: Histological analyses were performed on the bone tissues of \(^b\)Opg\(^\) knockout (\(^b\)Opg-KO) and wild-type (WT) mice of different ages. BMMSCs obtained from mice were cultured in vitro to evaluate their differentiation abilities. Results: With aging, the expression of \(^b\)Opg\(^\) in the bone marrow of WT mice markedly decreased, but that of the adipogenic specific transcription factor peroxisome proliferator-activated receptor γ (\(^b\)Ppar-γ) increased. Adipocytes formed in the bone marrow of \(^b\)Opg-KO mice at a relative young age, and the number of adipocytes increased dramatically with age. Compared with the WT control, the osteogenic differentiation of \(^b\)Opg-KO BMMSCs decreased, but the adipogenic differentiation increased. Moreover, exogenous OPG could inhibit the adipogenic differentiation and promote the osteogenic differentiation of \(^b\)Opg-KO BMMSCs in vitro. Conclusions: OPG plays an important role in regulating BMMSC differentiation and bone marrow adipogenesis.

L. Zhang and M. Liu contributed equally to this work.

© 2016 The Author(s)
Published by S. Karger AG, Basel
Introduction

During bone development and remodeling, bone marrow mesenchymal stem cells (BMMSCs) can differentiate into osteoblasts, chondrocytes, or adipocytes under the control of specific mechanisms [1]. Bone aging has been attributed to BMMSC aging and decreased osteoblast numbers and activities [2]. Interestingly, in the long bone marrow cavities, the number of adipocytes arising from BMMSCs gradually increases with age. This occurs because BMMSCs from aged bone marrow have a reduced capacity to differentiate into osteoblasts and an increased capacity to differentiate into adipocytes [3, 4]. In addition, osteoporosis is associated with increased bone marrow adipocytes [5]. Bone marrow fat has long been considered a simple filler tissue. However, an increasing amount of evidence demonstrates that bone marrow adipocytes are more than passive occupants [6], but direct evidence for the function of increasing adipocyte numbers in the bone marrow is lacking.

Receptor activator of nuclear factor kappa-B ligand (RANKL) is a member of the tumor necrosis factor (TNF) superfamily. RANKL affects remodeling by promoting osteoclast differentiation and activation [7]. On the osteoclast surface, RANKL binds to the receptor activator of nuclear factor kappa-B (RANK) to promote osteoclastogenesis. Osteoprotegerin (OPG) is a cytokine receptor that acts as a decoy receptor for RANKL. By binding to RANKL, OPG prevents RANK-mediated osteoclast proliferation and maturation. Thus, the OPG/RANKL ratio is an important determinant of bone mass and skeletal integrity [7]. In bone, OPG is mainly produced by osteoblasts and osteocytes [8]. In the long bones of Opg gene knockout (Opg-KO) mice, highly porous bone was identified by histological and micro-CT analysis. The increased number of active osteoclasts in the bone-remodeling ratio could explain the Opg-KO bone phenotypes [9].

Interestingly, in our recent study, we found that a number of adipocytes accumulated in the bone marrow of 12-week-old Opg-KO mice, much earlier than adipogenesis occurs in the bone marrow of WT mice. The marrow adipogenesis shifted to an earlier stage after the deletion of Opg. Usually, some adipocytes are observed in the bone marrow of six-month-old or older mice. In addition, when the Opg-KO mice aged, adipocytes accumulated in the bone marrow, especially in the trabecular bone remodeling area. In this study, we aimed to identify the original source of bone marrow adipocytes and the mechanism for producing them.

Materials and Methods

Animal models

Opg-KO mice and WT mice were obtained from the Shanghai Biomodel Organism Science & Technology Development Co., Ltd. (Shanghai, China). The generation and characterization of Opg-KO mice were described in a previous publication [9]. All animal experimental procedures followed the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publication No. 8023, revised 1978). This study was approved by the Ethics Committee of Tongji University (2014-NSFC003). WT and Opg-KO mice were housed in a temperature-controlled facility with a 12 h light/dark cycle.

