Lithium chloride enhances osteoblast differentiation and resists senile osteoporosis

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Research article

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Abstract

Background

Lithium chloride (LiCl) is commonly used in the clinic for the treatment of bipolar and other mental disorders. LiCl is an inhibitor of GSK-3β, and has been reported to modulate the balance of adipogenesis and osteogenesis. But, whether LiCl impacts bone formation and homeostasis in senile osteoporosis is still unclear.

Methods

Analysis of tibia in 2, 5, 7 and 10 months old C57BL/6 male mice were performed by MicroCT (μCT). 7 months old wild-type mice were treated with LiCl orally 0, 100 or 200 mg/kg for 3 months and then tested by μCT. The levels of osteogenesis marker genes and Wnt signaling target genes in bone marrow stromal cells (BMSCs) were detected by reverse transcription quantitative polymerase chain reaction and immunostaining. BMSCs were induced osteoblast differentiation and tested by Alizarin red S staining.

Results

μCT analyses of C57BL/6 mice showed that bone mineral density (BMD) and trabecular thickness (Tb.Th) increased until the bone mass peaked (5 months) and then began to fall subsequently. LiCl dramatically enhanced bone mass in the senile osteoporotic conditions, represented by increased ratio of bone volume to tissue volume (BV/TV), and decreased in trabeculae separation (Tb.Sp). Moreover, LiCl significantly increased both canonical osteoblastogenesis and Wnt signaling activity without affecting hormones.

Conclusion

This study uncovered the role of LiCl in canonical Wnt signaling and bone formation and have provided the evidence that LiCl may potentially repress senile osteoporosis.

Background

Osteoporosis is a systemic skeletal disease, characterized by a low bone mass and bone density, resulting in a decrease in bone strength. Globally, osteoporosis still has very high morbidity and mortality, and therefore necessitates a more effective treatment [1]. For the treatment of osteoporosis, the most commonly used drugs have been bone resorption inhibitors. However, micro-injuries of bone may result from use of resorption inhibitors, and has to be considered [2]. Similarly, due to its role in promoting osteogenesis, parathyroid hormone (PTH) has been approved by the Food and Drug Administration. But, prolonged administration of PTH has been shown to lead to an increased risk of osteosarcoma [3, 4]. Therefore, a pharmacologic approach to increase bone mass by stimulating osteogenesis remains a longstanding challenge in medicine.

It has been reported that several intracellular signaling pathways can regulate the differentiation of bone marrow mesenchymal stem cells (BMSCs) into osteoblasts [5]. Among them, the Wnt signaling increases the expression of runt-related transcription factor 2 (Runx2), and promotes osteoblast differentiation [6, 7]. Wnt signaling gets activated by growth factor stimulation, and by inactivated glycogen synthase kinase 3β (GSK-3β) [8, 9].

Lithium chloride is considered a safe drug for the early treatment of mental disorders and the prevention of bipolar disorder [10]. It up regulates Wnt/β-catenin signaling by inhibiting GSK-3β [11]. Interestingly, according to previous research, the patient's bone mass increased after taking lithium, whereas the bone transformation decreased [12]. We have found that LiCl inhibits lipogenesis by regulating Wnt and Hh signaling, thereby significantly promotion of osteogenesis of human BMSCs [13]. Moreover, LiCl enhanced bone mass and implant oseoientegration in rats with osteoporosis [14].

A decrease in bone mass accompanying advancing age is not specific to humans, but also in other mammals, such as mice and rat. Despite a clear role for the involvement of LiCl in Wnt signaling, its role in the osteogenic differentiation and bone formation is still elusive [15–18]. Here, we reported the role of LiCl in bone formation and homeostasis in senile osteoporosis. We used C57BL/6 mice as senile osteoporosis model to investigate the effects of LiCl on maintaining the bone homeostasis in adulthood through the Wnt signaling, with an objective to identify a compound that can target the osteoblast for senile osteoporosis.

Methods

Cell culture

We first washed the tibia and femur with DMEM medium and separated for bone marrow stromal cells (BMSCs). We washed the bone marrow cells twice with phosphate buffered saline (PBS), and pelleted the cells at 3,000 g for 10 min. Subsequently, we used 70% percoll to hold 2×10^8 cells. The cells in the low-density component were harvested and further cultured in DMEM supplemented with 10% FBS and 100 units/mL of penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. We then collected the low density cells and cultured them in DMEM medium with 100 units/mL penicillin/streptomycin and 10% FBS at 37 °C.

Mice

We purchased 2, 5, 7 and 10 months old C57BL/6 male mice from Shanghai SLAC Laboratory Animal Co. (Shanghai, China) and administered LiCl orally. We also administered 7 months old wild-type mice with LiCl orally with 0, 100 or 200 mg/kg for 3 months (n = 10 per group). Animal feeding environment consisted of relative humidity of 60%, temperature of 22–25 °C, and drink water available freely. All animals were housed and bred at Zhejiang University Animal Care Facility according to the institutional guidelines for laboratory animals, and the protocol was approved by the Zhejiang University Institutional Animal Care and Use Committee. The mice in all groups were sacrificed by intraperitoneal injection

sodium pentobarbital overdose (300 mg/kg) at indicated time and purchased the lumbar and tibia for μCt and histological detection.

