Cholesterol 25-Hydroxylase Promotes Osteogenesis and Suppresses Adipogenesis of Bone Marrow Stromal Cells via mTOR Signaling Pathway

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Abstract

Background

Increased adipogenesis and reduced osteogenesis of bone marrow stromal cells (BMSCs) are key features of glucocorticoid-induced osteoporosis (GIOP). However, the mechanism of controlling the differentiation balance of bone marrow stromal cells (BMSCs) is still unclear. Recent years, studies on the relationship between cholesterol metabolism and bone metabolism have been increasing. Various cholesterol metabolism-related molecules have been proved to be involved in BMSCs differentiation. Cholesterol-25-Hydroxylase (CH25H) is a multi-transmembrane endoplasmic reticulum related enzyme which participated in various metabolic process, including immunity, stem cell differentiation and inflammation. Here we discovered and proved its role in regulating stem cell differentiation and explored the mechanism.

Methods

RNAi sequences or cDNA were designed to knockdown or overexpress CH25H in cells. Alkaline phosphatase (ALP), alizarin red S (AR-S) staining and Oil Red O staining were used to identify osteogenic or adipogenic differentiation ability of different cells. Meanwhile, biomarkers of osteogenesis or adipogenesis were tested by quantitative real time polymerase chain reaction (qRT-PCR) to quantify the degree of cell differentiation. Western blot was performed to detect the protein expression of CH25H and the key molecules of mTOR signaling pathway. Subcutaneous ectopic osteogenesis experiment in nude mice and immunohistochemistry (IHC) were applied to verify the results of in vitro experiments.

Results

CH25H could increase osteogenesis and suppress adipogenesis. Besides, the mTOR signaling pathway was found upregulated when knocking down CH25H. When using rapamycin, a specific inhibitor of mTOR, the regulating effect of osteo-adipogenic regulation were partly reversed. These results indicated that CH25H was a key regulator of BMSCs osteo-adipogenic differentiation and the mTOR signaling pathway was the downstream mechanism. Animal experiments have also got consistent results.

Conclusion

CH25H could promotes osteogenesis and suppresses adipogenesis of bone marrow stromal cells via mTOR signaling pathway.

Introduction

Glucocorticoid-induced osteoporosis (GIOP) is one of the most severe complications of using glucocorticoids. But in diseases like chronic immune disease, the use of glucocorticoid is inevitable¹. Due to the unclear pathogenesis of GIOP, bisphosphonates are always the preferred drug for inhibiting the process of osteoclast to prevent and treat GIOP for more than 20 years. However, inhibiting the process of osteoclast alone is not enough for the treatment of GIOP². In the process of researching on GIPO, it's recognized that the abnormal differentiation of bone marrow stromal cells (BMSCs) is a main character. As the common progenitor of various cells, including osteoblasts, adipocytes, chondrocytes, neurocytes, and myoblasts, BMSCs maintain a delicate balance of differentiation to keep an appropriate bone mass. It's noteworthy that in the BMSCs of patients suffering from GIOP, there was a consistent trend of increased adipogenesis and reduced osteogenesis³⁻⁵. Therefore, seeking ways to promote osteogenesis as well as inhibit adipogenesis of BMSCs is important to control GIOP more effectively.

Recent years, successive clinical trials found that the metabolism disorder of cholesterol was associated with the decreased bone formation⁶⁻¹⁰. High level of serum cholesterol was negatively correlated with bone mineral density in patients¹¹. Whereas the use of cholesterol-lowering drugs increased bone mass and reduced osteoporosis-associated fracture risks^{12, 13}. However, related basic research about cholesterol in the differentiation of BMSCs has contradictory results¹⁴⁻¹⁶. One explanation is that not only cholesterol itself but some certain molecules related to cholesterol metabolism play a part in the process of BMSCs differentiation. Cholesterol-25-Hydroxylase (CH25H) is a multi-transmembrane endoplasmic reticulum related enzyme that catalyze cholesterol to produce 25-hydroxycholesterol (25HC). Recent studies about CH25H suggest it may participate in controlling the adipo-osteogenic differentiation of BMSCs. For instance, the overexpression of CH25H could improve systemic insulin sensitivity in mouse, while the insulin resistance induced by glucocorticoid was considered as a potential mechanism to explain the accumulation of fat and the decreased formation of bone^{17, 18}. In addition, CH25H could promote the expression of collagen, which plays an important part in osteogenesis¹⁹⁻²¹. But there is still no direct research on the function of CH25H in regulating BMSCs differentiation. Combined with studies about CH25H, we presume that CH25H might participate in regulated adipo-osteogenic differentiation of BMSCs and conducted an experimental demonstration in this study.

The mammalian target of rapamycin (mTOR) was discovered as a protein complex which is a member of protein kinase family related with Akt, integrating a variety of environmental cues to regulate organismal growth and homeostasis²². mTOR pathway contributed to maintain bone homeostasis and BMSCs lineage differentiation^{23, 24}. The activation of mTOR signaling pathway tends BMSCs to adipogenic differentiation and reduces osteogenic differentiation by regulating protein synthesis, adipose tissue morphogenesis and the activity of PPAR- γ ²⁵⁻²⁸. The inhibition of mTOR signaling pathway could promote osteogenesis and suppress adipogenesis²⁹.