Genotyping

Genomic DNA was obtained from the tails of WT and Opg-KO mice. Tissue was placed in 80 μl Buffer A (25 mM NaOH, 0.2 mM EDTA, pH 8.0) and dissolved at 97°C for 1 h. Next, 80 μl Buffer B (40 mM Tris-Hd, pH 8.0) was added and mixed well. After centrifugation for 3 min at 4,000 rpm, 1 μl supernatant was taken for genotyping. The PCR reaction components included 12.5 μl PCR mix (Lifefeng, Shanghai), 1 μl primer OPGJW (5’ - GTAAACGCCCTTCTCACACTACA - 3’), 1 μl primer OPGjD3 (5’ - GTTGGGTTGCTGATGATTAGATA - 3’), 1 μl primer OPGjDS (5’ - ATGGCATTGATGCAGCTGATG - 3’), 1 μl template DNA, and 8.5 μl sterile ddH2O. A reaction with water instead of template DNA was used as the negative control. PCR (Bio-Rad, USA) procedures were as follows: 94°C for 5 min; then 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec (repeated 50 times); and 72°C for 5 min. The products were analyzed by electrophoresis on 2% agarose. The WT band was 580 bp, and the Opg-KO band was 620 bp.
Histological analysis

Mice (female n=3, male n=3) were anesthetized by inhalation of isoflurane (USA) and perfused from the ascending aorta with saline and 4% paraformaldehyde (PFA). Histological analysis was performed as described previously [10]. Three samples were tested for each group. The hind legs were dissected and fixed for an additional 48 h at 4°C. Specimens were washed with phosphate buffered saline (PBS), decalcified in 10% EDTA (pH 7.4) for four weeks at 4°C, dehydrated in a graded series of ethanol, embedded in paraffin and cut into 5 μm sections. Hematoxylin and eosin (H&E) staining was used to evaluate the histological characteristics of 12-week-old and 6-month-old mice. With the 10X objective lens, fields of view were randomly selected in relevant observation areas of the bone marrow (proximal area, distal area, and secondary ossification center). Five views were randomly selected from each observation area; three sections from each mouse were employed; and three mice were included in each group. The sections were analyzed by two pathologists.

BMSC isolation and culture

Three four-week-old Opg-KO and WT male mice were sacrificed by cervical dislocation after inhalation anesthesia. The hind legs were dissected and further washed in phosphate buffer solution (pH 7.4) containing 1% penicillin (10,000 IU) and streptomycin (10,000 μg/ml). Using Dulbecco’s Modified Eagle’s Medium (DMEM)-low with 10% fetal bovine serum (FBS), bone marrow was flushed into 100 mm dishes and washed three to five times by adding complete media and centrifuging for 5 min at 1,000 rpm. The supernatant was removed, and cell number counts were performed using the Cedex XS cell count analyzer (Roche, Switzerland). Cells were then resuspended and seeded at a density of 10^6 cells in a 100 mm dish. The bone marrow cells were maintained at 37°C with 5% CO2. The medium was changed every three days. Cell passage after 90% confluence was achieved by treating cells with 0.25% trypsin/EDTA at 37°C. After three passages, the cells were utilized for cell proliferation and differentiation experiments. Cells of the third passage were stained with individual antibodies, including anti-mouse PE-CD45, PE-CD34, PE-Scal, and PE-CD29 (eBioscence, USA), at 4°C for 30 min. The corresponding mouse PE-ISO and the PBS served as the negative controls. The cells were suspended in 400 μl PBS and analyzed by flow cytometry (BD, USA). The BMSCs of both WT and Opg-KO mice were positive for Scal and CD29 and negative for CD45 and CD34. BMSC proliferation was assessed using Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) assays. WT and Opg-KO BMSCs at the third passage were collected and diluted to 20,000 cells/ml. Next, 100 μl cell suspension was plated in individual wells (n=6) of 96-well plates. After the cells were cultured for 1, 2, 3, 4, or 5 days, 10 μl of CCK-8 solution was added to the culture medium in each well. After incubation for one hour at 37°C, the optical density of each well was measured with an infinite M200 reader (Tecan, Australia) at 450 nm.