Osteogenic induction

We seeded the BMSCs in a 6-well plate, and induced osteoblast differentiation by adding 50 μM ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone in DMEM medium. In general, we found calcium nodes after 28 days of induction.

Alizarin red S staining

We then discarded the medium and then rinsed the cells twice with PBS. We then fixed the cells with 4% paraformaldehyde for 30 min at room temperature. We washed the cells 3 times with distilled water, and used 2% Alizarin Red S to stain the cells for 30 min at 37 °C. Finally, we examined the Alizarin stained cells under a microscope.

RT-PCR

We extracted total RNA by using Invitrogen using TRIzol, and performed reverse transcription by using an ABI kit (Grand Island, NY, USA). Primers corresponding to the target genes are as follows: ALP: AACAACCTGACTGACCCTTCG and ATGGGAGCCAGACCAAAGAT; Runx2: CCG CCT CAG TGA TTT AGGGC and GGG TCT GTA ATC TGA CTC TGTCC; OC: TCGTGTGTCTTCTCCACAGC and AGCCCTCTGCAGGTCATAGA; Lef1: GAAATCATCCCAGCCAGCAA and GCATCATTATGTAGCCAGAGTA; CyclinD1: AAAATGCCAGAGGCGGATGA and CAGTCCGGGTCACACTTGA; Dkk1: TTTGCCTGTTTGCGTCCTTC and TACTTGTTCCCGCCCTCA. β-actin was applied as internal control, and all experiments were repeated three times.

μCt scanning

After sacrificing the mice, we removed the tibias and fixed them in 4% formaldehyde for 3 days. We used μCT 40 (Scanco Medical AG, Brüttisellen, Switzerland) for μCT analyses and quantified one hundred 16-μm slices, with a threshold of 200.

H&E, trap staining and immunostaining

We first decalcified the tibia samples in 14% EDTA for 1 month, then embed it in paraffin. After the tissues were paraffinized, we subjected the 5 μm sections to H&E and trap staining. We then used the SP Rabbit HRP kit to perform immunohistochemistry staining (Kangwei Reagents, Beijing, China). Briefly, we decarboxylated the sections obtained with xylene and then treated with a series of fractionated ethanol. We then performed antigen recovery with 10 mM citric acid and sodium citrate, and treated the sections with 3% H_2O_2, blocked with normal serum for 1 h, and incubated with primary antibodies overnight at 4 °C. We then treated the sections with HRP-labeled secondary antibody for 30 min and the diaminobenzidine (DAB) until a brown color developed. Antibodies against ALP (ab224335), Lef (ab22569) and OC (ab93876) were purchased from Abcam.

ELISA assay

We collected the serum samples from the euthanized mice and stored them at -80 °C. We then measured serum T4, PTH and estrogen levels using ELISA kit (BioVision, San Francisco, USA). We carried out the analysis according to the manufacturer's protocol. We then extrapolated the absolute concentrations based on the standard curves.

Statistical analysis

Numerical data are expressed as means ± SD from six mice or three independent cultures. We used the SigmaStat software, Tukey-Kramer multiple comparison test and one-way ANOVA for statistical analyses. Comparisons were called statistically significant if $P < 0.05$ and ** $P < 0.01$.

Results

Mouse models of senile osteoporosis

According to previous studies, the bone mass of the laboratory mice with a life-span of 2-3years, reaches its maximum between 4 to 8 months, after which the bone mass decreases with time [19, 20]. We purchased 2, 5, 7 and 10 months old C57BL/6 female mice (n = 6) and measured their bone mineral density (BMD) by micro computed tomography (μCT). Three-dimensional reconstruction of these mice using μCT showed that the lumbar and the tibia BMD reached its highest value at 5 months and its lowest at 7 months. Within a five-month period, trabecular thickness (Tb.Th) and cancellous bone volume fraction (BV/TV) showed an increasing trend but then began to decline. The trabecular number (Tb.N) significantly decreased from 5 months, but trabecular separation (Tb.Sp) increased from 5 months (Fig. 1A -C).

It has been pointed out that the bone loss associated with age is based on decreased osteogenic potential. We also found that from 5 or 7 months in BMSCs, the mice showed decreased mRNA levels of osteogenesis marker genes, including osteocalcin (OC), alkaline phosphatase (ALP), and Runx2 (Fig. 2).

To assess the roles of canonical Wnt signaling in senile osteoporosis, we first assayed the mRNA levels of Wnt signaling markers in BMSCs. Based on Fig. 2, Wnt signaling markers including lymphoid enhancer-binding factor-1 (Lef1), CyclinD1 and Dickkopf-1 (Dkk1) were all increased at 5 months. After that, they declined with age.

At menopause, estrogen deficiency increases bone turnover. Since during bone turn over, the bone formation is less than bone resorption, bone loss and finally osteoporosis may result. We examined the levels of thyroxine (T4), parathyroid hormone (Pth), and estrogen in serum from above mice, and found that the levels of T4 and estrogen but not PTH declined with age (Fig. 3A).

Based on these results, we concluded that aged mice may be proper models for senile osteoporosis research.

