Low-density lipoprotein receptor deficiency impaired mice osteoblastogenesis in vitro

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Summary

Postmenopausal osteoporosis affected most elderly women with co-existence of lipid and bone metabolism disorders. However, the cellular and molecular mechanisms underlying the parallel progression and cross-talk of these systems remained unclear. In the present study, low-density lipoprotein receptor knockout (LDLR⁻⁾ mice were chosen to elucidate the effect of LDLR in regulating the differentiation of osteoblasts, which were responsible for bone formation and modulation of osteoclastogenesis. Primary osteoblasts were isolated from the calvarium of newborn LDLR⁻⁾ or wild-type mice followed by osteoblastic differentiation culture in vitro. Alkaline phosphatase activity was significantly decreased in LDLR⁻⁾ osteoblasts compared to wild-type controls, combined with calcium deposit formation delay, implying impaired osteoblastogenesis in vitro. Consistent with these findings, the expression of runt-related transcription factor 2 (Runx2) was decreased 3 days after differentiation in LDLR⁻⁾ osteoblasts compared to wild-type controls. Moreover, the expression of Osterix was decreased 7 days after differentiation in LDLR⁻⁾ osteoblasts compared to wild-type controls, later than Runx2. However, the osteoclastogenesis modulation role of osteoblasts was unaffected by the LDLR deficiency, evidenced by the same level of osteoprotegerin (OPG)/receptor activator of nuclear factor-κ B ligand (RANKL) axis between LDLR⁻⁾ and wild-type control osteoblasts. Our results provide a novel insight into the role of LDLR during osteoblastic differentiation and improve understanding of cross-talk between bone and lipid metabolisms.

Keywords: Postmenopausal osteoporosis, low-density lipoprotein receptor, osteoblast, osteoblastogenesis, alkaline phosphatase, runt-related transcription factor 2, Osterix

1. Introduction

Postmenopausal osteoporosis (PMO) affected most elderly women due to estrogen decline. Several epidemiological studies demonstrated the co-existence of lipid and bone metabolism disorders in postmenopausal females (1-3). Recent studies indicated a positive connection between cardiovascular disease and risk of osteoporosis, because high cholesterol levels and high low-density lipoprotein (LDL)-cholesterol levels had been described to correlate with low bone mineral density in PMO patients (4-7). Furthermore, the increased serum LDL-cholesterol level was indicated to be an index affecting the presence of prevalent non-vertebral fractures in postmenopausal females (8). Additionally, menopausal hormone therapy exerted benefit effects of reducing the risk of atherosclerosis (9-11). Therefore, hyperlipidemia may play an essential role in regulating osteoporosis progress.

Osteoporosis, a systemic metabolic skeletal disorder characterized by low bone mass and increased risk of fractures, is a consequence of bone turnover disruption caused by imbalance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Pre-
osteoblasts were derived from pluripotent mesenchymal stem cells (MSCs), which were also the progenitors of adipocytes and chondrocytes (12). Osteoblastogenesis could be defined by four sequential stages: lineage commitment, proliferation, extracellular matrix (ECM) synthesis, and matrix mineralization (13). Runt-related transcription factor 2 (Runx2) was the master transcription regulator of osteoblast lineage commitment that inhibited MSCs from differentiating into the adipocytic lineages (14). Null mutation of Runx2 in mice resulted in a cartilaginous skeleton with complete lack of osteoblasts (15-17). Runx2 regulated osteoblast-related molecules such as Osterix, type I collagen and alkaline phosphatase (ALP). Osterix was the second transcription factor for proliferative expansion of immature osteoblasts, and the expression of Osterix was absent in Runx2 null mice but Runx2 was expressed in Osterix null mice (18,19). Type I collagen was one of the major osteoblast-specific matrix proteins expressed by fully differentiated osteoblasts after complete mitosis, essential for both bone matrix synthesis and later mineralization (20,21). Moreover, osteoblasts took part in the regulation of osteoclastogenesis and bone resorption by producing receptor activator of nuclear factor-κB ligand (RANKL), the ligand of RANK present in osteoclast precursor cells, and its decoy receptor osteoprotegerin (OPG) (22). Precise regulation of osteoclastogenesis and osteostegastogenesis was required for the maintenance of normal bone metabolism.