In this study, to confirm CH25H participates in controlling the differentiation of BMSCs, we firstly detected the expression pattern of CH25H in the process of osteoblast and adipocyte differentiation. Then, through silencing or overexpressing CH25H in C3H10 α C2C12 and 3T3-L1 cell lines, we confirmed the impact of

expression levels of CH25H on osteogenesis and adipogenesis. The data of this study demonstrated CH25H could promote osteogenesis and inhibit adipogenesis by suppressing the mTOR signaling pathway, revealing a new function of CH25H in regulating BMSCs differentiation. These results may help further explain the pathogenesis of GIOP and give a new potential therapeutic target for this disease.

Materials And Methods

Reagents and antibodies

Dexamethasone (DXMS, D4902), L-Ascorbic acid (AA, A4403), β -Glycerophosphate (β -GP, G9422), Isobutylmethylxanthine (IBMX, I5879), Oil Red O (O1391), Indomethacin (ID, I7378), Alizarin red S (AR-S, A5533) and Rapamycin (553210) were purchased from Sigma Aldrich. Antibody to CH25H, mTOR, p-mTOR, p-P70S6K were from Absin. Antibodies to p-Akt and β -actin were purchased from Cell Signaling Inc. Antibody to ALP OCN, PPAR γ and CEBP α were purchased from Abclonal.

Culture of cells and osteogenic/ adipogenic induction

C3H10, C2C12, 3T3-L1 and 293 T cell lines were purchased from Cell Bank of the Chinese Academy of Science, Shanghai. All the four cell lines were cultured in fresh growth medium Which contains 90% high glucose DMEM (Gibco) and 10% FBS (Gibco). Cells were incubated in Cell incubator(ThermoFisher)at 37°C and 5% CO₂ humid atmosphere. Osteogenic induction medium was made from fresh growth medium containing 100mM DXMS, 50 μ M AA and 10 mM DXMS. Adipogenic induction medium was made from fresh growth medium containing with 500 mM IBMX, 200 mM ID, 1 μ M DXMS and 10 μ M insulin. The growth or induction medium was changed every two days.

Plasmids and viral infection

Three plasmids including pLKO.1-EGFP- puromycin, psPAX2, and pMD2.G (GeneChem, CHN) were involved in the lentivirus packing system for short hairpin RNA (shRNA) expression. Three CH25H RNAi sequences (Table 1) were chosen to knock down the expression of CH25H, which were annealed and inserted into the pLKO.1-EGFP- puromycin vector. Lentiviruses were packed in 293T cell line and infected targeted cells which seeded into 6 cm culture dishes at the density of around 60%. Stably transfected cells lines were screened out by puromycin (Sigma) for at least two weeks.

Table 1

	Sense strand	Antisense strand
shRNA1	5'-ACTCACCATCTTTACCTTT-3'	5'-AAAGGTAAAGATGGTGAGTGG-3'
shRNA2	5'-CATGCATCACTCTCAGTTT-3'	5'-AAACTGAGAGTGATGCATGTC-3'
shRNA3	5'-CTGTACACTTGATCCAGAA-3'	5'-TTCTGGATCAAGTGTACAGCG-3'
Control	5'-TTCTCCGAACGTGTCACGT-3'	

ALP, AR-S and Oil Red O staining

For osteogenic induction, alkaline phosphatase (ALP) and AR-S staining were chosen to show the formation of bone in cells. Differentiated cells were washed twice with PBS and fixed in 10% polyformaldehyde at 37°C for 10min. Then, for alkaline phosphatase (ALP) staining, an ALP staining kit (DE0004, Leagene) was applied in accordance with manufacturer's specifications. For AR-S staining, fixed cells were dyed with AR-S staining solution for 1 h at 37°C. Cells were rinsed in PBS to take residual ALP or AR-S solution away after staining completed, then airdried and photographed. For adipogenic induction, Oil Red O staining was used to measure the accumulation of lipid. Differentiated cells were rinsed with PBS and fixed in 10% polyformaldehyde for 10min in 37°C. The stock Oil Red O stain (0.5% in isopropanol) was diluted into 60% with ddH₂O. Then the diluted stain was applied to fixed cells for 1 h in room temperature. Then, Cells were rinsed in 75% ethanol to take residual Oil Red O solution away. Stained cells were observed and photoed by Inverted microscope (Nikon).

Quantitative real-time PCR

Cells were treated with RNAiso Plus (9108, Takara) to extract total RNA according to manufacturer's protocol. Tecan Infinity 200Pro multi-well plate reader was used to determine the concentration and quality of RNA samples. Then, the RNA samples were reverse transcribed into cDNA with the Primscript™ RT Master Mix (RR036A, Takara) for Quantitative real-time PCR. Reactions were implemented in a total volume of 10 µL, including 1 µL of 1:10 diluted cDNA, 5 µL 2×SYBR Premix Ex Taq and 0.2 µM of primers. The optimal amplification program was as follows: 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 34 s at 55°C, then the melting curve analysis of 15 s at 95°C, 1 min at 55°C, 15 s at 95°C and 15 s at 60°C. Each sample was established three holes and detected in three times repeatedly. The $2^{-\Delta\Delta CT}$ method was applied to analyze the results. β -actin was set as the internal reference to normalize the gene expression of samples. The primers used in qRT-PCR were searched from PrimerBank (<https://pga.mgh.harvard.edu/primerbank/>) and listed in Table 2.