Increases in bone mass after systemic treated with LiCl in vivo

We tested the effect of LiCl, a known GSK3β inhibitor on senile osteoporosis. We intragastrically administered LiCl into 7-months old mice for 3 months at 100 or 200 mg/kg daily. Because of the death in the process of intragastrically administered LiCl, we got data from six animals per group. As shown by the morphological analysis, LiCl at 200 mg/kg robustly increased the mass of the cancellous bone (Fig. 4A and B). This result was further confirmed by quantitative analysis of bone mass parameters. Compared to saline treatment, LiCl treatment at 100 mg/kg slightly increased BV/TV and Tb.N. There was no significant difference of Tb.Th between LiCl treatment at 100 mg/kg and vehicle treatment mice. LiCl significantly increased BV/TV and Tb.N at 200 mg/kg, and markedly reduced the Tb.Sp, without affecting Tb.Th (Fig. 4C). Moreover, there were more trabecular changes in the histology of lumbar sections from the LiCl group, whereas fewer trabecular number in the vehicle mice (Fig. 5A). Our data indicated that systemic treatment with LiCl resists the bone loss with advanced age.

The effect of LiCl on hormones

Lithium can cause the development of hypothyroidism by inhibiting the release of thyroid hormones [21]. In order to explore the effect of LiCl on the levels of serum hormones in our senile osteoporosis model, we examined the levels of T4, PTH and estrogen. As shown in Fig. 3B, LiCl treatment at 200 mg/kg daily for 3 months, increased the level of T4 but not PTH and estrogen. Thus, these data suggest that the increases in bone mass by treatment with LiCl is not due to hormones.

LiCl stimulates osteoblastic differentiation

To determine whether LiCl enhances bone mass via changes in bone formation or bone resorption, we conducted a study. We first examined the role of LiCl in osteoclastogenesis, by trap staining the lumbar vertebra sections. There was no apparent difference in the number of trap-positive osteoclasts between LiCl treated and vehicle mice (Fig. 5A). We then measured the bone nodules by alizarin-red S staining in BMSCs for bone formation activity. We found that LiCl treatment at both 100 mg/kg and 200 mg/kg, lead to significantly increased number of bone nodules (Fig. 5B and C).

Moreover, LiCl treatment at 100 and 200 mg/kg induced mRNA levels of Lef1 (1.4-fold and 2.8-fold), CyclinD1 (1.2-fold and 2.2-fold), and Dkk1 (2.1-fold and 5.7-fold, respectively) in BMSCs (Fig. 6A). In line with the induction of canonical signaling, LiCl treatment lead to a significant dose-dependent increase in the mRNA levels of osteogenic markers, including ALP (1.5-fold and 2.3-fold), Runx2 (3.1-fold and 12.7-fold), and OC (1.4-fold and 4.5-fold).

Finally, immunohistochemical staining of lumbar slices showed that LiCl robustly increased the numbers of Lef-, ALP- and OC-positive cells (Fig. 6B). In summary, these results demonstrate that LiCl may promote

osteogenesis and result in bone mass increase possibly by the activation of Wnt/β-catenin signaling but not hormones in aged mice.

Discussion

In this study, we have uncovered that LiCl stimulates osteoblastic differentiation in response to age-related bone loss by activating canonical Wnt signaling, further implicating an additional protective effect of LiCl on senile osteoporosis.

Age-related disorders, such as senile osteoporosis, is a disease that mostly affects the elderly, and often causes bone fractures and reduces the quality of life [22]. At present, there are more studies on ovariectomized (OVX) model, which is not representative of the characteristics of senile osteoporosis. We analyzed the BMD of 2, 5, 7, and 10 months old mice, and found that the bone mass reached its maximum amount at 5 to 7 months, after which displayed a decline with time. The expression of osteoblastogenic markers also showed a decreasing trend after 7 months of age. Our results demonstrate that C57BL/6 mice, which are widely used in the laboratory, have an age- related osteoporotic bone phenotype, with decreased bone mass and quality, and therefore might be a better model compared to the ovariectomized (OVX) model for the study of senile osteoporosis.

Previous studies have shown that LiCl promotes bone formation of hBMSCs by inhibiting lipogenesis by synergistically regulating Wnt and Hh pathways [23]. The homeostasic effect of LiCl on osteogenesis/adipogenesis has also been confirmed by others [24, 25]. LiCl has been reported to stimulate osteoblast differentiation in osteoblast-like MC3T3-E1 mouse cells [26]. Arioka et al. also demonstrated that LiCl enhanced the proliferation of osteoblast and impaired osteoclastogenesis by down-regulating GSK3β [27]. LiCl enhances osteoblast differentiation on hydrophilic titanium surfaces by inducing activation of Wnt signaling [26]. In line with the previous studies, we examined the expression of osteoblastic differentiation and the formation of bone nodules by alizarin-red S, and found that LiCl promoted osteogenic activity of BMSCs. But trap staining showed that LiCl did not regulate the osteoclast differentiation.