Several basic studies had established that oxidized-LDL inhibited osteoblastic differentiation and promoted adipocytic differentiation of MSCs in vitro (23,24). Moreover, oxidized-LDL increased RANKL expression and secretion in human osteoblast-like cells (24-26), indicating cross-talk between lipid and bone metabolism systems. LDL was transferred in particle form and internalized by binding to the receptor LDL receptor (LDLR) residing on the surface of liver cells (27). LDLR was the prototype member of the LDLR family that contained several key regulators involved in osteoblastic development. For example, human osteoblasts expressed low-density lipoprotein receptor-related protein (LRP) 1 with a capacity for osteocalcin carboxylation (28). LRP4, LRP5/6, and LRP8 were involved in the maintenance of bone (29-36). However, the role of LDLR in bone metabolism was unclear with controversial results in recent studies. LDLR deficient familial hypercholesterolemia patients showed normal bone mineral density at the femora neck (37). In an animal experiment, LDLR deficiency caused ectopic bone formation in an experimental osteoarthritis mouse model (38). Furthermore, Okayasu et al. showed that LDLR deficiency induced impaired pre-osteoclast fusion in vitro while increasing bone mass in LDLR deficient mice (39). However, decreased bone mass was detected in LDLR deficient mice with inhibition of osteoblastogenesis from bone marrow cells and enhanced osteoclastogenesis in vitro (40), indicating that LDLR might regulate bone metabolism in a complex manner.

Previous work from our group showed that most of the LDLR family members were up-regulated during osteoblastic differentiation in vitro, except for LDLR, which was down-regulated in the late stages of pre-osteoblast mineralization culture, indicating a unique role of LDLR in osteostegastogenesis (41). The function of LDLR in regulating osteoblastic development and related mechanisms remained to be further explored. In the present study, we made use of LDLR deficient mice and pre-osteoblasts in vitro culture system to explore the effect of LDLR deficiency on this process.

2. Materials and Methods

2.1. Mice

LDLR knockout (LDLR<sup>−/−</sup>) mouse strain (B6.129S7-Ldlrtm1Her/J) was purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China), originally from the Jackson Laboratory. Data obtained from C57BL/6 wild-type (WT) mice purchased from the Laboratory Animal Facility of Chinese Academy of Science (Shanghai, China) were used as controls. After being habituated to housing conditions for 3 days, mice were mated (one male with two females per cage) under standard housing conditions at 24°C on a reversed 12-12 hour light-dark cycle. Standard rodent food and water were provided ad libitum at room temperature. Primary osteoblasts were isolated from the calvarium of newborn mice. Experimental animal housing and handling were conducted in accordance with guidelines for the care and use of laboratory animals at Fudan University, Shanghai, China and in conformity with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Chemicals and reagents

Serum and phenol red-free minimal essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA). Dexamethasone, β-glycerophosphate disodium salt hydrate, ascorbic acid, and collagenase were obtained from Sigma-Aldrich Co (Saint Louis, MO, USA). Dispase was supplied by Hoffmann-La Roche Ltd (Basel, Switzerland). RNAiso Plus, PrimeScript<sup>TM</sup> RT Master Mix (Perfect Real Time) Kit and SYBR Premix Ex Taq II (Tli RNaseH Plus) Kit were obtained from TaKaRa biotechnology (Otsu, Japan). 2×GC rich PCR MasterMix was purchased from Tiangen Biotech (Beijing) Co., Ltd (Beijing, China). BCIP/NBT Alkaline Phosphatase Color Development Kit, Alkaline Phosphatase Assay Kit and penicillin-streptomycin were obtained from the Beyotime...
Institute of Biotechnology (Shanghai, China). Alizarin Staining kit was supplied by Genmed (Shanghai, China). All primers were synthesized by Shanghai Shenggong Company (Shanghai, China).