Osteogenic and adipogenic differentiation of BMSCs

The third passage BMSCs were seeded at a density of 5x10^4 cells in 24-well plates and cultivated in DMEM-low culture solution containing 50 μg/ml ascorbic acid, 5 mM β-glycerophosphate, and 10 nM dexamethasone (Sigma, USA) for the osteogenesis assay or containing adipogenesis differentiation supplements (Gibco, USA) for the adipogenesis assay. After 21 days of osteogenic induction, cells were used in ALP staining, Alizarin Red staining, von Kossa staining, immunofluorescence, RT-qPCR, and Western immunoblotting. After adipogenic induction, cells were used for Oil Red O staining and RT-qPCR.

Real-time quantitative PCR analysis

To assess the expression levels of osteogenesis and adipogenesis related markers, real-time quantitative PCR (RT-qPCR) was performed (listed in Table 1). Total RNA was extracted using TRizol® reagent (Invitrogen, USA). First-strand cDNA was synthesized from 1 μg total RNA using the Transcription First Strand cDNA Synthesis Kit (Roche, Switzerland) in a final volume of 20 μl with oligoDT primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the housekeeping gene. The expression of the target genes was evaluated with the Fast Start Essential DNA Green Master Kit (Roche, Switzerland) using a LightCycler®96 Instrument (Roche, USA). All reactions were performed in a total volume of 20 μl containing 1 μl cDNA. The thermocycler conditions were as follows: preincubation at 95°C for 600 sec; 45 cycles of 95°C for 10 sec, 60°C for 10 sec and 72°C for 20 sec; melting at 95°C for 10 sec; 65°C for 60 sec; and 97°C for 1 sec. Each reaction was run in triplicate and repeated three times. The primers (listed in Table 1) were designed and synthesized by Invitrogen (Shanghai, China). The relative mRNA expression level of the genes was calculated using 2^{-ΔΔCt}. 
Western immunoblotting analysis

First, 10⁶ cells were washed with PBS and lysed for 30 min at 4°C with lysis buffer as previously described. Electrophoresis of equal protein amounts under denaturing conditions was then performed on a 10% SDS-PAGE gel. The proteins were transferred to a PVDF membrane (Millipore, USA) at 4°C. The membrane was washed three times with TBST (0.1% Tween-20 in TBS, pH 7.4) and blocked for 1 h with 5% skim milk in TBST. Subsequently, membranes were probed at 4°C overnight with rabbit-anti-OPG (Abcam, USA, 1:1000), mouse-anti-PPAR-γ (Santa Cruz, USA, 1:1000), or mouse-anti-BSP (1:1000). After three washes, the blots were incubated with the secondary antibody, HRP-goat-anti-rabbit IgG or HRP-goat-at-anti-mouse IgG (Biotechwell, China), at a dilution of 1:2000 for 1 h at room temperature. The blots were incubated with the chemiluminescent substrate ECL (Millipore, USA) and visualized by the Smart Chem TM Image Analysis System (Sagecreation, China). For normalization purposes, the same blot was also probed with mouse-anti-rabbit β-actin antibody at 1:1000 (Abcam, USA).

Immunofluorescence staining

Cells were rinsed with PBS, fixed in 4% PFA, incubated in 0.25% Triton X-100, and then blocked in 5% BSA. Next, the proper antibody or IgG negative control was incubated with the cells overnight at 4°C. For the anti-OPG, anti-PPAR-γ, and anti-BSP antibodies, the dilution ratio for immunostaining was the same as that for Western immunoblotting. After three rinses, cells were incubated with the secondary antibody, including FITC-conjugated anti-rabbit IgG for OPG, CY3-conjugated anti-mouse IgG for PPAR-γ and BSP (Abcam, 1:1000). They were then incubated in 4% DAPI. Images were photographed with an inverted fluorescence microscope (Nikon, Japan).

