Synergistic Stimulation of Osteoblast Differentiation of Rat Mesenchymal Stem Cells by Leptin and 25(OH)D3 is Mediated by Inhibition of Chaperone-Mediated Autophagy

Qiting He
Shandong University Qilu Hospital
Ruixi Qin
Shandong University Qilu Hospital
Julie Glowacki
Harvard Medical School
Shuanhu Zhou
Harvard Medical School
Jie Shi
Shandong University Qilu Hospital
Shaoyi Wang
Shandong University Qilu Hospital
Yuan Gao
Shandong University Qilu Hospital
Lei Cheng (chenglei@email.sdu.edu.cn)
Shandong University Qilu Hospital

Research

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Abstract

Background

$25(OH)D_3$ is important for the osteoblast differentiation of bone marrow mesenchymual stem cells (BMMSCs), BMMSCs can directly hydroxylate $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ to induce osteoblast differentiation. Our previous research with human BMMSCs showed that the cell membrane receptor megalin is required for the $25(OH)D_3$-DBP complex to enter cells and thereby to stimulate osteoblast differentiation. Furthermore, leptin was shown to upregulate megalin in those cells. Leptin is a known inhibitor of PI3K/AKT-dependent chaperone-mediated autophagy (CMA). In this study, we tested the hypothesis that leptin acts synergistically with $25(OH)D_3$ to promote osteoblastogenesis in rat BMMSCs by a mechanism that entails inhibition of PI3K/AKT-dependent CMA.

Methods

The BMMSCs were isolated from rat bone marrow, qRT-PCR and western immunoblots were used to evaluate the expression of megalin, ALP, COL1A1, RUNX2 and CMA activity. The osteoblast differentiation ability was evaluated by ALP activity, ALP staining, and calcium deposition.

Results

After rBMMSCs were exposed to a combination of leptin and $25(OH)D_3$, osteoblast differentiation was significantly enhanced, the expression of osteoblastogenic genes ALP, COL1A1, and RUNX2 by qRT-PCR were up-regulated, and ALP activity, ALP staining, and calcium deposition were also significantly increased. The quantity of $25(OH)D_3$ entering rBMMSCs was increased through increased megalin receptors, and ELISA confirmed that the synthesis of $1\alpha,25(OH)_2D_3$ was increased. Addition of a PI3K/AKT inhibitor reduced the synergistic effect of osteoblast differentiation of rBMMSCs induced by combination leptin with $25(OH)D_3$; the inhibited CMA activity was partially rescued by a PI3K/AKT inhibitor, and the expression of megalin was down-regulated. Up-regulation of megalin expression by leptin played a synergistic role in osteoblast differentiation of rBMMSCs induced by $25(OH)D_3$; leptin promoted the expression of megalin by inhibiting the activity of CMA activity.

Conclusions

These studies indicate that leptin enhanced $25(OH)D_3$ stimulation of osteoblast differentiation of rBMMSCs by inhibiting CMA activity to increase megalin expression, and that PI3K/AKT signaling pathway is at least partially involved in the regulation of CMA activity and megalin expression.

Introduction

The maintenance of bone mass in the human body is the result of the dynamic balance of bone resorption and bone formation, osteoclasts absorb calcified bone matrix and osteoblasts synthesize new bone matrix. When the rate of bone resorption is faster than bone formation, the bone mass decreases gradually, which eventually leads to osteoporosis and increases the risk of fracture, which usually occurs in middle-aged and elderly postmenopausal women [1, 2]. Bone marrow mesenchymal stem cells (BMMSCs) are a kind of multifunctional stem cells, which can differentiate into osteoblasts, adipocytes, and chondrocytes in a suitable culture environment, and are the precursor cells of osteoblasts, the differentiation of BMMSCs into osteoblasts involves a series of complex mechanisms [3, 4]. Due to the BMMSCs are easy to obtain and culture as seed cells for bone tissue regeneration, so the research of BMMSCs on osteoporosis has been a hot spot [5].

Vitamin D is very important for the overall mineralization of bones and the speed of bone conversion [6]. Vitamin D deficiency will reduce bone mineral density and increase the risk of osteoporosis, even result in a fracture. 1α,25-dihydroxy-cholecalciferol (1α,25$(OH)_2D_3$) is an active metabolite of vitamin D, and bone is one of the important target organs, in which 1α,25$(OH)_2D_3$ can induce BMMSCs to differentiate into osteoblasts [7]. 25-hydroxycholecalciferol (25$(OH)D_3$) needs to be hydroxylated to 1α,25$(OH)_2D_3$ under the action of 25$(OH)D_3$-1α-hydroxylase, so it can plays a physiological role [8]. 25$(OH)D_3$ is more stable than 1α, 25$(OH)_2D_3$ in blood, the half-life period of 25$(OH)D_3$ is about 3 weeks, but 1α, 25$(OH)_2D_3$ is only about 4 hours. Vitamin D mainly in the form of 25$(OH)D_3$ exists in the blood, 25$(OH)D_3$ had a high affinity with vitamin D binding protein (DBP) [9–11]. It's reported that BMMSCs contain 25$(OH)D_3$-1α-hydroxylase, which hydroxylates 25$(OH)D_3$ into the more active form, 1α, 25$(OH)_2D_3$. The level of 1α-hydroxylase of 25$(OH)D_3$ is related to osteogenesis [12, 13]. 25$(OH)D_3$ is very important for osteoblast differentiation of BMMSCs, it's worthy for us to think about how to enhance the ability of 25$(OH)D_3$ on the osteoblast differentiation of BMMSCs.

Our previous studies showed that megalin (lipoprotein-related protein 2; LRP2; gp330) is one of the key receptors for 25$(OH)D_3$ into BMMSCs [14]. *In vitro*, 25$(OH)D_3$ entered cells as the 25$(OH)D_3$-DBP complex *via* megalin receptors on the cell membrane and hydroxylated to 1α, 25$(OH)_2D_3$ under the action of 25$(OH)D_3$-1α-hydroxylase. We also found that there were significant differences in constitutive expression of megalin in samples from different subjects. The samples with low expression of megalin were less sensitive to 25$(OH)D_3$. It was found that leptin could up-regulate the expression of megalin receptor [14]. Chaperone-mediated autophagy (CMA) is a selective autophagy, which can degrade soluble cellular proteins in time through the lysosome, the proteins were degraded in the lysosomal cavity through lysosomal Associated Membrane Protein 2A (LAMP2A), LAMP2A is the key rate-limiting receptor of CMA activity and can affect the activity of CMA [15]. It's reported that geldanamycin can induce the activity of CMA to promote the expression of LAMP2A and reduce the expression of ryanodine receptor type-2 in cardiomyocytes [16]. As a receptor on the cell membrane, whether the metabolism of megalin is

associated with CMA activity is our interest. In this study, we set CMA activity as the major part of megalin metabolism and investigate how CMA activity affects BMMSCs osteoblast differentiation.

We put forward and tested some hypotheses: 1. leptin could enhance the action of 25(OH)D_3 on osteoblast differentiation of rBMMSCs via promoting the expression of megalin; and 2. leptin affected the expression of megalin by regulating the activity of CMA. In this study, we tested how leptin acts to up-regulate the expression of megalin thereby enhancing the extent of osteoblast differentiation of rBMMSCs induced by 25(OH)D_3. We determined the role of CMA in regulating megalin expression.

Materials And Methods

Ethics Statement

All procedures were approved by the Shandong University Committee on the Use and Care of Animals and conducted per the Guidelines for the Care and Use of Laboratory Animals. Sprague Dawley (SD) rats were purchased from SPF (Beijing) Biotechnology Co. Ltd. (Beijing, China).