2.3. Genomic DNA isolation and genotyping

Genotypes of LDLR<sup>-/-</sup> mice were confirmed by polymerase chain reaction (PCR) analyses of genomic DNA. Generally, tissue samples were collected and incubated in 50mM NaOH at 95°C for 15 min. Then 1/10 of total volume 1M Tris HCl (PH = 8.0) was added and mixed well. After centrifugation at 12,000g for 15 min at room temperature, the supernatant was used for further PCR analyses with 2×GC rich PCR MasterMix. Touchdown PCR reaction was performed using the following conditions according to the protocol from the Jackson Laboratory website: 94°C for 2 min, followed by 10 cycles of 94°C for 20 sec, annealing temperature for 15 sec decreased 0.5°C every second cycle from 65°C, 68°C for 10 sec, then another 28 cycles of 94°C for 15 sec, 60°C for 15 sec, and 72°C for 10 sec, and finally an additional step of 72°C for 2 min before the end of the program. Primer mixture was made of 3 primers and the sequences are listed in Table 1. PCR product was electrophoresed on 1.5% low melting point agarose gel. The homozygote mutant showed a product of 179bp length with 351 bp for wild-type. The heterozygote mouse strain showed two separated bands of 179bp and 351bp length (Figure 1).

2.4. Primary osteoblasts isolation

Primary osteoblasts isolated from the calvarium of newborn mice were separated two days before the Osteogenic Induction Media treatment as described previously (41). Briefly, skull bones were extracted and digested in α-MEM medium with 0.1% collagenase and 0.2% dispase for 10 min at 94°C vortexed at 180 rpm, and the supernatant was discarded, then the digestion treatment was repeated 4 more times. Cells from the second to fifth digestion fraction were collected and resuspended in Growth Medium, which were supplemented with 10% serum, 10 units/mL penicillin and 10 μg/mL streptomycin in α-MEM medium without phenol red. 1 × 10<sup>5</sup> cells were sowed into each well of 24-well cell culture plates. The isolated primary osteoblasts were treated with Osteogenic Induction Media containing 10% serum, 20 mM ascorbic acid, 1M β-glycerophosphate disodium salt hydrate, and 1 mM dexamethasone in phenol red-free α-MEM at 37°C and 5% CO<sub>2</sub>, after reaching 80% confluence cultured in Growth Medium (42). Medium was refreshed once every two days. The differentiated osteoblasts were analyzed at specific time points in the following experiments.

2.5. Osteoblastic mineralization culture

Mineralization of osteoblasts is the process during which differentiated osteoblasts are induced to produce extracellular calcium deposits in vitro. In the osteoblastic mineralization culture system, primary osteoblasts were treated with Osteogenic Induction Media containing 10% serum, 20 mM ascorbic acid, 1M β-glycerophosphate disodium salt hydrate, and 1 mM dexamethasone in phenol red-free α-MEM at 37°C and 5% CO<sub>2</sub>, after reaching 80% confluence cultured in Growth Medium (42). Medium was refreshed once every two days. The differentiated osteoblasts were analyzed at specific time points in the following experiments.