Table 2

Gene	Forward	Reverse
CH25H	5'- CTGGGACACCATAAGGACAAG - 3'	5'- AAGCCCACGTAAGTGATGATAG - 3'
Runx2	5'- TTCAACGATCTGAGATTTGTGGG - 3'	5'- GGATGAGGAATGCGCCCTA - 3'
Col1α1	5'- GTGCGATGACGTGATCTGTGA - 3'	5'- CGGTGGTTTCTTGGTCGGT - 3'
OCN	5'- CACTCCTCGCCCTATTGGC - 3'	5'- CCCTCCTGCTTGGACACAAAG - 3'
CEBPα	5'- GCGGGAACGCAACAACATC - 3'	5'- GTCACTGGTCAACTCCAGCAC - 3'
PPARγ	5'- GGAAGACCACTCGCATTCTT - 3'	5'- GTAATCAGCAACCATTGGGTCA - 3'
SREB1	5'- TGACCCGGCTATTCCGTGA - 3'	5'- CTGGGCTGAGCAATACAGTTC - 3'
β-actin	5'- GGGACCTGACTGACTACCTC-3'	5'- TCATACTCCTGCTTGCTGAT-3'

Western blot

Western blotting was performed to compare the relative expression of genes at protein level. The medium of cells was removed and the adherent cells was washed with cold PBS twice. 60 µl RIPA(p0013b, Byotime) buffer with 1% PMSF(st506, Beyotime) was added to the 60mm dishes and the cells were scraped into EP tubes. Lysates were vibrated 10s every 5 min for 30 min at 0°C, and centrifuged at 12000 rpm at 4°C for 10 min to harvest the supernatant. The BCA kit(P0010, Beyotime)was used to determine the concentration of total protein. The protein sample was subjected to gel electrophoresis and transferred to PVDF membranes (Milipore, USA), the later were blocked using 5% non-fat milk for 2 h at room temperature. The PVDF membrane then incubated by primary antibodies at 4°C overnight, the membranes were rinsed by TBST three times for 10 min, and then probed with goat anti-rabbit or mouse secondary antibodies (7074, 4410, CST, USA) at room temperature for 2 h, and detection with an ECL kit (Share-bio, CHN). Images were read by Fluor Chem E system (Proteinsimple, USA).

In vivo ectopic bone formation assay

3× 10⁶ control or CH25H-overexpressed C3H10 cells was injected subcutaneously to skin under the right front leg of male BALB/c nude mice aged 4 weeks. 6 replicates were set in each group. Implants were harvested and fixed in 10% formaldehyde for Oil Red O, HE, Masson's staining and Immunohistochemistry (IHC) after six weeks' observation. All animal care and experimental protocols were approved by the Animal Care and Use Committee of Fudan University and complied with the Animal Management Guidelines of China.

Statistical analyzes

All the experiments were performed independently for three times at least. The results were analyzed with the Statistical Package for Social Sciences (SPSS) 20.0 software for Windows which was provided by Fudan university. The results were presented as the mean ± SD. Statistical analysis of the differences

between the groups was performed by analysis of variance (oneway ANOVA). $P < 0.05$ indicates that the difference was statistically significant. The value of $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ was considered statistically significant. The graphs were made by ggplot2 R package in the environment of R 4.0.2 for windows.

Results

3.1. CH25H expression was upregulated during osteogenesis and downregulated during adipogenesis.

We chose mouse mesenchymal stem cells C3H10 and mouse myoblast precursor cells C2C12 for osteoblasts induction, C3H10 and mouse adipose progenitor cells 3T3-L1 for adipocytes induction. All these three cell lines had been proved having osteogenic or adipogenic differentiation potential^{30,31}. Total protein and mRNA of the cells was collected after osteogenic induction for 0d, 7d and 14d, adipogenic induction for 0d, 5d, and 10d. As shown in Fig. 1A with the time for osteogenic induction extends, osteogenesis and mineralization degree enhanced as the ALP and AR-S staining deepen. The biomarkers Runx2, OCN and Col1 α 1 increased (Fig. 1C, D). Meanwhile, the mRNA and protein levels of CH25H were promoted in a gradual manner along with the osteogenic process (Fig. 1B-D). While during the process of adipogenic induction, fat droplets stained by Oil Red O in C3H10 and 3T3-L1 cell lines increased as shown in Fig. 1E, and the classic biomarkers CEBP α , PPAR γ and SREBP1 were increased as well (Fig. 1G-I). But the protein and mRNA levels of CH25H were decreased during this period (Fig. 1F). All of these findings suggested that CH25H might play an important role in the process of adipo-osteogenic differentiation.

3.2. Establishment of CH25H knocking down cell lines

Lentivirus transfection system were used to knockdown CH25H in C3H10, C2C12 and 3T3-L1 cell lines to deeply assess the effect of CH25H in adipo-osteogenic differentiation. To ensure the effect of knockdown, we designed three shRNA sequences and evaluated the efficiency of knockdown in both protein and mRNA levels respectively. Western blotting and qRT-PCR result as shown in Fig. 2. A and B suggested that CH25H expression was most significantly silenced in the CH25H-shRNA3 group, consistent in the three cell lines.