Reagents

Recombinant rat leptin (CYT-227) was purchased from Prospec-Tany TechnoGene Ltd. (Israel). 25(OH)D_3 was purchased from Aladdin (Shanghai, China). PI3K/AKT signaling pathway inhibitor LY294002 was purchased from Sigma-Aldrich (Shanghai, China). Antibodies against phosphorylated-p38 (p-p38, #4511), total-p38 (t-p38, #8690), β-actin (#3700), phosphorylated STAT3 (p-STAT3, #9145) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, #5174), anti-mouse IgG (H+L) (DyLight™ 680 Conjugate or DyLight™ 800 4X PEG Conjugate), and anti-rabbit IgG (H+L) (DyLight™ 680 Conjugate or DyLight™ 800 4X PEG Conjugate) secondary antibody were purchased from Cell Signaling Technology (CST, Shanghai, China). Antibodies against phosphorylated-AKT (p-AKT, sc-293125), total-AKT (t-AKT, sc-81434), total STAT3 (t-STAT3, sc-293151) and megalin (sc-515772) were purchased from Santa Cruz Biotechnology (Shanghai, China). Antibodies against runt-related transcription factor 2 (RUNX2, ab23981), collagen type I (COL1A1, ab34710) and *LAMP2A (ab125068)* were obtained from Abcam (Abcam, Shanghai, China). Antibody against HSC70 (AF5187) was obtained from Affinity Biosciences (USA). Fluorescent second antibody Rhodamine (TRITC)-Conjugated Goat anti-Mouse IgG (H+2) was purchased from ZSGB-BIO (Beijing, China). Alkaline phosphatase (ALP) activity kit, BCIP/NBT alkaline phosphatase (ALP) color development kit, Enhanced Cell Counting Kit-8 (CCK-8) and Alizarin Red Staining (ARS) were purchased from Beyotime (Shanghai, China). Dexamethasone, β-glycerophosphate, and ascorbate-2-phosphate were purchased from Solarbio Science

& Technology Co., Ltd. (Beijing, China). Rat 1α,25$(OH)_2D_3$ ELISA Kit was purchased from Bioswamp Life Science Lab (Wuhan, China). All primers were synthesized by BioSune (Shanghai, China).

Isolation and culture of primary rBMMSCs

Four-week-old (100 - 150 g) normal male SD rats were selected as the source of primary rBMMSCs for culture *in vitro*. The rBMMSCs were isolated from whole bone marrow method as follows. SD rats were euthanized with pentobarbital sodium (35 mg/kg, intraperitoneal injection) followed by cervical dislocation and bathed in 75% ethyl alcohol for 15 minutes. The muscles were removed from all femurs and tibias, and the bones were soaked and rinsed with aseptic phosphate-buffered saline (PBS; Beijing Dingguo Changsheng Biotechnology, China). Both epiphysis were removed to expose the marrow cavity. The contents of the marrow were flushed into a sterile 100 mm tissue culture dish (NEST Biotechnology Co.LTD, Jiangsu, China) with Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). The flushing was repeated 3 times; the process was repeated in the opposite direction. The pooled mixture was centrifuged at 1000 RPM for 5 minutes, the supernatant was discarded, and the cells were suspended with complete culture medium and plated into a 100 mm tissue culture dish (NEST Biotechnology Co.LTD). The rBMMSCs were cultured in a saturated humidity incubator with a volume fraction of 5% CO_2 at 37 °C. Non-adherent cells were removed by medium change after 48 h to 72 h, then the medium was replaced every 3 days. When the cell density reached 80 - 90% confluency, the cells were trypsinized and subcultured at the ratio of 1 : 3. Passage 3 to 5 rBMMSCs were used in this study.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted from rBMMSCs with Trizol reagent (SparkJade, Shandong, China) according to the manufacturer's instructions. Complementary DNA was synthesized with ReverTra Ace Qpcr RT Kit (Toyobo Life Science, Shanghai, China) according to the manufacturer's instructions. The qRT-PCR was performed on ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, USA) using SYBR Green Realtime PCR Master Mix (Toyobo Life Science) to evaluate the expression levels of megalin, RUNX2, ALP, COL1A1, *LAMP2A,* HSC70 and β-actin (internal control). The $2^{-\triangle\triangle Ct}$ method was used to calculate gene relative expression levels. The primers for qRT-PCR are shown in Table 1.

Table 1 Sequences of primers

Gene	Forward(5'-3')	Reverse(5'-3')
ALP	AGATGGATGAGGCCATCGGA	CCAAACGTGAAAACGTGGGA
COL1A1	CACTGCAAGAACAGCGTAGC	AAGTTCCGGTGTGACTCGTG
RUNX2	CAGACCAGCAGCACTCCATA	AGACTCATCCATTCTGCCGC
Megalin	ACAACTCGGATGAACGGGAC	AGTAGGTGCCGTTGGGAAAG
LAMP2A	AAGAGCAGGTGGTTTCCGTG	ATGGGCACAAGGAAGTTGTCT
HSC70	CTCCATTACCCGTGCTCGAT	GAACCACCCACCAGGACAAT
β-actin	CTCTGTGTGGATTGGTGGCT	CGCAGCTCAGTAACAGTCCG

Abbreviations: ALP, alkaline phosphatase; COL1A1, collagen type I; RUNX2, runt-related transcription factor 2; LAMP2A, lysosomal Associated Membrane Protein 2A; HSC70, heat shock cognate 71 kDa protein.

Western immunoblots

Total protein was extracted from rBMMSCs with radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) and protease inhibitor *cocktail* (MedChemExpress, Shanghai, China). Protein concentration was measured with the BCA kit (Boster Biological Technology, Wuhan, China). After denaturation, 40 ug aliquots of protein were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Shanghai, China). Membranes were blocked with 5% nonfat milk at room temperature for 1 - 2 h. Membranes were sequentially incubated overnight at 4 °C with the following primary antibodies: GAPDH (1:2000; CST), β-actin (1:10000; CST), RUNX2 (1:1000; Abcam), COL1A1 (1:1000; Abcam), t-AKT (1:400; Santa Cruz Biotechnology), p-AKT (1:1000; Santa Cruz Biotechnology), t-p38 (1:1000; CST), p-p38 (1:1000; CST), p-STAT3 (1:1000; CST), t-STAT3 (1:400; Santa Cruz Biotechnology), *LAMP2A (1:2000;* Abcam*)* or HSC70 (1:1000; Affinity Biosciences). The membranes were washed with TBST three times, and incubated with the anti-mouse IgG (H + L) (DyLight™ 680 Conjugate or DyLight™ 800 4X PEG Conjugate) or anti-rabbit IgG (H + L) (DyLight™ 680 Conjugate or DyLight™ 800 4X PEG Conjugate) secondary antibody for 30 minutes at room temperature. The fluorescent signal on membranes was detected by **Odyssey** two-color infrared fluorescence imaging system (LI-COR, USA); band grey values were quantified with Image J software.

Cell viability

Effects of leptin, 25(OH)D_3, and the combination of leptin and 25(OH)D_3 on the viability of rBMMSCs were assessed with the CCK-8 kit (Beyotime, China). Leptin (300 ng/ml), 25(OH)D_3 (100 nM), or the combination of leptin and 25(OH)D_3 was added to each well, with 5 replicate wells at 24 h after seeding in 96-well plates (5000 cells/well). After 24, 48, and 72 h, 10 ul CCK-8 solution was added to each well according to the manufacturer's instructions. Absorbance at 450 nm was measured with Varioskan flash (Thermo Scientific, USA).

Osteoblast differentiation of rBMMSCs

The rBMMSCs were plated into 6-well plates (2×10^5/well), after reaching confluence in basal medium, cultures were changed to osteoblast differentiation medium (ODM; basal medium with 10% FBS, 10 nM dexamethasone, 5 mM β-glycerophosphate, and 50 μg/mL ascorbate-2-phosphate), leptin (300 ng/mL), 25(OH)D_3 (100 nM), and leptin (300 ng/mL) + 25(OH)D_3 (100 nM) were added to the ODM after 24 h. The concentration of 25(OH)D_3 used in the present study was chosen according to the optimal physiological concentrations [17]. The ODM was replaced twice weekly.