2.6. RNA isolation and quantitative real-time PCR

Total RNA was isolated from primary osteoblasts in 6-well cell culture plates using the RNAiso Plus according to the manufacturer’s instruction at specific time points after Osteogenic Induction Media treatment. Briefly, media was removed and 1 mL of RNAiso Plus was added into the well to lyse the cells. After homogenization by pipetting up and down, 0.2 mL chloroform per 1mL of RNAiso Plus was added to the mixture and mixed thoroughly. After incubation at room temperature for 15 min, the mixture was centrifuged for 15 min at 12,000 g at 4°C. The upper aqueous phase containing RNA was transferred to a clean tube carefully and precipitated by adding an equal volume 100% isopropanol. The RNA was concentrated by centrifugation at 12,000 g at 4°C for 10 min and washed with 75% ethanol. Then the RNA pellet was air-dried.
for 5 min at room temperature and dissolved in RNase-free water. The reverse transcription reaction was conducted immediately after RNA isolation in a final volume of 10 μL containing 500 ng RNA sample, and 2 μL 5×PrimeScript™ RT Master Mix (Perfect Real Time) according to the protocol from the reagent kit. Afterwards, quantitative real-time PCR was performed in a 20 μL mixture of 2 μL reverse transcription product, 10 μL 2×SYBR Premix Ex Taq II (Tli RNaseH Plus), 1.6 μL forward/reverse primer mixture for a final concentration of 0.4 μM and DNase free water up to the final volume. The reverse transcription reaction was conducted immediately after RNA isolation in a final volume of 10 μL containing 500 ng RNA sample, and 2 μL 5×PrimeScript™ RT Master Mix (Perfect Real Time) according to the protocol from the reagent kit. Afterwards, quantitative real-time PCR was performed in a 20 μL mixture of 2 μL reverse transcription product, 10 μL 2×SYBR Premix Ex Taq II (Tli RNaseH Plus), 1.6 μL forward/reverse primer mixture for a final concentration of 0.4 μM and DNase free water up to the final volume. The reverse transcription reaction was conducted immediately after RNA isolation in a final volume of 10 μL containing 500 ng RNA sample, and 2 μL 5×PrimeScript™ RT Master Mix (Perfect Real Time) according to the protocol from the reagent kit. Afterwards, quantitative real-time PCR was performed in a 20 μL mixture of 2 μL reverse transcription product, 10 μL 2×SYBR Premix Ex Taq II (Tli RNaseH Plus), 1.6 μL forward/reverse primer mixture for a final concentration of 0.4 μM and DNase free water up to the final volume. The reverse transcription reaction was conducted immediately after RNA isolation in a final volume of 10 μL containing 500 ng RNA sample, and 2 μL 5×PrimeScript™ RT Master Mix (Perfect Real Time) according to the protocol from the reagent kit. Afterwards, quantitative real-time PCR was performed in a 20 μL mixture of 2 μL reverse transcription product, 10 μL 2×SYBR Premix Ex Taq II (Tli RNaseH Plus), 1.6 μL forward/reverse primer mixture for a final concentration of 0.4 μM and DNase free water up to the final volume. The reverse transcription reaction was conducted immediately after RNA isolation in a final volume of 10 μL containing 500 ng RNA sample, and 2 μL 5×PrimeScript™ RT Master Mix (Perfect Real Time) according to the protocol from the reagent kit. Afterwards, quantitative real-time PCR was performed in a 20 μL mixture of 2 μL reverse transcription product, 10 μL 2×SYBR Premix Ex Taq II (Tli RNaseH Plus), 1.6 μL forward/reverse primer mixture for a final concentration of 0.4 μM and DNase free water up to the final volume. The reverse transcription reaction was conducted immediately after RNA isolation in a final volume of 10 μL containing 500 ng RNA sample, and 2 μL 5×PrimeScript™ RT Master Mix (Perfect Real Time) according to the protocol from the reagent kit. Afterwards, quantitative real-time PCR was performed in a 20 μL mixture of 2 μL reverse transcription product, 10 μL 2×SYBR Premix Ex Taq II (Tli RNaseH Plus), 1.6 μL forward/reverse primer mixture for a final concentration of 0.4 μM and DNase free water up to the final volume.

2.7. Alizarin Red S staining

The deposited calcium deposits of differentiated primary osteoblasts were stained using Alizarin Red S according to the manufacturer's instructions as follows. After 7 days of Osteogenic Induction Media treatment in 24-well plates, cells were washed with 500 μL Cleaning Buffer and fixed with Fixation Buffer for 10 min at room temperature, then washed with Cleaning Buffer again. After fixation, 500 μL Alizarin Red S Staining Buffer was added to each well and the plate was incubated in the dark for 10 min at room temperature. The Staining Buffer was removed carefully when mineralized osteoblasts appeared bright orange-red while undifferentiated cells were slightly red or colorless. The plate was air-dried, and results were viewed by a HP scanner and recorded.

2.8. ALP staining and activity analyses

The ALP activity was greatly enhanced during osteoblastic differentiation in vitro. BCIP/NBT is the preferred staining substrate for ALP detection. After 7 days of Osteogenic Induction Media treatment in 24-well plates, cells were washed with 500 μL PBS and fixed with 4% paraformaldehyde for 20 min at room temperature, then washed with 500 μL PBS three times. The fixed cells were incubated in BCIP/NBT buffer mixed according to the protocol from the kit: 3 mL ALP Staining Buffer, 10 μL 300×BCIP Buffer and 20 μL 150×NBT Buffer for more than 30 min at room temperature avoiding light until the ALP positive differentiated osteoblasts appeared blue-violet. The reaction was stopped by adding excess deionized water. The results were visualized by a HP scanner and recorded. ALP activity in cell lysate was quantitated by using an Alkaline Phosphatase Assay kit according to the manufacturer's protocol. Absorbance was measured at 405 nm.

2.9. Statistical analyses

All values were presented as the mean ± SEM. Data differences were assessed by student’s t-test with the aid of IBM SPSS software. A p value less than 0.05 was accepted as statistically significant. All experiments were repeated more than three times.