3.3. CH25H knockdown inhibited osteogenesis and promoted adipogenesis

We next examined the effect of CH25H in the process of adipo-osteogenic differentiation. The control and shRNA treated C3H10 and C2C12 cells were cultured in the osteogenic differentiation medium. We evaluated the difference of their osteogenesis ability by the means of ALP, AR-S staining and the biomarkers of osteogenesis (Fig. 2C, E-G). All the results proved that the ability of osteogenesis in CH25H knockdown cell lines was significantly weakened. In the aspect of regulating adipogenic differentiation, we cultured control or shRNA treated C3H10 and 3T3-L1 cell lines in adipogenic medium and used Oil

Red O staining to show the degree of lipid droplet formation at 0d, 5d and 10d. As shown in Fig. 2D, CH25H-knockdown cell lines had greater fat droplets formation capability, and the classic biomarkers of adipogenesis expressed more than control cells (Fig. 2H-J). Data above proved that CH25H knockdown inhibits osteogenesis and promotes adipogenesis.

3.4. CH25H knockdown activated the mTOR signaling pathway

For the purpose of exploring the potential mechanism involved, we then identified differences of key molecules in common osteogenesis related signaling pathways between the two groups. We found that mTOR signaling pathway, including Akt, p-Akt, mTOR, p-mTOR and p-P70S6K were upregulated in CH25H knockdown cell lines (Fig. 3A). what's more, we collected the protein samples in 0d and 14d during the process of osteogenic induction, 0d and 10d during the process of adipogenic process to test the key molecules of mTOR signaling pathway expression trends. As Fig. 3B and C shown, the mTOR signaling pathway was not only reflected different expression trends in the process of osteogenic or adipogenic differentiation but also highly expressed in CH25H knockdown cell lines in both three cell lines. As an important signaling pathway for the regulation of cell differentiation and development, there have been researches proved that mTOR pathway could regulate lineage differentiation between osteoblasts and adipocytes^{23, 32}. We hypothesized that mTOR signaling play a role in CH25H-induced change in MSCs differentiation.

3.5. Rapamycin rescued the shift of adipo-osteogenic balance in MSCs caused by CH25H knockdown

To certify the function of mTOR signaling pathway in CH25H induced change of differentiation direction, we used rapamycin, a specific inhibitor of mTOR, finding that with rapamycin treatment, the osteogenesis inhibition effect caused by CH25H knockdown was rescued (Fig. 4A-D) and the expression levels of mTOR, p-mTOR, p-P70S6K were suppressed as well (Fig. 4E). In the process of adipogenic differentiation, the using of rapamycin also rescued the adipogenesis enhancement caused by CH25H knockdown (Fig. 5A-D), and the proteins of mTOR, p-mTOR, p-P70S6K were suppressed before and after adipogenic induction (Fig. 5E).

3.6. Overexpression of CH25H promotes osteogenesis and suppresses adipogenesis through inhibiting the mTOR signaling pathway.

In order to validate the results above, we established CH25H overexpression C3H10 cell lines (C3H10-cDNA), and test expression effect of CH25H in both protein and mRNA levels (Fig. 6A-B). As Fig. 6C showed, contrary to C3H10-blank cells, the mTOR signaling pathway is inhibited in C3H10-cDNA cell lines. ALP and AR-S and Oil Red O staining shows that in C3H10-cDNA cells, the differentiation of osteoblasts was upregulated, while adipogenesis was suppressed (Fig. 6D and E). The express changes of biomarker in mRNA levels was consistent with the results of staining (Fig. 6F-K).

3.7. The function of CH25H in regulating osteo-adipogenic differentiation in vivo

To confirm the results in vitro, we performed animal assays with CH25H coexpression C3H10 cell lines and blank C3H10 cell lines. 4×10^6 of these two kinds of cells were injected into nude mice aged four weeks respectively (6 independent repeats in each group). After six weeks feeding, implants were taken out. HE, Masson, Oil Red O staining and IHC were used to confirm the degree of osteogenic or adipogenic differentiation. As shown in Fig. 7A, CH25H-overexpressed C3H10 cells had an increased tendency of osteogenesis and a decreased tendency of adipogenesis. The results of IHC (Fig. 7B) indicated that in CH25H-overexpressed cells, the expression ALP and OCN were upregulated and the expression of PPAR- γ and CEBP α were downregulated. The results of animal experiments furtherly verified the conclusions of experiments in vitro.

Discussion

Glucocorticoid-induced osteoporosis (GIOP), which is a common form of secondary osteoporosis, characterized as abnormal decreased osteogenic differentiation and increased adipogenic differentiation, has been relatively well established^{33, 34}. However, the underlying mechanisms are not fully understood. Bone marrow surrounds the trabecular elements of the skeleton and is made up of bone marrow mesenchymal stem cells (BMSCs). As a type of non-hematopoietic stem cell with strong potential for multipotent differentiation, BMSCs differentiate under the regulation of multiple pathways. These signaling pathways eventually effect on Runx2, which controls osteogenic differentiation, or nuclear receptor PPAR γ , which controls adipogenic differentiation, and eventually determine how stem cells mature. For the osteoblasts and adipocytes have a common origin, a switching mechanism in BMSCs might help to explain previous observations that enhanced bone marrow adipogenesis seemed to be at the cost of osteoblast differentiation^{35, 36}. For example, the BMP pathway could regulate the commitment of BMSCs to the osteoblast lineage. While Akt pathway could enhance adipogenesis by activation CEBPs. However, BMSCs lineage differentiation could be regulated at various molecular levels, when they are stimulated by multiple micro-environmental factors. The lineage switch between osteoblasts and adipocytes might be regulated by multiple molecular pathways, such as mTOR and Wnt/ β -Catenin signaling.

The mTOR, a conserved serine-threonine protein kinase, belongs to the Akt related kinase family. Recent evidence has indicated that mTOR could modulate cell proliferation and differentiation in various of cell types. Studies in vitro and in vivo have demonstrated that the expression of mTOR is induced in several types of cells, including adipocytes^{37–39}. It is reported that mTOR signaling plays a crucial role in both initiating and maintaining adipogenesis by promoting the expression of adipogenesis related gene PPAR γ , which is crucial for the feedback of CEBP α and other adipogenesis related gene expression program^{37, 39}. The use of rapamycin could inhibit mTOR-activated adipogenesis⁴⁰. In osteogenesis, the use of rapamycin could rescue the reduced capacity to form new bone caused by mTOR activation²⁹.

Based on the results above, it is reasonable to speculate that mTOR signaling pathway is a key switch to determine the lineage differentiation of BMSCs.

As a kind of hydroxylase, the main function of CH25H is to oxidize cholesterol to be 25-hydroxycholesterol(25HC)⁴¹. In regulating cholesterol, CH25H are traditionally regarded as an important regulator that maintain cholesterol homeostasis by inhibiting sterol regulator-binding protein (SREBP) and activating liver X receptor (LXR), which are two key transcriptional regulators^{42, 43}. These years, furtherly studies about CH25H had revealed that CH25H is not only an enzyme involved in cholesterol metabolism but has many other biological functions like immunomodulatory and participating in inflammation^{44, 45}. But there has no report whether CH25H could regulate the differentiation of BMSCs. In this study, to strengthen the reliability of our data, three cell lines including C3H10, C2C12 and 3T3-L1 were used. Among them, C3H10 was used in both adipogenic and osteogenic induction, C2C12 and 3T3-L1 cell lines were respectively used for osteogenic and adipogenic induction. The corresponding inductivity of each cell line has been widely used by researchers^{46–49}. Our design insured that there was data of two kinds of cells in each experiment. We demonstrated that CH25H could promote osteogenesis and inhibit adipogenesis through suppressing the mTOR signaling pathway. For the first time, to our knowledge, we revealed the function of CH25H in regulating BMSCs differentiation. The data we provide may help furtherly understand the adipo-osteogenic differentiation regulation, and give a potential therapeutic target in treating disease caused by disordered differentiation of BMSCs like GIOP.

Conclusion

In this study, we demonstrated that CH25H could increase osteogenesis and suppress adipogenesis for the first time. Besides, the mTOR signaling pathway was found to be the downstream signaling pathway of CH25H in regulating osteo-adipogenic differentiation of MSCs. Taken together, our results indicate that CH25H promotes osteogenesis and suppresses adipogenesis via the mTOR signaling pathway.

Abbreviations

BMSCs

bone marrow stromal cells

GIOP

glucocorticoid-induced osteoporosis

CH25H

cholesterol-25-Hydroxylase

25HC

25-hydroxycholesterol

mTOR

the mammalian target of rapamycin

shRNA

short hairpin RNA
ALP
alkaline phosphatase
AR-S
alizarin red S
qRT-PCR
quantitative real time polymerase chain reaction
DXMS
Dexamethasone
AA
L-Ascorbic acid
 β -GP
 β -Glycerophosphate
IBMX
isobutylmethylxanthine
ID
indomethacin
IHC
immunohistochemistry

Declarations

Consent for publication:

Not applicable.

Data Statement

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

Ethical Approval and Consent to participate:

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Competing interests:

The authors declare that there are no conflicts of interest.

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Author Contribution Statement:

HR Li and L Zhou: The acquisition, analysis, interpretation of the data for the work. SW Sun and TQ Zhang: Substantial contributions to the conception and design of the work.

WL Zhou and TL Zhang: Revising this work critically for important intellectual content.

MH Wang: Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

All authors have read and approved the manuscript.

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References

1. Adami G, Saag KG. Glucocorticoid-induced osteoporosis update. *Curr Opin Rheumatol*. 2019;31(4):388-393. <https://doi.org/10.1097/BOR.0000000000000608>
2. Chiodini I, Merlotti D, Falchetti A, Gennari L. Treatment options for glucocorticoid-induced osteoporosis. *Expert opinion on pharmacotherapy*. 2020;21(6):721-732. <https://doi.org/10.1080/14656566.2020.1721467>
3. Masaki H, Miki T. [Biochemical markers of bone in steroid (glucocorticoid)-induced osteoporosis (GIOP)]. *Clinical calcium*. 2006;16(11):1823-1832.
4. Komori T. Glucocorticoid Signaling and Bone Biology. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme*. 2016;48(11):755-763.
5. Han L, Wang B, Wang R, Gong S, Chen G, Xu W. The shift in the balance between osteoblastogenesis and adipogenesis of mesenchymal stem cells mediated by glucocorticoid receptor. *Stem cell research & therapy*. 2019;10(1):377. <https://doi.org/10.1186/s13287-019-1498-0>
6. Alagiakrishnan K, Mereu LE, Tsuyuki RT, Kalisiak M, Sclater A, Hervas-Malo M. Cholesterol and osteoporosis in postmenopausal women: a pilot study. *Journal of the American Geriatrics Society*. 2003;51(10):1510-1511.

7. Leutner M, Matzhold C, Bellach L, et al. Diagnosis of osteoporosis in statin-treated patients is dose-dependent. *Annals of the rheumatic diseases*. 2019;78(12):1706-1711.
<https://doi.org/10.1136/annrheumdis-2019-215714>
8. Reed RM, Wise RA, Dobs AS, Lechtzin N, Girgis RE. Elevated HDL cholesterol levels are associated with osteoporosis in lung transplant candidates with chronic obstructive pulmonary disease. *Respiratory medicine*. 2010;104(12):1943-1950. <https://doi.org/10.1016/j.rmed.2010.08.004>
9. Sun S, Adyshev D, Dudek S, Paul A, McColloch A, Cho M. Cholesterol-dependent Modulation of Stem Cell Biomechanics: Application to Adipogenesis. *Journal of biomechanical engineering*. 2019;<https://doi.org/10.1115/1.4043253>
10. Viccica G, Vignali E, Marcocci C. Role of the cholesterol biosynthetic pathway in osteoblastic differentiation. *Journal of endocrinological investigation*. 2007;30(6 Suppl)
11. Mandal CC. High Cholesterol Deteriorates Bone Health: New Insights into Molecular Mechanisms. *Frontiers in endocrinology*. 2015;6:165. <https://doi.org/10.3389/fendo.2015.00165>
12. Meier CR, Schlienger RG, Kraenzlin ME, Schlegel B, Jick H. HMG-CoA reductase inhibitors and the risk of fractures. *JAMA*. 2000;283(24):3205-3210.
13. Edwards CJ, Hart DJ, Spector TD. Oral statins and increased bone-mineral density in postmenopausal women. *Lancet (London, England)*. 2000;355(9222):2218-2219.
14. Ghosh-Choudhury N, Mandal CC, Choudhury GG. Statin-induced Ras activation integrates the phosphatidylinositol 3-kinase signal to Akt and MAPK for bone morphogenetic protein-2 expression in osteoblast differentiation. *The Journal of biological chemistry*. 2007;282(7):4983-4993.
15. Huang P, Nedelcu D, Watanabe M, et al. Cellular Cholesterol Directly Activates Smoothed in Hedgehog Signaling. *Cell*. 2016;166(5)<https://doi.org/10.1016/j.cell.2016.08.003>
16. Luchetti G, Sircar R, Kong JH, et al. Cholesterol activates the G-protein coupled receptor Smoothed to promote Hedgehog signaling. *eLife*. 2016;5<https://doi.org/10.7554/eLife.20304>
17. Almon RR, Dubois DC, Jin JY, Jusko WJ. Temporal profiling of the transcriptional basis for the development of corticosteroid-induced insulin resistance in rat muscle. *J Endocrinol*. 2005;184(1):219-232.
18. Noebauer B, Jais A, Todoric J, Gossens K, Sutterlüty-Fall H, Einwallner E. Hepatic Cholesterol-25-Hydroxylase Overexpression Improves Systemic Insulin Sensitivity in Mice. *J Diabetes Res*. 2017;2017:4108768. <https://doi.org/10.1155/2017/4108768>
19. Raselli T, Wyss A, Gonzalez Alvarado MN, et al. The Oxysterol Synthesising Enzyme CH25H Contributes to the Development of Intestinal Fibrosis. *J Crohns Colitis*. 2019;13(9):1186-1200.

<https://doi.org/10.1093/ecco-jcc/jjz039>

20. Bardai G, Lemyre E, Moffatt P, et al. Osteogenesis Imperfecta Type I Caused by COL1A1 Deletions. *Calcif Tissue Int.* 2016;98(1):76-84.
21. Besio R, Maruelli S, Battaglia S, et al. Early Fracture Healing is Delayed in the Col1a2 Osteogenesis Imperfecta Murine Model. *Calcif Tissue Int.* 2018;103(6):653-662. <https://doi.org/10.1007/s00223-018-0461-x>
22. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell.* 2012;149(2):274-293. <https://doi.org/10.1016/j.cell.2012.03.017>
23. Chen C, Akiyama K, Wang D, et al. mTOR inhibition rescues osteopenia in mice with systemic sclerosis. *J Exp Med.* 2015;212(1):73-91. <https://doi.org/10.1084/jem.20140643>
24. Hadji P, Coleman R, Gnant M. Bone effects of mammalian target of rapamycin (mTOR) inhibition with everolimus. *Crit Rev Oncol Hematol.* 2013;87(2):101-111. <https://doi.org/10.1016/j.critrevonc.2013.05.015>
25. Lynch CJ, Patson BJ, Anthony J, Vaval A, Jefferson LS, Vary TC. Leucine is a direct-acting nutrient signal that regulates protein synthesis in adipose tissue. *American journal of physiology Endocrinology and metabolism.* 2002;283(3):E503-E513.
26. Fox HL, Kimball SR, Jefferson LS, Lynch CJ. Amino acids stimulate phosphorylation of p70S6k and organization of rat adipocytes into multicellular clusters. *The American journal of physiology.* 1998;274(1):C206-C213. <https://doi.org/10.1152/ajpcell.1998.274.1.C206>
27. Roh C, Han J, Tzatsos A, Kandror KV. Nutrient-sensing mTOR-mediated pathway regulates leptin production in isolated rat adipocytes. *American journal of physiology Endocrinology and metabolism.* 2003;284(2):E322-E330.
28. Kim JE, Chen J. regulation of peroxisome proliferator-activated receptor-gamma activity by mammalian target of rapamycin and amino acids in adipogenesis. *Diabetes.* 2004;53(11):2748-2756.
29. Liu Y, Kou X, Chen C, et al. Chronic High Dose Alcohol Induces Osteopenia via Activation of mTOR Signaling in Bone Marrow Mesenchymal Stem Cells. *Stem Cells.* 2016;34(8):2157-2168. <https://doi.org/10.1002/stem.2392>
30. Sun S, Zhou L, Yu Y, Zhang T, Wang M. Knocking down clock control gene CRY1 decreases adipogenesis via canonical Wnt/ β -catenin signaling pathway. *Biochem Biophys Res Commun.* 2018;506(3):746-753. <https://doi.org/10.1016/j.bbrc.2018.10.134>
31. Zhou L, Sun S, Xu L, Yu Y, Zhang T, Wang M. DExH-Box helicase 58 enhances osteoblast differentiation of osteoblastic cells via Wnt/ β -Catenin signaling. *Biochem Biophys Res Commun.*

2019;511(2):307-311. <https://doi.org/10.1016/j.bbrc.2019.02.039>

32. Xiang X, Zhao J, Xu G, Li Y, Zhang W. mTOR and the differentiation of mesenchymal stem cells. *Acta Biochim Biophys Sin (Shanghai)*. 2011;43(7):501-510. <https://doi.org/10.1093/abbs/gmr041>
33. Adami G, Saag KG. Glucocorticoid-induced osteoporosis: 2019 concise clinical review. *Osteoporos Int*. 2019;30(6):1145-1156. <https://doi.org/10.1007/s00198-019-04906-x>
34. Li G-W, Xu Z, Chen Q-W, Chang S-X, Tian Y-N, Fan J-Z. The temporal characterization of marrow lipids and adipocytes in a rabbit model of glucocorticoid-induced osteoporosis. *Skeletal Radiol*. 2013;42(9):1235-1244. <https://doi.org/10.1007/s00256-013-1659-7>
35. Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *J Cell Sci*. 1992;102 (Pt 2):341-351.
36. Gimble JM, Robinson CE, Wu X, Kelly KA. The function of adipocytes in the bone marrow stroma: an update. *Bone*. 1996;19(5):421-428.
37. Yu W, Chen Z, Zhang J, et al. Critical role of phosphoinositide 3-kinase cascade in adipogenesis of human mesenchymal stem cells. *Mol Cell Biochem*. 2008;310(1-2):11-18.
38. Martin SK, Fitter S, Dutta AK, et al. Brief report: the differential roles of mTORC1 and mTORC2 in mesenchymal stem cell differentiation. *Stem Cells*. 2015;33(4):1359-1365. <https://doi.org/10.1002/stem.1931>
39. Zhang HH, Huang J, Düvel K, et al. Insulin stimulates adipogenesis through the Akt-TSC2-mTORC1 pathway. *PLoS One*. 2009;4(7):e6189. <https://doi.org/10.1371/journal.pone.0006189>
40. Yeh WC, Bierer BE, McKnight SL. Rapamycin inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells. *Proc Natl Acad Sci U S A*. 1995;92(24):11086-11090.
41. Walther TC, Farese RV. Lipid droplets and cellular lipid metabolism. *Annual review of biochemistry*. 2012;81:687-714. <https://doi.org/10.1146/annurev-biochem-061009-102430>
42. Brown MS, Dana SE, Goldstein JL. Cholesterol ester formation in cultured human fibroblasts. Stimulation by oxygenated sterols. *The Journal of biological chemistry*. 1975;250(10):4025-4027.
43. Lehmann JM, Kliewer SA, Moore LB, et al. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *The Journal of biological chemistry*. 1997;272(6):3137-3140.
44. Ouyang W, Zhou H, Liu C, et al. 25-Hydroxycholesterol protects against acute lung injury via targeting MD-2. *Journal of cellular and molecular medicine*. 2018;22(11):5494-5503. <https://doi.org/10.1111/jcmm.13820>

45. Park K, Scott AL. Cholesterol 25-hydroxylase production by dendritic cells and macrophages is regulated by type I interferons. *Journal of leukocyte biology*. 2010;88(6):1081-1087. <https://doi.org/10.1189/jlb.0610318>
46. Hashimoto Y, Matsuzaki E, Higashi K, et al. Sphingosine-1-phosphate inhibits differentiation of C3H10T1/2 cells into adipocyte. *Mol Cell Biochem*. 2015;401(1-2):39-47. <https://doi.org/10.1007/s11010-014-2290-1>
47. Chen S-Z, Xu X, Ning L-F, et al. miR-27 impairs the adipogenic lineage commitment via targeting lysyl oxidase. *Obesity (Silver Spring)*. 2015;23(12):2445-2453. <https://doi.org/10.1002/oby.21319>
48. Guan X, Gao Y, Zhou J, et al. miR-223 Regulates Adipogenic and Osteogenic Differentiation of Mesenchymal Stem Cells Through a C/EBPs/miR-223/FGFR2 Regulatory Feedback Loop. *Stem Cells*. 2015;33(5):1589-1600. <https://doi.org/10.1002/stem.1947>
49. Tang Z, Xu T, Li Y, Fei W, Yang G, Hong Y. Inhibition of CRY2 by STAT3/miRNA-7-5p Promotes Osteoblast Differentiation through Upregulation of CLOCK/BMAL1/P300 Expression. *Molecular therapy Nucleic acids*. 2020;19:865-876. <https://doi.org/10.1016/j.omtn.2019.12.020>