ALP staining, Alizarin red S staining, and von Kossa assay

After 21 days of osteogenic induction, culture plates were rinsed once with PBS and fixed with 4% PFA for 30 min. To measure the ALP activity of WT and Opg-KO BMSCs, the appropriate culture plates were stained with BCIP/NBT (Beyotime, China) alkaline phosphatase (ALP) liquid for 30 min and rinsed three times with distilled water. The ALP⁺ nodules on three different images were counted and plotted. For Alizarin red S staining, the cells were stained with 2% Alizarin red solution for 15 min and rinsed three times with distilled water. In addition, cell culture dishes were incubated with von Kossa staining solution (GenMed Scientifics Inc, USA) for 1 h and rinsed three times. Then, images were obtained using an inverted microscope (Nikon, Japan). For Alizarin red S staining and the von Kossa assay, three different images were selected to quantify the calcium deposition area and to calculate the calcified area/total area ratio.
Oil Red O staining

After adipogenic induction, culture plates were fixed with 4% PFA. Then, the cells were incubated with Oil Red O staining solution (Sigma, USA) for 20 min and rinsed three times, and images were captured using a Nikon Ti inverted microscope (Nikon, Japan). Three different images were taken to quantify the number of lipid droplets.

The role of OPG in the adipogenic and osteogenic differentiation of BMSCs

To investigate the role of OPG in the adipogenic differentiation of BMSCs, 50 ng/ml OPG protein was added to the culture medium of WT and Opg-KO BMSCs (n = 3). After 7 days or 21 days of adipogenic induction, the cells were stained with Oil Red. By using RT-qPCR, the expression of Ppar-γ was compared in WT and Opg-KO BMSCs after 21 days of adipogenic induction. To investigate the role of OPG in the osteogenic differentiation of BMSCs, 50 ng/ml OPG protein was added to the culture medium of the WT and Opg-KO BMSCs. After 3 days, 7 days, 14 days, or 21 days of osteogenic induction, the cells were stained with Alizarin Red. Three different images were selected to quantify the ratio of the calcified area to the total area.

Statistical analysis

For both the animals and cell samples used in this study, triplicate samples were employed. Student’s T-tests were used to compare differences between the WT control and the Opg-KO groups. P < 0.05 was considered significant. All analyses were performed using SPSS20.0 and GraphPad software.

Results

More adipocytes were identified in the bone marrow of Opg-KO mice compared to WT mice

An obvious bone phenotype in the Opg-KO mice was the porous cortical bone. The cortical bone was thick due to the rapid remodeling of the bone matrix. In addition to the bone morphological changes, there were many more adipocytes in the bone marrow of the Opg-KO mice compared to the WT mice, and the adipocytes appeared much earlier than in the WT mice (Figs 1A & 1B). Starting from 12 weeks of age, a number of adipocytes were observed in the secondary ossification center and the proximal and distal regions of the Opg-KO femur (Figs 1C & 1D). The black arrows indicate the adipocytes in the WT and Opg-KO bone marrow. The differences between the Opg-KO and WT fat cell numbers at six months were even greater than those at younger ages (Figs 1C & 1D). The statistical analysis revealed that the biggest differences in adipocyte numbers appeared in the secondary ossification center at 12 weeks of age (Fig. 1E). In contrast, at six months, the biggest difference was observed in the proximal area (Fig. 1F). Based on RNA extracted from bone marrow, the level of Alp in 8-week-old or 1-year-old WT mice was higher than that in Opg-KO mice (Fig. 1G), and the WT Ppar-γ level was lower than that in Opg-KO mice (Fig. 1H). In other words, Opg-KO bone marrow cells could produce more Ppar-γ but less Alp. These data suggested that the bone marrow accumulated more adipocytes at earlier ages after the deletion of Opg. In addition, age-related changes of bone marrow OPG/PAR-γ were observed. Interestingly, in the WT bone marrow, expression of Opg decreased with age, and expression of Ppar-γ increased (Fig. 1I).

Expression of BMSC differentiation markers in WT and Opg-KO mice BMSCs

Using multiple analyses (RT-qPCR, immunofluorescent assays, and Western immunoblotting), OPG was identified in WT BMSCs but not in Opg-KO BMSCs (Figs 2A & 2B). There was no significant difference in cell proliferation between WT and Opg-KO mice (Figs 2C & 2D). Compared with WT mice, the Opg-KO BMSCs produced more Ppar-γ (Figs 2E & 2F) but fewer bone formation markers, including Alp and Runx2 (Figs 2G & 2H). Thus, compared with WT BMSCs, the Opg-KO BMSCs had a different differentiation tendency.
Changes of osteogenic differentiation properties in Opg-KO BMMSCs