In recent years, the roles of LiCl on bone homeostasis in vivo have also been studied. LiCl promotes implant osseointegration and osteogenesis in osteoporotic rats [28, 29]. LiCl treatment increased the alveolar bone mass during orthodontic retention [30]. Combination of LiCl and LY294002 increased osteoblastic differentiation but decreased osteoclast differentiation [31]. But the impact of LiCl on bone formation and homeostasis in senile osteoporosis is still unclear. Here we report that BMD, Tb.N, and BV/TV can be significantly increased by LiCl treatment in 7-months old mice. Tb.Sp decreased compared with vehicle treatment, suggesting that LiCl postpones age-related bone loss. Bone homeostasis is maintained by coordinated cycles of bone resorption and formation. In this study, we tested the activity of osteoclast by trap staining, and found there was no obvious difference between vehicle and LiCl treatment. We have no idea about the role of LiCl in osteoclastogenesis.

Lithium was used in the current study as a GSK-3β inhibitor, which increases the concentration of β-catenin [32]. The Wnt/β-catenin signaling promotes osteogenic differentiation and reduces adipogenic differentiation; therefore, Wnt signaling lead to an increase in expression of target genes, including Lef1 and CyclinD1. The above mentioned role of LiCl in the regulation of the Wnt signaling suggests that LiCl has an important role in the differentiation of BMSC. Furthermore, we found that LiCl also increased the expression of Dkk1, which is a canonical upstream inhibitor of Wnt signaling. We also found that prolonged administration of LiCl did not change the hormones. But previously study have demonstrated that LiCl reversed the trabecular bone damage in OVX rat, we did not examined the function of LiCl in OVX mice and had no idea about the role of LiCl in postmenopausal osteoporosis. The above results demonstrate that bone formation is regulated by LiCl in an Wnt signaling-dependent but hormone-independent manner and may be a therapeutic candidate for the treatment of senile osteoporosis.

Conclusion

LiCl significantly enhances the osteogenesis of BMSCs through activation of Wnt signaling, leading to a significant increase of bone mass in senile osteoporosis mice.

Abbreviations

LiCl
Lithium chloride
BMSCs
Bone marrow-derived mesenchymal stem cells
μCT
Micro computed tomography
BMD
Bone mineral density
BV/TV
Bone volume fraction
Tb.Th
Trabecular thickness
Tb.Sp
Trabecular separation
Tb.N
Trabecular number
PTH
Parathyroid hormone
Runx2
Runt-related transcription factor 2
ALP

Alkaline phosphatase
GSK-3β
Glycogen synthase kinase 3β
OC
Osteocalcin
Dkk1
Dickkopf-1
Lef1
Lymphoid enhancer-binding factor-1

Declarations

Ethics approval and consent to participate

The animal protocol was approved by the Animal Care and Use Committee of the Zhejiang University City College.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YB and YT performed the experiments. XL analyzed and interpreted the data. XR and JW contributed to the conception of the study, and JW was a major contributor to writing the manuscript. JY contributed reagents and helped write the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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Figures