ALP staining and activity assay

For ALP staining and activity assays, rBMMSCs were seeded into 12-well plates (1×10^5/well) with ODM ± leptin (300 ng/ml) ± 25(OH)D_3 (100 nM) for 7 days, the ODM was changed every 3 days. For ALP staining, the cells were washed with PBS 3 times and fixed with 4% paraformaldehyde (Solarbio Life Sciences, Beijing, China) for 20 min. They were washed 3 times with PBS and stained with BCIP/NBT ALP color development kit (Beyotime). For the measurement of ALP activity, the rBMMSCs were collected and incubated at 37 °C for 30 min according to the manufacturer's instructions of ALP Activity Assay Kit (Beyotime). The ALP activity was detected at 405 nm with Varioskan flash (Thermo Scientific, USA).

Assessment of calcium deposition

After the rBMMSCs were cultures with ODM for 21 days, calcium deposition was quantified with the ARS Kit (Beyotime). In brief, cells were washed 3 times with distilled water, fixed with 4% paraformaldehyde (Solarbio) for 20 min, and washed 3 times with distilled water. Cells were then incubated with 1% ARS solution for 30 min at room temperature, and rinsed with distilled water. For measure of the relative value of ARS, the stain was dissolved with 10% cetylpyridinium chloride (Sangon Biotech, Shanghai, China) for 1 h. Aliquots (200 μl) were transferred to 96-well plates, and detected at 405 nm with Varioskan flash (Thermo Scientific, USA).

Immunofluorescence (IF) analysis of cells

The rBMMSCs (5×10^4/well in 24-well plates) were seeded onto alcohol-washed coverslips (Diameter: 14 mm) with 500 ul basal medium, after reaching 60% - 70% confluence, mediums were changed to basal medium with different concentrations of leptin (0, 50, 100, 300 ng/ml), and changed to ODM ± leptin (300 ng/ml) ± 25(OH)D_3 (100 nM) for 24 h. The coverslips were washed with PBS 3 times, each time for 3 minutes, then rBMMSCs were fixed with 4% paraformaldehyde for 20 minutes at room temperature, and were washed with PBS 3 times, each time for 3 min. Triton Xmuri 100 (0.2% in PBS, 20 min, room temperature) was used to permeate the cells. They were washed 3 times with PBS, and blocked with 5% BSA for 30 min at room temperature. First megalin antibody (1:80; Santa Cruz Biotechnology) was applied to each coverslip and incubated overnight at 4 °C in a humidified chamber. Thereafter, they were washed 3 times, and incubated with fluorescent second antibody Rhodamine (TRITC)-Conjugated Goat anti-Mouse IgG (H+2) (1:50; ZSGB-BIO, Beijing, China), in the humidified chamber at room temperature for 1 h. Subsequent steps were carried out in darkness. The coverslips were washed with PBS 3 times for 3 minutes each time. For staining nuclei the coverslips were incubated with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; Beyotime) for 5 minutes; images were collected with the Lionheart FX (Bio Tek, USA). The optical density of more than 6 cells was randomly measured with Image J software, and the average optical density was calculated to represent the fluorescence intensity.

ELISA assay of biosynthesis of 1α,25(OH)$_2$$D_3$ by rBMMSCs

The rBMMSCs were cultured in 6-well plates in growth medium until confluent, when medium was changed to ODM with 25(OH)D_3 (100 nM), with or without leptin (300 ng/ml) for 24 h. Supernatants were

collected and stored at -80°C for subsequent determination of 1α,25$(OH)_2D_3$. The cells were collected for western immunoblotting. Biosynthesis of 1α,25$(OH)_2D_3$ was measured by a Rat 1α,25$(OH)_2D_3$ ELISA Kit (Bio-Swamp, Wuhan, China) according to the manufactures' protocol.

Statistical analysis

All experiments were repeated at least three times. All data are presented as means ± standard deviation, and statistical analyses were performed with SPSS 20. Significant differences between the groups were determined by Student's *t*-test. A value of $P < 0.05$ was considered significant.

Results

Leptin enhanced the expression of megalin of rBMMSCs

As tests of whether leptin and 25$(OH)D_3$ can promote the expression of megalin in rBMMSCs, qRT-PCR and IF were performed to assess the relative expression levels of megalin mRNA and protein. We found that different concentrations of leptin stimulated rBMMSCs after 8 h, with the increase of leptin concentration, the expression of megalin was dose-dependently up-regulated (Fig. 1A). At 300 ng/ml, leptin significantly stimulated megalin expression, so this concentration was optimal for follow-up experiments. However, 25$(OH)D_3$ had no significant effect on the expression of megalin with or without leptin, and there was no synergistic or inhibitory effect in combination with leptin (Fig. 1D). IF data confirmed the above results after the rBMMSCs were stimulated 24 h (Fig. 1B-C,1E-F).

Leptin promoted cell proliferation of rBMMSCs

As a test of whether leptin or 25$(OH)D_3$ had cytotoxicity on rBMMSCs proliferation, CCK-8 analysis was used to measure cell proliferation. The results showed no effect on the proliferation of rBMMSCs with 25$(OH)D_3$ alone at 24 h, 48 h, and 72 h. Leptin with or without 25$(OH)D_3$, however, promoted the proliferation of rBMMSCs on 48 h and 72 h, but had no obvious effect on the first day (Fig. 2A).

Leptin enhanced the ability of 25$(OH)D_3$ to induce osteoblast differentiation of rBMMSCs.

As a test whether leptin has a synergistic effect on osteoblast differentiation of rBMMSCs induced by 25$(OH)D_3$, we measured mRNA expression level of osteoblast genes (ALP, COL1A1, RUNX2) after rBMMSCs were transferred to ODM for 12 hours (Fig. 2B), and also detected the expression level of osteoblast proteins 3 days later (Fig. 2C-D). The results showed that leptin or 25$(OH)D_3$ used alone had no significant effect on osteoblast differentiation of rBMMSCs at cetain concentrations, but that osteoblast differentiation was significantly enhanced when a combination of two agents, compared with 25$(OH)D_3$ treatment alone. The synthesis of 1α,25$(OH)_2D_3$ was increased significantly compared with 25$(OH)D_3$ group with ODM for 24 h, as assessed by ELISA (Fig. 2G).

25$(OH)D_3$ and leptin enhanced ALP activity and calcium deposit formation

ALP Activity Assay Kit and ALP staining were used to evaluate the effects of 25(OH)D_3 with or without leptin on the ALP activity of rBMMSCs. The results showed that there was no significant change in ALP Activity of rBMMSCs on day 7 after 25(OH)D_3 or leptin was added to ODM alone. A combination of 25(OH)D_3 and leptin, resulted in significantly increased ALP on day 7 (Fig. 2E-F). Similar results were obtained from ARS and relative quantification after 21 days (Fig. 2H-I). The results showed that leptin enhanced the osteoblastic differentiation ability of rBMMSCs induced by 25(OH)D_3

Leptin inhibited CMA activity of rBMMSCs.

To verify whether leptin can inhibit the CMA activity or not, we used qRT-PCR and western blot to detect the mRNA and protein expression levels of CMA-related genes LAMP2A and HSC70. The results showed that different concentrations of leptin stimulated rBMMSCs for 8h in the growth medium, the mRNA relative expression levels of LAMP2A and HSC70 decreased significantly (Fig. 3A), and the protein relative expression levels were similar with mRNA after 24 hours (Fig. 3B-C). However, after stimulating rBMMSCs in ODM for 8 hours, 25(OH)D_3 did not affect CMA activity, and there was no promoting or inhibitory effect when it combined with leptin (Fig. 3D, 3E-F).

Leptin inhibited CMA activity through PI3K/AKT signal pathway.

To explore the signal pathways involved in the inhibition of CMA activity by leptin, we used Western immunoblotting to quantify several signal pathway proteins related to leptin, including JAK2/STAT3, mitogen-activated protein kinase (MAPK)/p38 and PI3K/AKT signaling pathways. The results showed that with the increase of leptin concentration, the expression level of p-AKT protein increased significantly after rBMMSCs was stimulated in ODM for 1 h, but there were no significant differences in p-STAT3, t-STAT3, p-P38, t-P38 and AKT (Fig. 3G-H).

The effect of leptin promoted the expression of megalin was partially blocked by PI3K/AKT signal pathway inhibitorLY294002 (20 μM).