Figures

Figure 1

Association of CH25H with osteoblast and adipocyte differentiation. A. ALP and AR-S staining during osteogenesis of C3H10 and C2C12 cells. B. Western blotting results indicated the expression of CH25H in C3H10 and C2C12 was upregulated during the time osteogenesis. C and D. The relative mRNA levels of CH25H and classic biomarkers including Runx2, Col1α1 and OCN tested by qRT-PCR. E. Oil Red O staining showed the increase of lipid droplet formation during adipogenic induction in C3H10 and 3T3-L1 cells. F. The protein levels of CH25H decreased during the process of adipogenesis in C3H10 and 3T3-L1 cells. G-I. The mRNA levels of CH25H and biomarkers including CEBPα, PPARγ and SREB1. Values are expressed as the mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001

Figure 2

Knockdown of CH25H suppressed osteogenesis and promoted adipogenesis. A and B. the effects of CH25H knockdown detected by Western blotting and qRT-PCR in protein and mRNA levels of three cell lines. C. AR-S and ALP staining showed the osteogenesis and extracellular mineralization were suppressed in CH25H knockdown cells. G. The fat droplet formation was increased in CH25H knockdown cells during adipogenic inducing. E-G. The relative mRNA levels of Runx2, Col1a1 and OCN were

downregulated in CH25H knockdown cells during the process of osteogenic induction. H-J. Relative mRNA levels of CEBP α , PPAR γ and SREB1 were upregulated in CH25H knockdown cells during the process of adipogenic induction. Values are expressed as the mean $\pm$ SD. *P < 0.05, **P < 0.01 and ***P < 0.001

Figure 3

Knockdown upregulated the mTOR signaling pathway. A. Akt, p-Akt, mTOR, p-mTOR and p-P70S6K were promoted after CH25H knockdown in C3H10, C2C12 and 3T3-L1 cell lines. B and C. The expression levels of Akt, p-Akt, mTOR, p-mTOR and p-P70S6K at the 0d, 7d and 14d in CH25H knockdown and control cell lines during the process of osteogenic induction. C. The expression levels of Akt, p-Akt, mTOR, p-mTOR and p-P70S6K at the 0d, 5d and 10d in CH25H knockdown and control cell lines during the process of adipogenic induction

Figure 4

The suppression of mTOR reversed the suppression effect of osteogenesis in CH25H knockdown cells. A. AR-S and ALP staining showed the osteogenesis and extracellular mineralization were enhanced when added rapamycin on the 14th day of osteogenic induction compared to CH25h knockdown cells. B-D. The expression of the biomarkers of osteogenesis including Runx2, Col1 α 1, OCN in control, shRNA and shRNA+Rapamycin groups on the tenth day of adipogenic induction. E. The expression levels of mTOR, p-mTOR and p-P70S6K in control, shRNA and shRNA+Rapamycin groups on the 0d and 14d of osteogenic induction.

Figure 5

The suppression of mTOR reversed the enhancement of osteogenesis in CH25H knockdown cells. A. Red Oil O staining showed the fat droplet formation was decreased when added rapamycin on the tenth day of adipogenic induction compared to CH25h knockdown cells. B-D. The expression of the biomarkers of osteogenesis including CEBPα,PPARγ and SREB1 in control, shRNA and shRNA+Rapamcyin groups on the tenth day of adipogenic induction. E. The expression levels of mTOR, p-mTOR and p-P70S6K in control, shRNA and shRNA+Rapamcyin groups on the 0d and 10d of adipogenic induction.

Figure 6

The overexpression of CH25H in C3H10 suppressed the mTOR signaling pathway, promoted osteogenesis and suppressed adipogenesis. A and B. the protein and mRNA levels of CH25H in C3H10-Blank and C3H10-cDNA groups. C. the key moleculars of mTOR signaling pathways were suppressed in CH25H overexpressed cells. D. AR-S and ALP staining showed the osteogenesis and extracellular mineralization were enhanced in CH25H overexpress cells. E. The Oil Red O staining indicated the adipogenic differentiation was suppressed in CH25H overexpress cells. G-K. the relative mRNA levels of osteogenesis and adipogenesis biomarkers during the process of osteogenic and adipogenic induction between C3H10-Blank and C3H10-cDNA groups.

Figure 7

The overexpression of CH25H promoted osteogenesis and suppressed adipogenesis in vivo. A. HE, Masson and Oil Red O staining shown that CH25H-overexpressed C3H10 cells has an increased tendency of osteogenesis and a decreased tendency of adipogenesis. B. ALP and OCN were highly expressed while PPAR γ and CEBP α were downregulated in CH25H-overexpressed C3H10 cell lines.