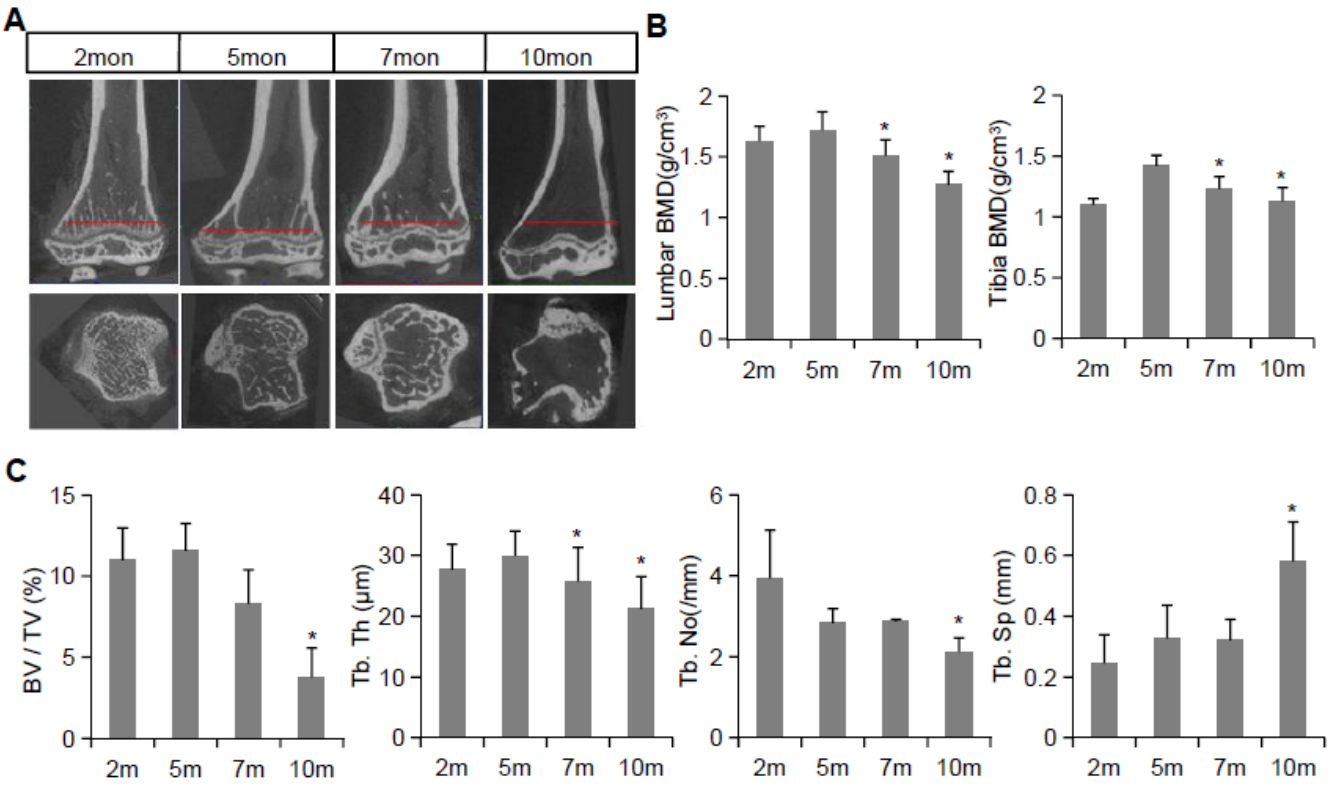

Figure 1

Senile osteoporosis mice model. (A) μCT of trabecular bone of the femur of C57BL/6 mice at 2, 5, 7, and 10-months of age. (B) The BMD of lumbar and femur. (C) Quantification of μCT of femur. *P<0.05 vs 2 months. n = 6 for each group.

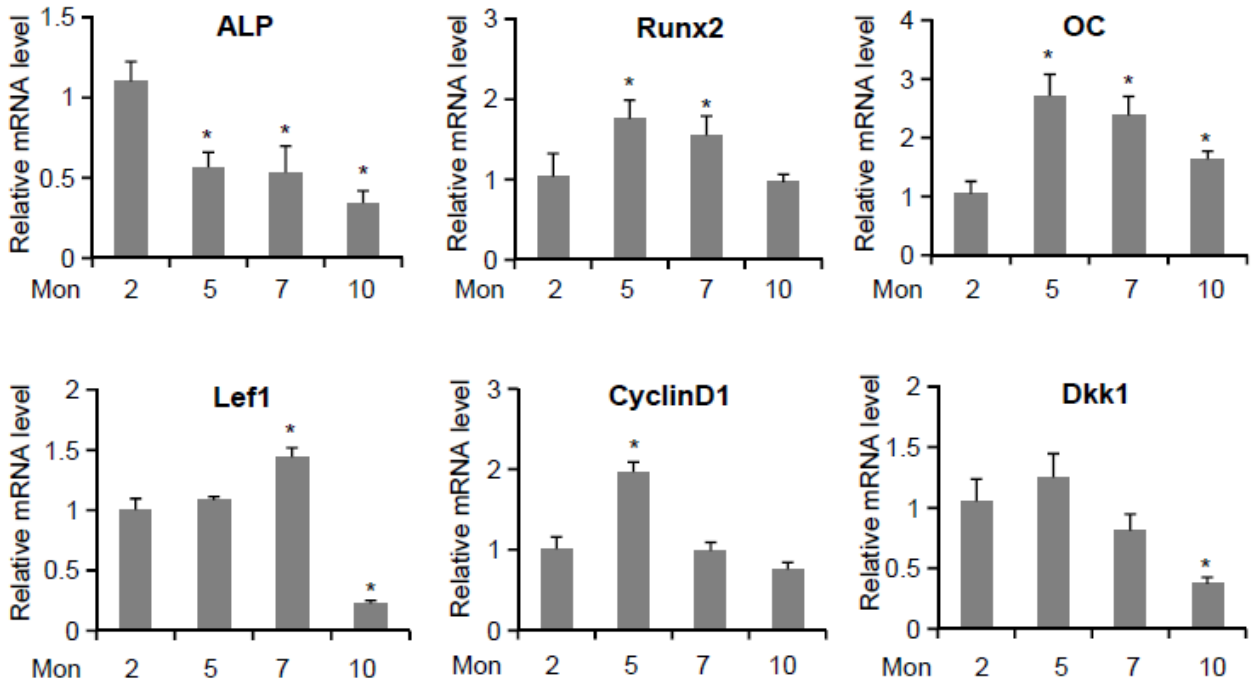

Figure 2

LiCl induced the expression of osteoblast- and Wnt pathway-specific genes in BMSCs. BMSCs were cultured for 24 h and tested by real-time PCR for mRNA levels of AP, Runx2, OC, Lef, CyclinD1, and Dkk1. *P<0.05 vs 2 months. n = 3 for each group.