To test whether PI3K/AKT signal pathway was involved in the expression of megalin, we used a PI3K/AKT signal pathway inhibitor to inhibit the expression of p-AKT, and to measure the expression of CMA-related genes and megalin by qRT-PCR, IF, and Western immunoblot. The PI3K/AKT signal pathway inhibitor was added to the ODM 2 h earlier. The results showed that the level of p-AKT decreased significantly after the addition of leptin (300 ng/mL) + LY294002 (20 μM) in ODM for 1 h (Fig. 4A-B), that mRNA relative expression of CMA related gene (LAMP2A and HSC70) inhibited by leptin with 25(OH)D_3 was partially rescued in ODM for 8 h (Fig. 4C), that protein relative expression levels were similar with mRNA after 24 h (Fig. 4D-E). and that mRNA expression level of megalin enhanced by leptin with 25(OH)D_3 in ODM for 8 h was also significantly decreased compared with the level in leptin with 25(OH)D_3 group without the inhibitor (Fig. 4F). Moreover, IF analysis confirmed decreased expression of megalin in ODM for 24 h compared with the level in leptin with 25(OH)D_3 group without the inhibitor (Fig.4G-H).

The ability of leptin to enhance 25(OH)D_3 promoted osteoblast differentiation of rBMMSCs was weakened by the PI3K/AKT signal pathway inhibitor.

To test whether 25(OH)D_3 stimulation of osteoblast differentiation was enhanced by leptin through PI3K/AKT signal pathway, we used PI3K/AKT signal pathway inhibitor (LY294002, 20 μM). The increased mRNA expression levels of ALP, COL1A1, and RUNX2 induced by leptin with 25(OH)D_3 were significantly lower after the addition of PI3K/AKT signal pathway inhibitor (LY294002, 20 μM) for 12 h (Fig. 5A). Similarly, the protein expression levels of COL1A1, and RUNX2 were lower after 3 days (Fig. 5B-C), and the synthesis of 1α,25$(OH)_2D_3$ was significantly lower compared with leptin + 25(OH)D_3 group (Fig. 5D). Moreover, ALP activity, ALP staining, and ARS also showed that the ability of leptin to enhance 25(OH)D_3 promoted osteoblast differentiation of rBMMSCs was reduced after the addition of PI3K/AKT signal pathway inhibitor (LY294002, 20 μM) (Fig. 5E-H). A summary diagram for the role of leptin to promote megalin expression by inhibiting CMA to enhance osteoblast differentiation of rBMMSCs induced by 25(OH)D3 is shown in the figure 6.

Discussion

1α,25$(OH)_2D_3$ plays an important role in the osteoblast differentiation of BMMSCs, it can prevent the age-related osteoporosis [18]. There are two main sources of vitamin D in the human body, one is intake and absorption from diet, and the other is skin exposure to ultraviolet rays. Vitamin D from both sources is hydroxylated to 25(OH)D3 by 25-hydroxylase (CYP2R1) in the liver, and thereafter to active 1α,25$(OH)_2D_3$ by 1α-hydroxylase (CYP27B1) in the kidney [19]. Many studies found various extrarenal tissues such as skin, lymph nodes, colon, pancreas, adrenal medulla, dendritic cells, endothelial cells, brain, hypothalamus, placenta, bone cells, BMMSCs have 1α-hydroxylase (CYP27B1) [20-23]. Evidence shows that 25(OH)D_3 binds to vitamin D binding protein (DBP) in blood and enters into proximal tubular cell via the megalin receptor [24]. Megalin, a member of the low-density lipoprotein family, 600 kd, was found to be expressed in renal proximal convoluted tubular epithelial cells. It reabsorbs glomerular filtered proteins and acts as a non-specific receptor for many proteins, including hormones, enzymes, drugs, and the 25(OH)D_3-DBP complex [25]. Many extrarenal tissues also express megalin receptor, such as BMMSCs, alveoli, gallbladder, and placenta [26]. Our previous research showed that megalin is one of the key receptors for 25(OH)D_3 in human BMMSCs [14]. The expression level of megalin determined the osteoblast differentiation potential of BMMSCs induced by 25(OH)D_3. We found that the megalin expression level of BMMSCs in some subjects was relatively low, and 25(OH)D_3 has lower effects on the MSCs with low megalin.

In our study, we found that neither 25(OH)D_3 (100 nM) nor leptin (300 ng/mL) could significantly stimulate the osteoblast differentiation of rat BMMSCs, however, the co-stimulation of leptin and 25(OH)D_3 could significantly promote the osteoblast differentiation of rBMMSCs, the results of qRT-PCR and western blots indicated that the expression levels of osteoblastogenic genes ALP, COL1A1, and RUNX2 were increased. ALP activity and ARS enhanced significantly. Other research reported that

BMMSCs can be induced to differentiate into osteoblasts by 25(OH)D_3 at least 250 nM in vitro experiments [27]. The Institute of Medicine (IOM) of USA, now called the National Academy of Medicine commissioned a report that 20 ng/mL (50 nM) 25(OH)D_3 appeared to be sufficient for skeletal health in the general population, a high concentration of 25(OH)D_3 (> 125 nM) has many side effects in vivo, they recommend the sufficient concentration is 20 ng/ml (50 nmol/liter) [28]. Other groups disputed that recommendation, the Endocrine Society recommended the sufficient serum concentration of 25(OH)D_3 is 30 ng/mL (75 nM) [29]. Subsequently, an international group reported that 25(OH)D_3 concentrations between 20-50 ng/mL (50-125 nM) appear to be safe and sufficient in the general population for skeletal health [30]. The concentration of 25(OH)D_3 used in our study accorded with the recommendation of international group, but could not significantly induce osteoblast differentiation of BMMSCs at the concentration of 100 nM in vitro, however, after the addition of leptin, the osteoblast differentiation ability of BMMSCs induced by 25(OH)D_3 (100 nM) was significantly enhanced.

Moreover, we explored the mechanism of leptin enhanced the ability of 25(OH)D_3 to induce osteoblast differentiation of rBMMSCs. The results of qRT-PCR and IF showed that leptin could enhance the expression of megalin receptor in rBMMSCs, but 25(OH)D_3 had no effect on megalin receptor, the ELISA results showed that leptin promoted 25(OH)D_3 to enter into rBMMSCs. Our studies show the importance of megalin expression in rBMMSCs for their osteoblast differentiation potential. Synergistic actions of 25(OH)D_3 with leptin on osteoblast differentiation required upregulation of megalin, the key receptor of the 25(OH)D3-DBP complex. When the megalin receptor was up-regulated, more 25(OH)D_3 was activated to 1α,25(OH)$_2D_3$ and induced more osteoblast differentiation of rBMMSCs. These findings are in accord with our previous report with human BMMSCs, showing relatively reduced osteoblast differentiation with 25(OH)D_3 in cells with lower constitutive or experimentally reduced megalin expression, and increased osteoblastogenesis upon addition of leptin [14]. The studies reported here show synergy of leptin and 25(OH)D_3 to stimulate osteoblast differention in rBMMSCs and add further information about the mechanism of that synergy. Leptin was discovered in 1994 in a group of spontaneous obese mice [31]. It regulates energy metabolism and appetite through the hypothalamic leptin receptor, and regulates immune cell function and maintains bone mass [31]. The evidence shows that leptin induces osteoblast differentiation of human BMMSCs [32], but others showed that leptin could not stimulate the differentiation of rBMMSCs osteoblasts [33]. Leptin receptor is highly expressed in BMMSCs, which include precursor cells of osteoblasts [34].

Most importantly, we discovered that leptin inhibited CMA activity through the PI3K/AKT signal pathway to promote the expression of megalin receptor, the expression level of genes (LAMP2A and HSC70) was suppressed. The results of qRT-PCR and western blots showed that PI3K/AKT inhibitor could reverse the inhibited effect of CMA activity by leptin, and the expression of megalin receptor was down-regulated. ELISA results showed that the entry of 25(OH)D_3 into rBMMSCs was decreased, and the synergistic effect of leptin and 25(OH)D_3 on osteoblast differentiation of rBMMSCs was inhibited. CMA is a selective autophagy, similar to the results of our study, leptin is a inhibitor of autophagy to promote apoptosis of

chondrocytes through PI3K/AKT signal pathway during osteoarthritis pathogenesis [35]. Moreover, in a chronic overpressure mouse model, leptin repressed the cardiac autophagy by the PI3K/AKT signal pathway and leaded to cardiac dysfunction [36]. These results indicated that leptin is a inhibitor of PI3K/AKT-dependent CMA, leptin promoted the expression of megalin receptor by inhibiting CMA activity.