3. Results

3.1. The LDLR deficiency inhibited the ALP activity in differentiated primary osteoblasts

Data from our previous study showed that the expression of LDLR decreased during osteoblastic differentiation (41). To gain an insight into the role of LDLR in osteoblastic differentiation, we first examined the effect of LDLR deficiency on ALP activity of differentiated primary osteoblasts 7 days after Osteogenic Induction Media treatment. ALP staining showed that the ALP activity in LDLR-/- osteoblasts obviously decreased compared to osteoblasts from wild-type newborn mice (Figure 2A). The results demonstrated by ALP activity analyses showed that the ALP activity dramatically decreased in the LDLR-/- osteoblast cell lysate compared to wild-type controls (Figure 2B, p < 0.001), suggesting that LDLR might play a critical role in osteoblastic differentiation.

3.2. The LDLR deficiency inhibited mineralization of differentiated primary osteoblasts

To further test the effect of LDLR deficiency on mineralized matrix formation in osteoblasts, Alizarin Red S (ARS) staining was performed 7 days after Osteogenic Induction Media treatment. As shown in Figure 3A, calcium deposit formation labeled by ARS staining was impaired by LDLR deficiency. A marked difference in

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Table 2. Summary of oligonucleotide primers for cDNA amplification

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin forward primer</td>
<td>CCTCTATGCACACACAGT</td>
</tr>
<tr>
<td>β-actin reverse primer</td>
<td>AGCCACACATCACACAG</td>
</tr>
<tr>
<td>Col1a1 forward primer</td>
<td>TGACTGGAAGAGGGAGAGTA</td>
</tr>
<tr>
<td>Col1a1 reverse primer</td>
<td>GACGGCTAGTGGGAGAAC</td>
</tr>
<tr>
<td>OPG forward primer</td>
<td>CTCCTGCCGGCCTCTCTTAT</td>
</tr>
<tr>
<td>OPG reverse primer</td>
<td>CCCTCTTCTCTACTCAC</td>
</tr>
<tr>
<td>Otx forward primer</td>
<td>GCTGCTAATTGTATCTTCG</td>
</tr>
<tr>
<td>Otx reverse primer</td>
<td>CTATGTAGCTGCTAAACAGA</td>
</tr>
<tr>
<td>RANKL forward primer</td>
<td>CAAGATGGCTTATACCTGT</td>
</tr>
<tr>
<td>RANKL reverse primer</td>
<td>TTGAAGCTGGTTAACGAC</td>
</tr>
<tr>
<td>Runx2 forward primer</td>
<td>GACAGTCACAACTTCTG</td>
</tr>
<tr>
<td>Runx2 reverse primer</td>
<td>GCGGAGTATCTCATTAC</td>
</tr>
</tbody>
</table>

Col1a1: collagen type I alpha 1; OPG: osteoprotegerin; Otx: Osterix; RANKL: receptor activator of nuclear factor-κ B ligand; Runx2: runt-related transcription factor 2
mineralization level was also observed when comparing the number of mineralized nodules per well between the LDLR−/− and wild-type groups (Figure 3B, *p < 0.05), indicating that LDLR might be involved in the regulation of osteoblast mineralization.

3.3. The LDLR deficiency decreased Runx2 and Osterix expression in differentiated primary osteoblasts

All the results above showed a potential role of LDLR in regulating osteoblastic differentiation. Runx2 was the master transcription factor and regulated a series of osteoblast-related gene expressions. We therefore inspected the mRNA level of Runx2. In the wild-type group, Runx2 expression was normal at day 3 of Osteogenic Induction Media treatment and dramatically increased at day 7 compared to day 0 (Figure 4A, *p = 0.333, *p < 0.01). In LDLR−− osteoblasts, Runx2 expression decreased at day 3 of Osteogenic Induction Media treatment and increased at day 7 compared to day 0 (Figure 4A, *p < 0.05, *p < 0.001). When compared to the wild-type group, the Runx2 mRNA level was slightly reduced in LDLR−− osteoblasts at day 0, was significantly impaired at day 3, and slightly reduced again at day 7 (Figure 4A, *p = 0.488, *p < 0.05, *p = 0.306), indicating LDLR might regulate osteoblastic differentiation through the effect on Runx2 expression in the early stages of differentiation in vitro.

Osterix was the second key factor in osteoblastic differentiation regulated down-stream of Runx2. In the wild-type group, Osterix expression increased at day 3 of osteoblastic differentiation and decreased at day 7 compared to day 0 (Figure 4B, *p < 0.001, *p < 0.001). In LDLR−− osteoblasts, the Osterix expression also significantly increased at day 3 and decreased at day 7 compared to day 0 of osteoblastic differentiation (Figure 4B, *p < 0.05, *p < 0.01). Compared to wild-type controls, Osterix expression was normal at day 3 of Osteogenic Induction Media treatment in the LDLR−− osteoblasts, but severely decreased at day 7 (Figure 4B, *p = 0.922, *p < 0.01), suggesting that the effect of LDLR deficiency on Osterix might be later than Runx2 or LDLR might regulate Osterix expression during osteoblastic differentiation through Runx2.

We also analyzed the expression level of functional factor Collagen-1 during osteoblastic differentiation in vitro. Collagen-1 expression significantly increased at day 7 of osteoblastic differentiation compared to day 0 in both LDLR−− and wild-type groups (Figure 4C, *p < 0.001, *p < 0.01) and there was no significant difference at each time point between the two groups (Figure 4C, *p = 0.305, *p = 0.243, *p = 0.074).
The OPG/RANKL level in osteoblast was not affected by LDLR deficiency

Besides the bone formation role, osteoblasts could regulate osteoclastogenesis by expressing OPG and RANKL. We isolated primary osteoblasts to examine the effect of LDLR deficiency on this process. Although the expression of OPG significantly decreased in LDLR⁻/⁻ osteoblasts compared to wild-type controls (Figure 5A, p < 0.01), the expression of RANKL was similar between the two groups (Figure 5B, p = 0.149). Actually, the ratio of OPG/RANKL slightly decreased in LDLR⁻/⁻ osteoblasts compared to wild-type controls with no significance (Figure 5C, p = 0.636), suggesting that the deficiency of LDLR might have no influence on the modulation role of osteoblasts to osteoclastogenesis.

4. Discussion

Several members of the LDLR family had been shown to take part in the regulation of osteoblastogenesis. Loss-of-function mutation in LRPS was associated with decreased bone mass in osteoporosis-pseudoglioma syndrome (OPPG; MIM259770) patients (30), while gain-of-function was associated with increased bone mass in other healthy patients (43,44). Moreover, mutation in LRP4 had been identified with high-bone-mass disorders in patients with sclerosteosis or van Buchem diseases (33,45,46). Our previous study showed that most of the LDLR family members increased during osteoblastic differentiation in vitro, such as LRP4/5/6, but the expression of LDLR decreased 25 days after differentiation in vitro, indicating LDLR might function in a unique way during osteoblastogenesis. To explore the effect of LDLR on osteoblastic development and function, we used a LDLR deficient mouse model and isolated pre-osteoblasts from calvarium of newborn mice. Our results showed that ALP activity of differentiated primary osteoblasts dramatically decreased in the LDLR⁻/⁻ group compared to wild-type controls. The mineralization level was also impaired in the LDLR⁻/⁻ group. By analyzing the expression of key transcriptional factors during osteoblastic differentiation, we confirmed that the expression of Runx2 decreased, followed by impaired expression of Osterix, the direct factor regulated downstream of Runx2, in the LDLR⁻/⁻ group compared to wild-type controls. Our current study indicated that
LDLR might affect the differentiation and function of osteoblasts. Osteoblasts originate from MSCs, which are also progenitors of adipocytes and chondrocytes. The Wnt signaling pathway played an essential role during development of osteoblasts. Activation of the Wnt signaling pathway promotes osteoblastic differentiation and inhibited expression of peroxisome proliferator-activated receptor-(PPAR)-γ. PPAR-γ was commonly considered as the key regulator of adipogenesis, and absence of PPAR-γ caused adipogenesis depletion. LRP5/LRP6 affected osteoblastogenesis by inducing the Wnt signal as co-receptor in the dual receptor complex with frizzled (FZD) (32). LRP4, the third member of the LRP5 and LRP6 subfamily of LDLRs, inhibited the Wnt signaling pathway by facilitating sclerostin action, the antagonist of Wnt signaling pathway (29,36). Dimerization of LRP6 through its LDLR domain induced canonical Wnt signaling pathway (47). LDLR may induce the Wnt signal through the LDLR domain as well. Moreover, LRP6 could regulate LDLR-mediated LDL uptake (48). Is it possible that LDLR could affect the LRP6-mediated Wnt signaling pathway activation in turn? More experiments should be conducted to explore the Wnt signaling pathway activity and PPAR-γ level in the LDLR deficient osteoblasts in vitro and in vivo. The potential that LDLR deficiency might promote development of adipocytes should be further explored.

There had been controversial results in recent studies regarding the role of LDLR in bone metabolism. Okayasu et al. cultured bone marrow cells from LDLR-/- mice and detected reduced osteoclast formation from bone marrow cells in vitro with increased bone mass in these mice compared with wild-type controls. These authors attributed the reduction in osteoclast formation to a defect in cell-cell fusion of preosteoclasts. Furthermore, these authors demonstrated that the LDLR-/- derived preosteoclasts contained less osteoclast fusion regulator molecules such as Atp6v0d2 and DC-STAMP in the plasma membrane than those from wild-type preosteoclasts (39). However, Chen and colleagues detected decreased bone mass in the LDLR-/- mice, which was associated with decreased Runx2 and Collagen-1 expression during osteoblastogenesis and increased TRAP levels during osteoclastogenesis from bone marrow cells in vitro, implying that the decreased bone mass in LDLR-/- mice was associated with decreased osteoblastic function and increased osteoclastic function in these mice (40). In the present study, we isolated pre-osteoblasts from the calvarium of newborn LDLR-/- mice and detected ALP activity, calcium deposit formation, and osteoblastogenesis related factor expression 0, 3, and 7 days after differentiation. The activity of ALP decreased in LDLR-/- osteoblasts 7 days after differentiation compared to wild-type controls, combined with impaired calcium deposit formation that indicated inhibited mineralization of differentiated osteoblasts. Consistent with impaired osteoblastogenesis, the expression of Runx2 decreased 3 days after differentiation of LDLR-/- osteoblasts compared to wild-type controls, followed by decreased Osterix expression 7 days after differentiation, implying that LDLR might regulate Osterix expression during osteoblastic differentiation through Runx2. However, the modulation role of osteoblasts on osteoclastogenesis was not affected by LDLR deficiency, evidenced by the same level of the OPG/RANKL ratio between LDLR-/- and wild-type control osteoblasts. The bone mass and bone microarchitecture of LDLR-/- and wild-type mice in vivo should be analyzed in further studies.

The specific function of LDLR was to remove LDL-cholesterol particles from the circulation (49,50). Once entering endosomes, the LDL-LDLR complex dissociated due to the local low PH and the receptor was recycled to the plasma membrane for clearance of more cholesterol and cholesterol ester-containing LDL particles from the circulation. Then LDL was transported to lysosomes and degraded (51). Moreover, the degradation of LDLR was mediated by binding to proprotein convertase subtilisin/kexin type 9 (PCSK9). Interestingly, 17α-ethinyl estradiol treatment increased the binding of LDL to liver cell membranes in vitro. Moreover, the removal rate of rat and human LDL from blood plasma increased in estrogen-treated rats (52,53). Additionally, 17α-ethinyl estradiol at a pharmacologic dose increased LDLR number and mRNA level in livers of rabbits (54). In pituitary GH3 somatolactotropes, LDLR expression level and LDL uptake were up-regulated by estrogen treatment (55). Furthermore, the absence of PCSK9-triggered LDLR degradation led to a sex- and tissue-dependent subcellular distribution of LDLR that depends on estrogens (56). All the results indicated that LDLR may contribute to estrogen-mediated bone metabolism. Further studies are required to explore the effect of LDLR deficiency on ovariectomy-induced bone loss and its corresponding bone metabolism changes.

In conclusion, osteoblastic differentiation was disrupted by LDLR deficiency in vitro. Alkaline phosphatase activity significantly decreased in LDLR-/- osteoblasts in vitro compared to wild-type controls, combined with calcium deposit formation delay. LDLR deficiency reduced the expression of Runx2 in early stages and down-regulated Osterix in later stages during osteoblastic development in vitro, but the OPG/RANKL level remained unaffected in LDLR-/- osteoblasts compared with wild-type controls in vitro.

Acknowledgements
This work was supported by the National Natural Science Foundation of China No. 31571196 (to Ling Wang), the Science and Technology Commission of Shanghai Municipality 2015 YIXUEYINGDAO
References