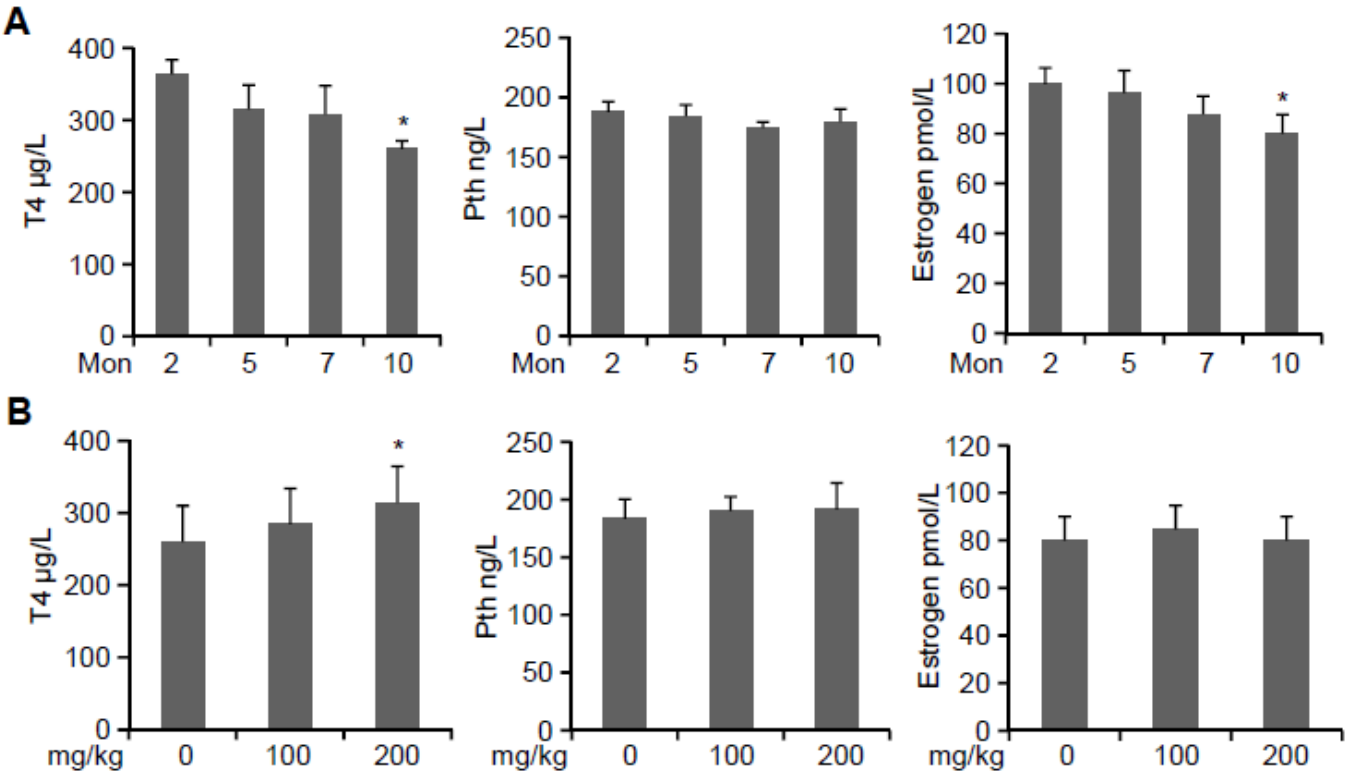

Figure 3

The levels of hormones. (A) The serum levels of T4, PTH and estrogen in senile osteoporosis model mice and (B) in LiCl treated mice, examined by ELISA.