CMA is a selective autophagy, which can degrade specific cellular proteins in time through the lysosome. These proteins contain specific amino acid sequences KFERQ, about 40% of the proteins in the mammalian contain typical KFERQ sequences. Heat shock protein HSC70 specifically recognizes and binds the specific amino acid sequence of these proteins, and unfolds the substrate protein, then enters the lysosome through the LAMP2A receptor on the lysosomal membrane for subsequent degradation [37]. LAMP2A receptor is not only the key rate-limiting part of CMA, but also the specific marker of CMA. The number and activity of LAMP2A determine the level of CMA. CMA is involved in the regulation of glucose and lipid metabolism, DNA repair, cell reprogramming and cell response to stress [16]. In a study by Yin Li et al, inhibition of the PI3K/AKt/mTOR signaling pathway played a protective role in acute liver failure by promoting CMA activity [38]. Our results showed that leptin significantly reduced the expression of LAMP2A and that the PI3K/AKT signal pathway inhibitor (LY294002) partially rescued the inhibited CMA activity, this indicates that leptin induced the expression of megalin by inhibiting CMA activity through PI3K/AKT signal pathway. Our studies do not identify protein substrates involved in CMA, but they support continued investigations to study the protein substrate involved in CMA that affects the expression of megalin.

Conclusion

In sum, these data reveal the mechanism by which there is synergy between leptin and 25(OH)D_3 to promote osteoblast differentiation. Leptin promoted the expression of megalin by inhibiting the activity of CMA, increased the utilization of 25(OH)D_3 by rBMMSCs, and enhanced the ability of 25(OH)D_3 to induce osteoblast differentiation of rBMMSCs. PI3K/AKT is at least partially involved in the regulation of CMA activity. These data indicate the importance of megalin in BMMSCs for vitamin D's role in skeletal health.

Abbreviations

BMMSCs: Bone marrow mesenchymal stem cells; 1α,25$(OH)_2D_3$: 1α,25-dihydroxy-cholecalciferol; (25(OH)D_3: 25-hydroxycholecalciferol; ARS: Alizarin Red Staining;

ALP: alkaline phosphatase; ODM: osteogenic differentiation medium; qRT-PCR: quantitative real-time polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase, RUNX2: runt-related transcription factor 2; COL1A1, collagen type I; LAMP2A, lysosomal Associated Membrane Protein 2A; HSC70, heat shock cognate 71 kDa protein.

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Declarations

Ethics approval and consent to participate

The experimental procedures of this study were conducted in accordance with Shandong University Committee on the Use and Care of Animals and conducted per the Guidelines for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

QTH, YG, LC: study design; QTH: developed the study, performed the research, analyzed the data; RXQ: assisted some of the experiments, wrote the paper; JG, SHZ: provided acquisition, analysis of data and revised the manuscript; JS, SYW: assisted some of the experiments.

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Author details

[1]Department of Orthopedic Surgery, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, China

[2]Department of Pathology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, China

[3]Department of Orthopedic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

[4]Department of Oral and Maxillofacial Surgery, Harvard School of Dental Medicine, Boston, Massachusetts, USA

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Figures

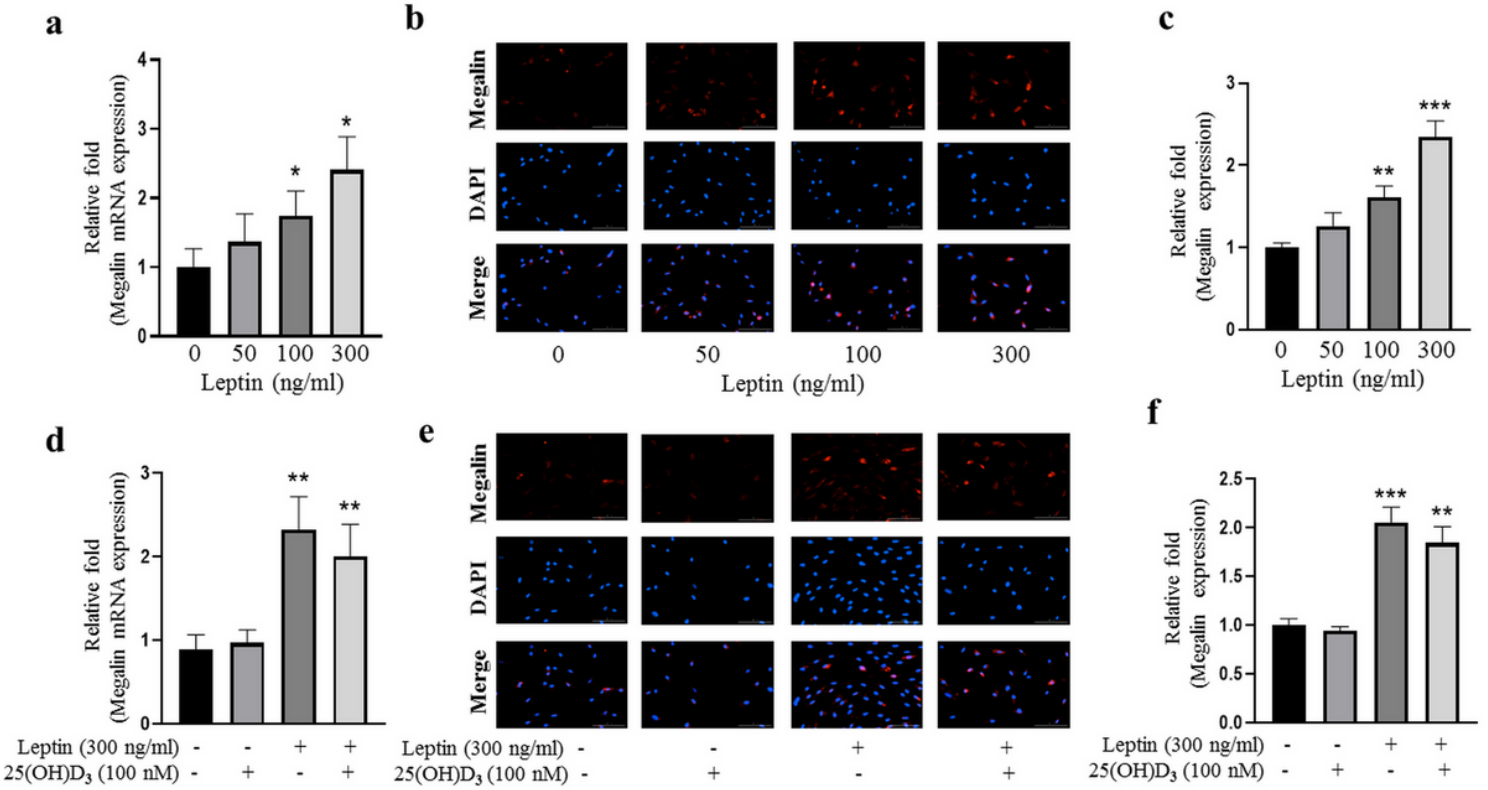

Figure 1

Leptin enhanced the expression of megalin of rBMMSCs. a Relative mRNA expression of rBMMSCs megalin in growth medium after 8 h; b IF showed leptin enhanced the protein expression of rBMMSCs megalin; c Relative quantitative analysis of megalin IF staining; d 25(OH)D3 had no effect on the relative mRNA expression of megalin with or without leptin in osteoblast differentiation medium (ODM) after 8 h; e IF showed 25(OH)D3 did not affect the protein expression of megalin; f Relative quantitative analysis of megalin IF staining. (Scale bars = 100μm. *P < 0.05, **P < 0.01 in comparison with the control group.)

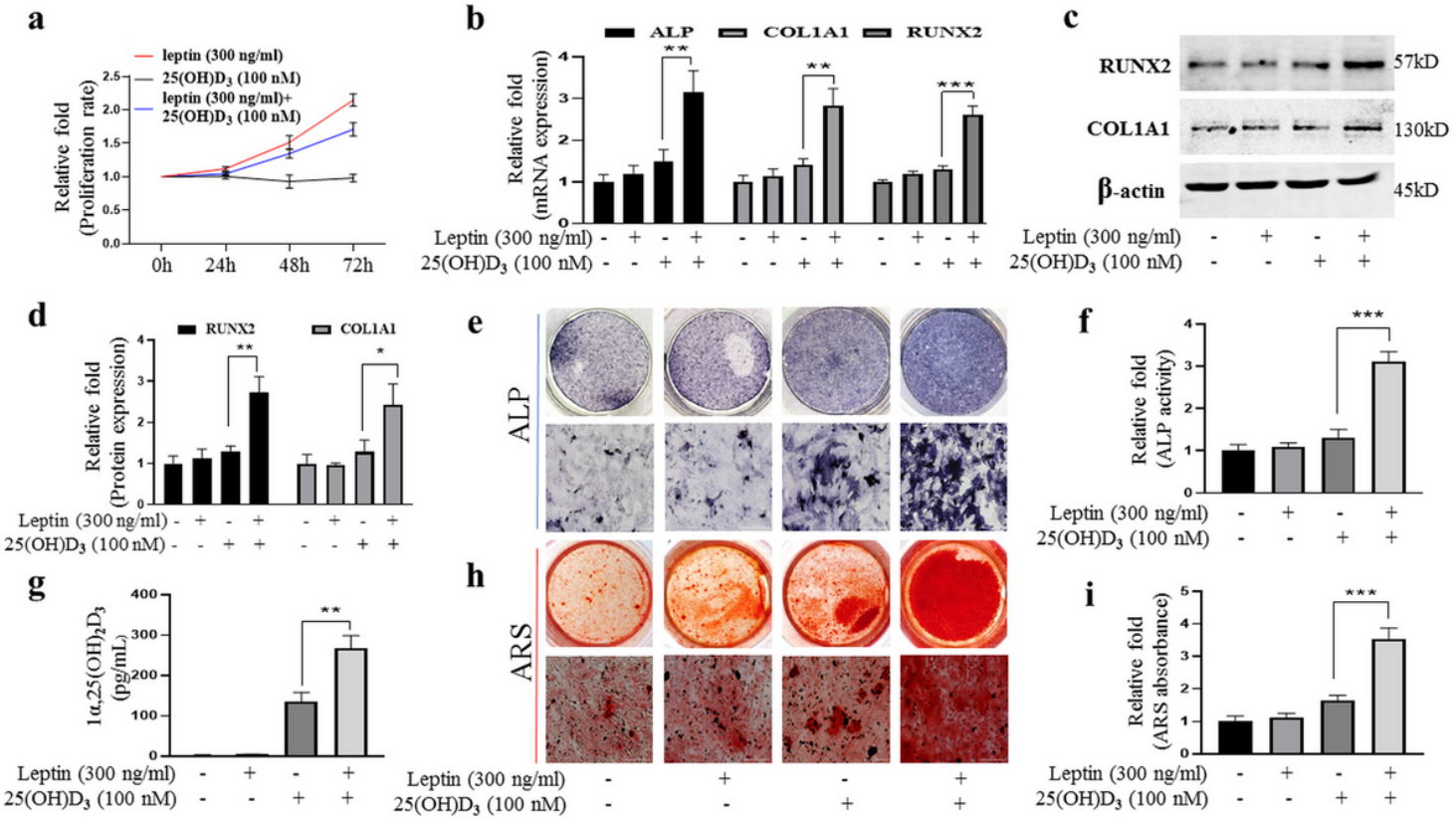

Figure 2

Leptin enhanced the ability of 25(OH)D3 to induce osteoblast differentiation of rBMMSCs. a The effects of leptin or 25(OH)D3 on rBMMSCs proliferation at 24 h, 48 h and 72 h; b Relative mRNA expression of osteoblastogenic genes (ALP, COL1A1, and RUNX2) at 12 h of osteoblast differentiation; c Western blot bands of proteins of RUNX2 and COL1A1 on day 3 of rBMMSCs osteoblast differentiation; d Relative quantitative analysis of proteins of RUNX2 and COL1A1; e Results of ALP staining of osteoblast differentiation of rBMMSCs on day 7; f Relative ALP activity fold of osteoblast differentiation of rBMMSCs on day 7; g The synthesis of 1α,25(OH)2D3 by rBMMSCs with ODM for 24 h; h Results of ARS staining of osteoblast differentiation of rBMMSCs on day 21; i Relative quantitative analysis of ARS of osteoblast differentiation of rBMMSCs on day 21. (Scale bars = 200 μm. *P < 0.05 , **P < 0.01, ***P < 0.001 in comparison with the control group.)

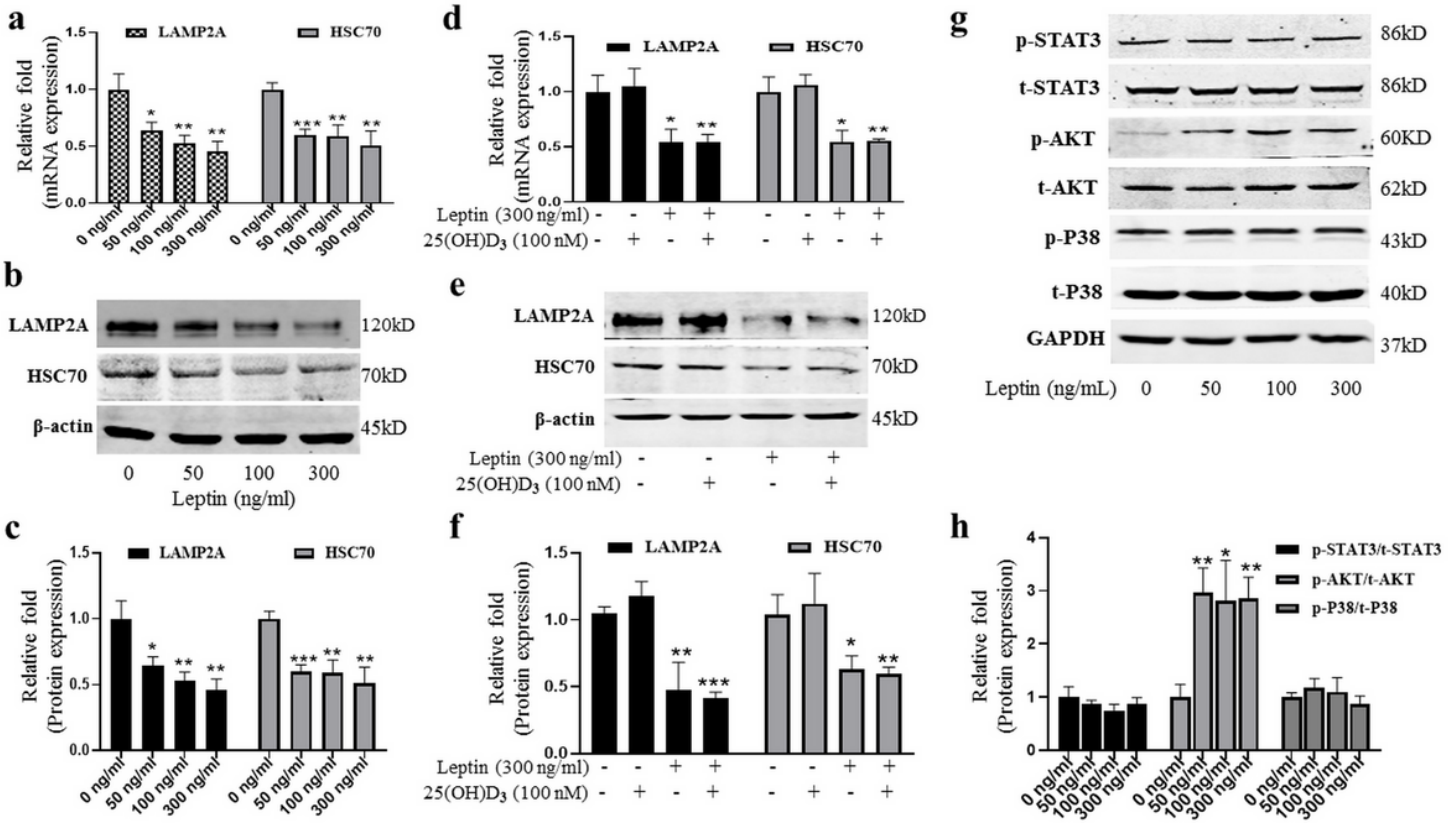

Figure 3

Leptin inhibited CMA activity of rBMMSCs by activating PI3K/AKT signal pathway. a Relative mRNA expression of CMA related genes (LAMP2A, and HSC70) of rBMMSCs in growth medium for 8 h; b Western blot bands of proteins of LAMP2A and HSC70 of rBMMSCs in growth medium for 24 h; c Relative quantitative analysis of proteins of LAMP2A and HSC70; d 25(OH)D3 with or without leptin did not affect the relative mRNA expression of LAMP2A, and HSC70 in ODM for 8 h; e 25(OH)D3 did not affect the expression of proteins of LAMP2A, and HSC70 in ODM with or without leptin for 24 h; f Relative quantitative analysis of proteins of LAMP2A and HSC70; g Western blot analyses of JAK2/STAT3, PI3K/AKT, and MAPK/p38 in ODM for 1 h; h Relative quantitative analysis of related signal pathway proteins. (*P < 0.05, **P < 0.01, ***P < 0.001 in comparison with the control group.)

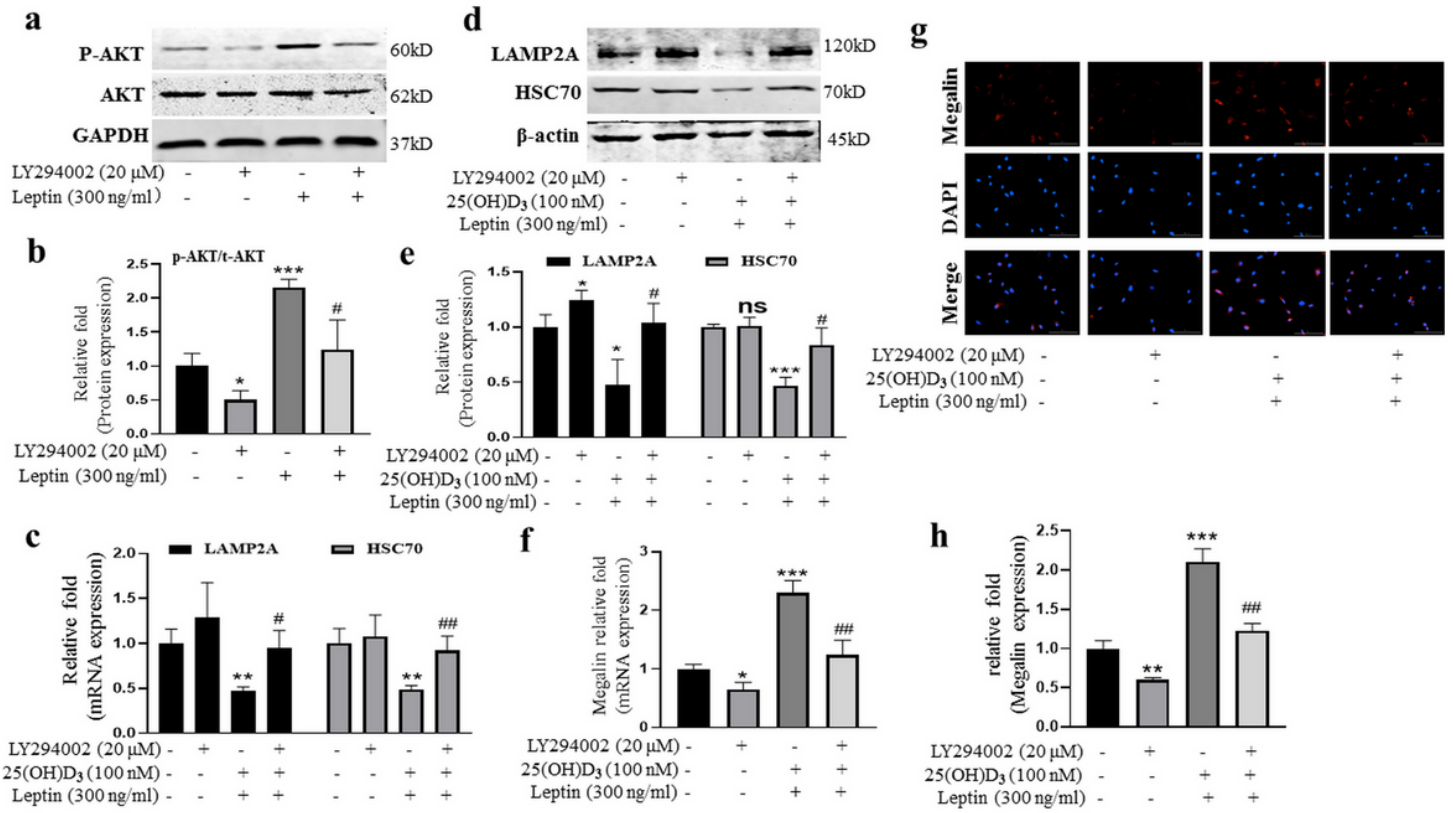

Figure 4

The effect of leptin promoted the expression of megalin was partially blocked by PI3K/AKT signal pathway inhibitor. a Western blot analyses showed that the level of p-AKT was significantly decreased in leptin treated rBMMSCs with the inhibitor in ODM for 1 h; b Relative quantitative analysis of western blot analyses; c The mRNA relative expression of LAMP2A and HSC70 inhibited by leptin combined with 25(OH)D3 was partially rescued in ODM for 8 h; d The decreased protein levels of LAMP2A and HSC70 induced by leptin with 25(OH)D3 treatment were significantly increased following the addition of LY294002 (20 μM) for 24 h; e Relative quantitative analysis of western blot analyses; f The mRNA relative expression of megalin by leptin with 25(OH)D3 + LY294002 was significantly decreased; g IF showed that the expression level of megalin enhanced by leptin with 25(OH)D3 was significantly decreased following the addition of LY294002 (20 μM) compared with the level in leptin with 25(OH)D3 group without the inhibitor in ODM for 24 h; h Relative quantitative analysis of megalin IF staining. (scale bar = 100 μm, *P < 0.05, **P < 0.01, ***P < 0.001 in comparison with the control group; #P < 0.05, ##P < 0.01 in comparison with Leptin + 25(OH)D3 group.)

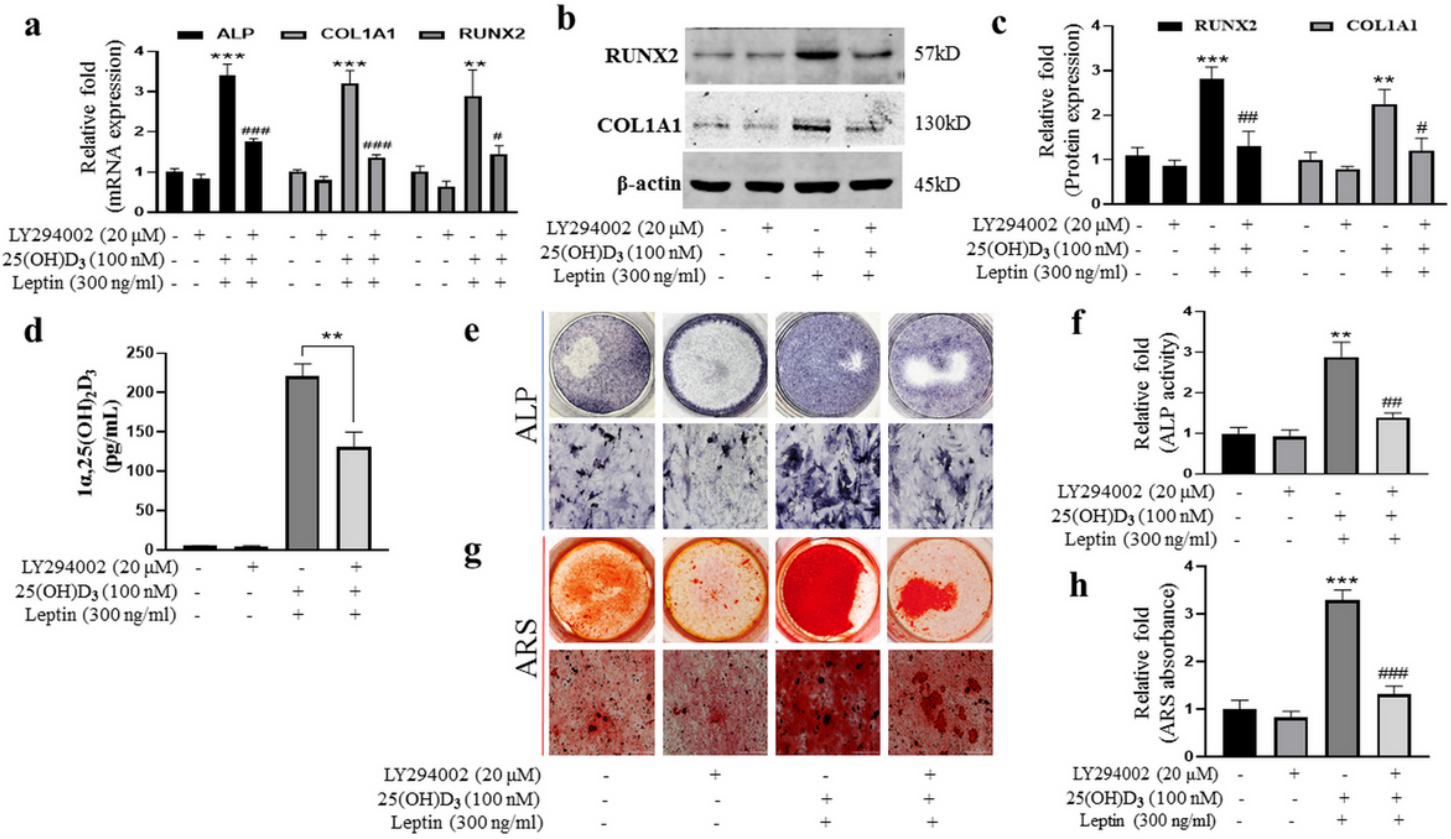

Figure 5

The ability of leptin to enhance 25(OH)D3 promoted osteoblast differentiation of rBMMSCs was weakened by the PI3K/AKT signal pathway inhibitor. a The increased mRNA levels of ALP, COL1A1, and RUNX2 induced by 25(OH)D3 combined with leptin were significantly decreased after the addition of LY294002 (20 μM) for 12 h; b The protein levels of RUNX2 and COL1A1 induced by leptin with 25(OH)D3 were significantly decreased following the addition of LY294002 (20 μM) for 3 days; c Relative quantitative analysis of western blot analyses; d The synthesis of 1α,25(OH)2D3 by rBMMSCs with ODM for 24 h; e Results of ALP staining; f The relative expression level of ALP activity; g Results of ARS staining; h The relative expression level of ARS. (scale bar = 200 μm, **P < 0.01, ***P < 0.001 in comparison with the control group; #P < 0.05, ##P < 0.01, ###P < 0.001 in comparison with Leptin + 25(OH)D3 group.)

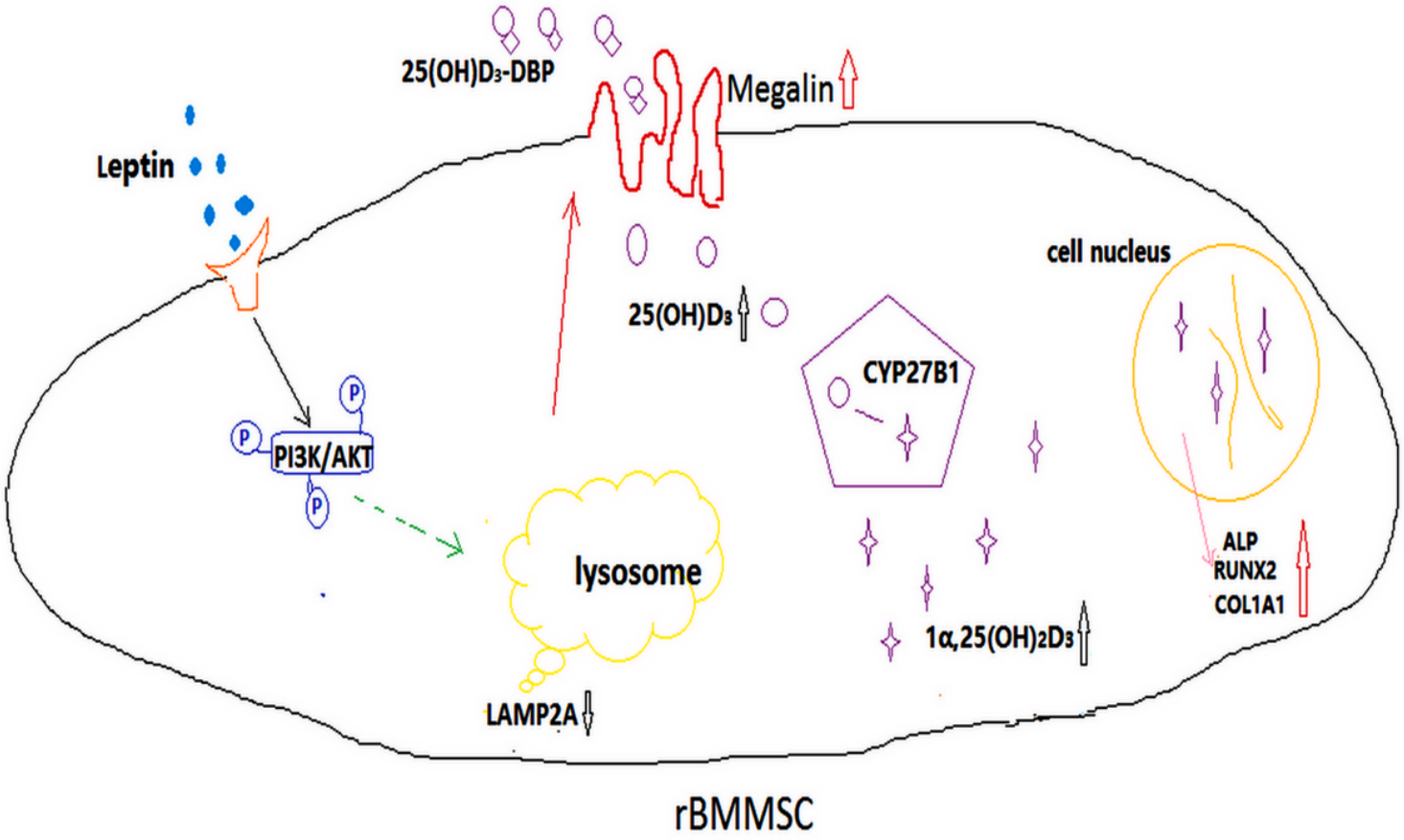

Figure 6

A summary diagram for the role of leptin to promote megalin expression by inhibiting chaperone-mediated autophagy to enhance osteoblast differentiation of rat bone marrow mesenchymal stem cells induced by 25(OH)D3. As indicated by these studies, Leptin inhibited the activity of CMA by activating PI3K/AKT signaling pathway to promote the expression of megalin, and then the quantity of 25(OH)D3 entering rBMMSCs was increased through increased megalin receptors. 25(OH)D3 was converted to 1α,25(OH)2D3 by 1α-hydroxylation of mitochondrial CYP27B1, the synthesis of 1α,25(OH)2D3 was up-regulation and stimulated osteoblast differentiation of rBMMSCs.