After inducing osteogenic differentiation for 21 days, the ALP activity of Opg-KO BMMSCs was lower than that of WT BMMSCs based on ALP staining (Fig. 3A). The calcification rate of Opg-KO BMMSCs was slower than that of WT BMMSCs, as determined by Alizarin-red S staining (Fig. 3B) and von Kossa staining (Fig. 3C). Furthermore, compared with WT BMMSCs, the mRNA expression levels of the osteogenic transcription factors Alp, Runx2, and Osterix were much lower in Opg-KO BMMSCs (Fig. 3D). Compared with the WT control, the
expression of the mature osteoblast-specific markers *Bsp, Ocn*, and *Dmp1* was lower (Figs. 3E & 3F), but the expression of both the bone mineralization inhibitor *Ocn* and the bone formation inhibitor *Dkk1* was upregulated (Figs. 3E & 3G). Additionally, the expression of the Wnt signaling pathway molecules *Wnt5a, β-catenin*, and *Lrp5* was downregulated in *Opg-KO* mice (Fig. 3G). These data suggest that the osteogenic differentiation abilities of *Opg-KO* BMSCs are weaker than those of WT BMSCs.

**Changes of adipogenic differentiation in Opg-KO BMSCs**

The adipocyte formation rate was tested after adipogenic induction culture for 21 days. Compared to the adipogenic induced WT BMSCs, the number of lipid droplets was increased in *Opg-KO* BMSCs (Fig. 4A). Additionally, compared to the control, the mRNA expression levels of the adipogenic markers *Ppar-γ* and *Ap2* in induced *Opg-KO* BMSCs were upregulated (Fig. 4B). In contrast, the levels of Wnt signaling molecules *Wnt5a, β-catenin*, and *Lrp5* in *Opg-KO* BMSCs were downregulated, and the Wnt inhibitor *Dkk1* was upregulated after 21 days of adipogenic induction (Fig. 4C). Thus, the adipogenic differentiation ability of *Opg-KO* BMSCs was markedly increased.
**Fig. 3.** Osteogenic differentiation of Opg-KO BMSCs. (A-C) The osteogenic differentiation of BMSCs was characterized by ALP staining, Alizarin red S staining and von Kossa staining. (D) The expression of bone formation markers (Opg, Alp, Runx2, and Osteonectin) in osteogenic-induced BMSCs was compared by RT-qPCR. (E) The expression of bone mineralization-related markers (Bsp, Ocn, Dmp1, and Opn) was compared by RT-qPCR. (F) BSP protein expression was determined by immunofluorescence staining and Western immunoblotting. (G) The expression of WNT signaling related factors (Wnt5a, β-catenin, Lrp5, and Dkk1) was compared by RT-qPCR. Error bars indicate the mean ± SEM, n=3. *P<0.05; **P<0.01; ***P<0.005.

**OPG inhibits the adipogenic differentiation and promotes the osteogenic differentiation of BMSCs**

BMSCs cultured with or without OPG show different adipogenic differentiation characters. With added ectogenic OPG, the adipogenic differentiation of BMSCs was inhibited at 7 days and 21 days (Fig. 5A). Compared with the untreated groups, the expression levels of Ppar-γ decreased in both WT and Opg-KO BMSCs treated with OPG (Fig. 5B). However, the calcification levels in both cultured WT and Opg-KO BMSCs were increased after osteogenic differentiation with ectogenic OPG (Fig. 5C).

**Discussion**

In the aging bone marrow, adipogenesis is mainly caused by an inversion of BMSCs from osteogenic to adipogenic differentiation [11-13]. For osteoporosis patients, the
**Fig. 4.** Adipogenic differentiation of Opg-KO BMMSCs. (A) Adipogenesis was determined by Oil Red O staining. The number of lipid droplets was quantified and plotted as shown. (B) The expression of Ppar-γ and Ap2 was compared by RT-qPCR. (C) The expression of WNT signaling molecules (Wnt5a, β-catenin, Lrp5, and Dkk1) was compared by RT-qPCR. The scale bar represents 100 μm. Error bars indicate the mean ± SEM, n=3. *P<0.05; **P<0.01.

**Fig. 5.** The role of the ectogenic OPG in the adipogenic and osteogenic differentiation of BMSCs. (A) OPG protein inhibited the adipogenic differentiation of WT and Opg-KO BMMSCs with or without OPG treatment. (B) The expression levels of Ppar-γ in WT and Opg-KO BMMSCs after 21 days of adipogenic induction were compared by using RT-qPCR. (C) Osteogenic differentiation of BMMSCs was tested by Alizarin red S staining after 3 days, 7 days, 14 days and 21 days of induction. The scale bar represents 100 μm. Error bars indicate the mean ± SEM, n=3, *P<0.05; **P<0.01.

Volume of bone marrow adipose tissue is higher than normal [14]. Thus, bone marrow fat could be used as a marker of bone aging [15]. Recently, in addition to filling the marrow space and energy storage, new functions of marrow adipocytes have been revealed, including inducing adipogenic differentiation and supporting osteoclast formation [16-18]. In bone marrow, adipocytes could produce key adipokines,
including leptin and adiponectin [19, 20]. Leptin can inhibit the adipogenic differentiation of BMSCs [21, 22], and it regulates bone absorption [23]. Adiponectin stimulates osteoblast differentiation via MAPK, BMP and COX2 signaling [24–27]. Moreover, bone marrow adipocytes also regulate the hematopoietic microenvironment [28]. Thus, the mechanism of adipocyte generation in bone marrow should be thoroughly studied.

In this study, a close relationship between bone marrow adipogenesis and OPG was identified. Based on our investigation, it is difficult to see significant marrow adipogenesis in WT mice younger than 12 weeks of age. In WT mice six months old and older, the number of adipocytes increases, and some of the cell bodies become larger. Interestingly, adipocyte formation is accelerated in Opg-KO marrow. Adipocytes were observed in the bone marrow cavity at approximately 12 weeks in Opg-KO mice, which is much earlier than in WT mice. In vitro studies also showed that Opg-KO BMSCs could be induced to form adipocytes faster than WT BMSCs, but they were more difficult to induce into osteoblasts. In addition, exogenous OPG protein could inhibit adipogenic differentiation and promote osteogenic differentiation in both WT and Opg-KO BMSCs in vitro. Therefore, the increased numbers of adipocytes in Opg-KO bone marrow are related to the abnormal differentiation of BMSCs with Opg ablation.

The adipogenic determination of BMSCs includes specific pathways (Wnt, Hh, Notch, etc) and related transcription factors [29, 30]. Among them, PPAR-γ is a key transcription factor for adipogenic signaling, and it can facilitate the differentiation and maturation of adipocytes and regulate energy metabolism [31–34]. The suppression of PPAR-γ transactivation switches the differentiation of bone marrow stem cells from adipocytes to osteoblasts [35–37]. In Opg-KO mice, the expression of PPAR-γ is upregulated in BMSCs, and adipogenesis is notably accelerated. We believe that the upregulation of PPAR-γ in Opg-KO BMSCs is the direct reason for the abnormal adipogenesis in Opg-KO marrow. In addition, in the marrow of aged WT mice, the expression of PPAR-γ is upregulated, and the expression of OPG is downregulated. This information reminds us that there might be a direct or indirect relationship between OPG and PPAR-γ in BMSCs.

Further questions include the following: (1) how does OPG regulate BMSC differentiation and which pathways are involved; (2) what are the specific roles of adipocytes in regulating bone remodeling and the microenvironment of bone marrow? Answering these questions could help us explain the relationship between bone remodeling and adipogenesis in the bone marrow environment [38–40].

**Abbreviations**

BMSC (bone marrow mesenchymal stem cells); OPG (osteoprotegerin); TNFR (tumor necrosis factor receptor); Opg-KO (Osteoprotegerin knockout); WT (wild-type); Ppar-γ (peroxisome proliferator-activated receptor γ).

**Acknowledgements**

This investigation was supported by NSFC (81300840, 81470715 SY; 81370971 WXG); Guangdong NSF for Distinguished Young Scholar (S2013050013880, 2013072120024, SY; 2016YFC102705, SY; 2013M530213, SY; Xyq2013080; NSFC 81371141, 81570966 2Q.

**References**


34 Lecka-Czernik B: Marrow fat metabolism is linked to the systemic energy metabolism. Bone 2011;50:534-539.