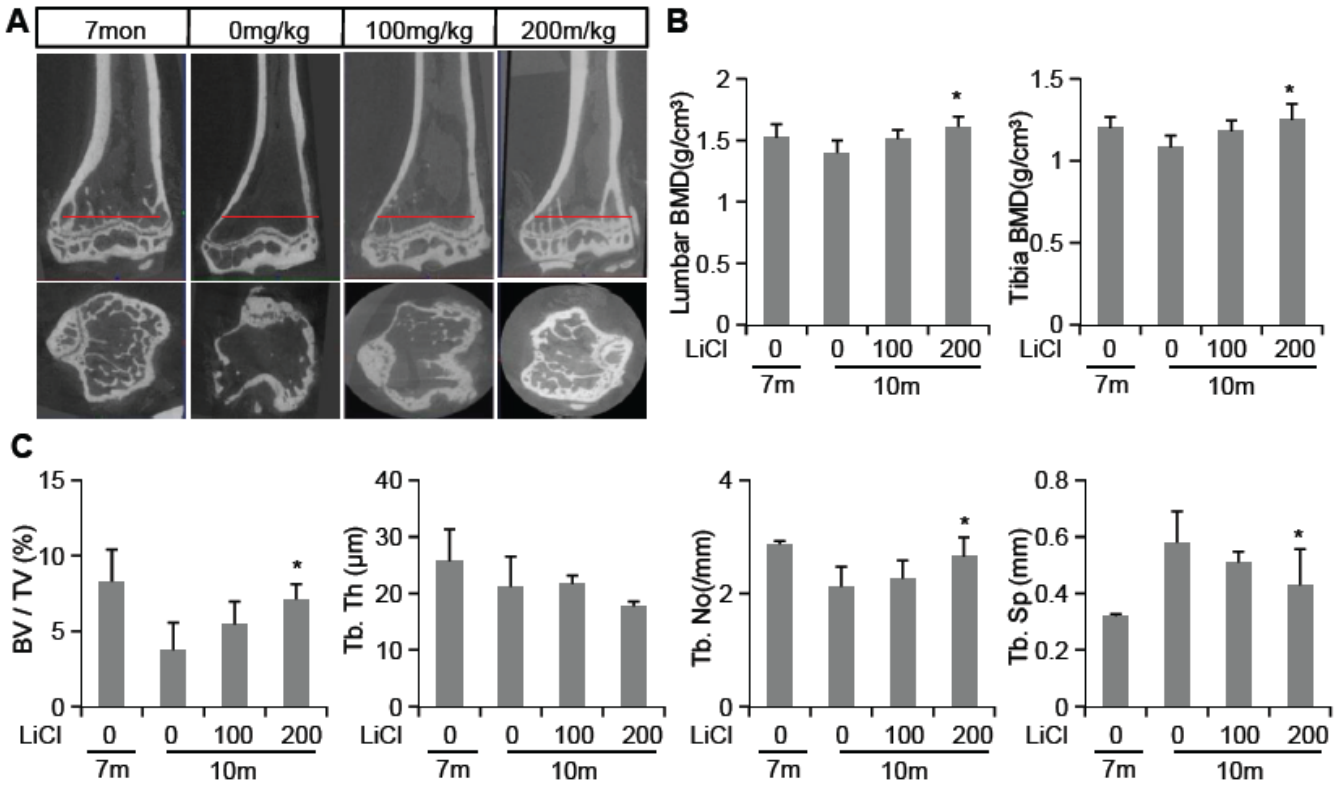

Figure 4

The promotion of LiCl to the bone mass of the senile osteoporosis mice model. (A) μCT analysis of femur in mice administered with saline or indicated concentration of LiCl for 3 months. (B and C) Quantitative analysis of 3D reconstruction of μCT bone parameters. *P<0.05 vs vehicle treatment. n = 6 for each group.

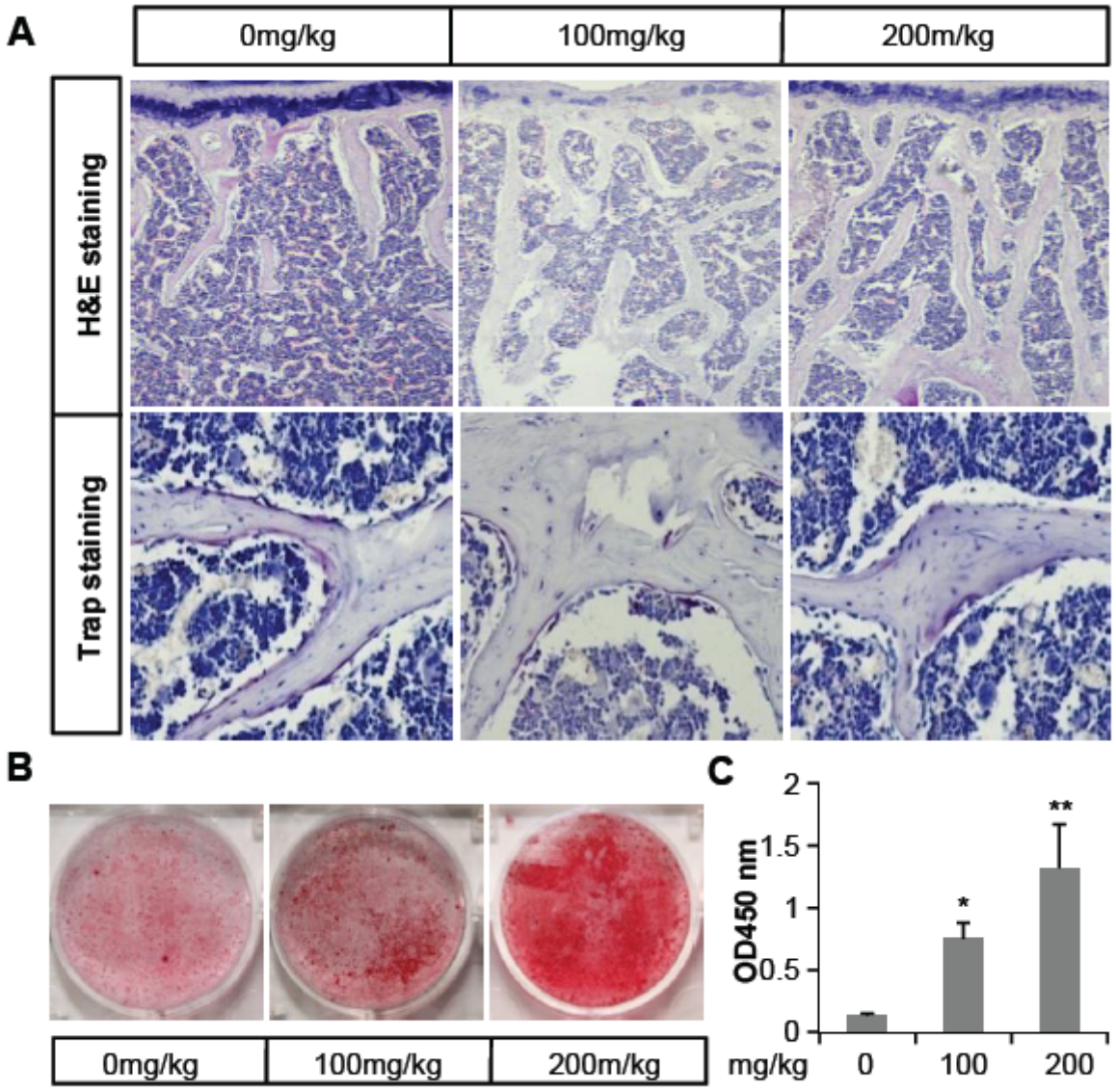

Figure 5

LiCl promoted the differentiation of osteoblast. (A) H&E and Trap staining of paraffin sections of lumbar from mice administrated with LiCl. (B and C) BMSCs were cultured in the medium of osteoblast differentiation for 4 weeks, then stained with alizarin-red staining to test the bone nodules. *P<0.05, **P<0.01vs vehicle treatment.

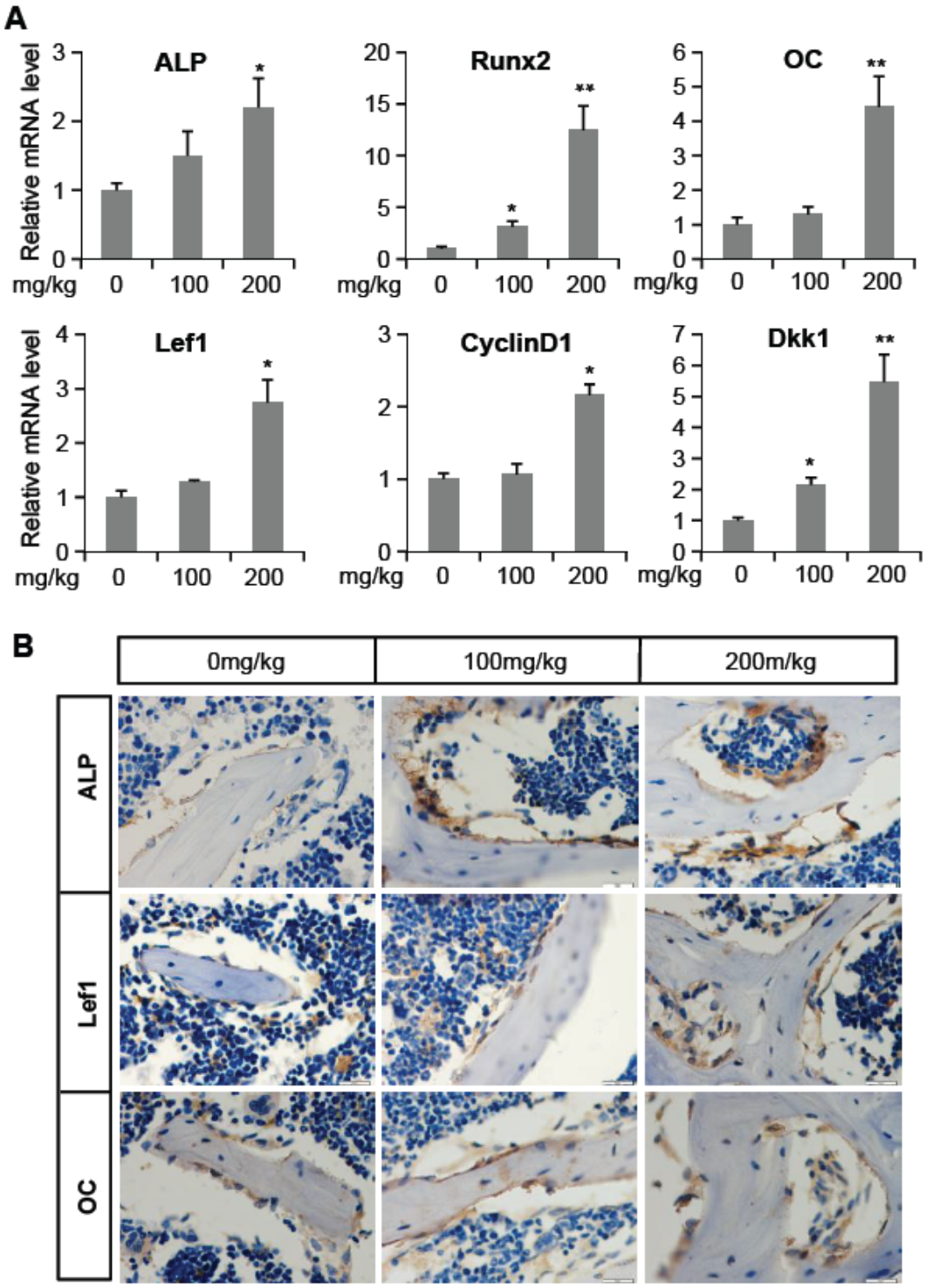

Figure 6

LiCl increased the expression of osteoblastogenic and Wnt signaling markers. (A) BMSCs cultured for 24 h and tested by real-time PCR for mRNA levels of AP, Runx2, OC, Lef, Dkk1 and CyclinD1 from LiCl treated and control mice. (B) Immunohistochemistry used to analyze the expression of OC, ALP and Lef1 in the distal femur of mice. *P<0.05, **P<0.01 vs vehicle treatment.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsARRIVEGuidelinesChecklist2014.pdf