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Original Paper

Lithium Chloride Modulates Adipogenesis and Osteogenesis of Human Bone Marrow-Derived Mesenchymal Stem Cells

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Key Words
Lithium Chloride • Adipogenesis • Osteogenesis • Human bone meshenchymal stem cells • Wnt signaling • Hedgehog signaling

Abstract
Background/Aims: Lithium chloride (LiCl) has long been used as a psychiatric medication; however, its role in the differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) remains largely unknown. The aim of this study is to explore the effect of LiCl on the differentiation of BMSCs. ***Methods:*** The roles of LiCl in osteogenic and adipogenic processes were observed using alizarin red staining and oil red O staining, respectively. The effects of LiCl on the Wnt and Hedgehog (Hh) pathways were investigated. ***Results:*** Our data showed that LiCl effectively promoted osteogenesis and inhibited adipogenesis by simultaneously affecting the Wnt and Hh pathways. ***Conclusion:*** These results suggest that LiCl influences the differentiation of BMSCs directly through the Wnt and Hh pathways and thus may be a candidate drug for the treatment of osteoporosis.

Introduction

Osteoporosis is a disease characterized by decreased bone mass and density [1, 2]. At present, osteoporosis still has a high incidence and mortality rate worldwide, and more effective treatments for osteoporosis have yet to be developed. The most commonly used medicines for the treatment of osteoporosis are designed to repress bone resorption; however,

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these medicines have been shown to cause micro-injuries in bone. Parathyroid hormone (PTH) is an osteogenesis-promoting medicine approved by the Food and Drug Administration; however, long-term treatment with PTH increases the risk of osteosarcomas[3]. Therefore, the greatest challenge in this field is to develop medicines that can increase bone mass by stimulating osteogenesis.

Lithium is a well-known treatment for depression and, in particular, for mania, and can be used for either short-term or long-term treatment [4]. Interestingly, data from previous studies have shown that bone mass was increased in patients taking lithium for the treatment of mental disorders, and bone transformation was also decreased in those patients[5]. These data further suggest that lithium can be used clinically as a candidate drug for the treatment of osteoporosis. Lithium was originally approved as a safe medicine for the treatment of mental disorders; however, the role of lithium chloride in BMSC differentiation is not clear.

Previous studies have shown that the decrease in bone mass caused by osteoporosis is accompanied by an increase in adipocytes in bone [6]. Recent *in vivo* and *in vitro* assays indicated that osteoporosis is caused by an increased adipogenic differentiation and a decreased osteogenic differentiation of BMSCs [6]. Therefore, work in this field has sought to clarify the mechanism for the reverse differentiation of osteogenesis and adipogenesis and to explore targets and potential candidate drugs that can promote osteogenic differentiation and inhibit adipogenic differentiation.

The Wnt/β-catenin signaling pathway is involved in many physiological processes via regulating downstream gene expression [7-10]. Also this axis is very important for regulating osteogenic-adipogenic reverse differentiation [11, 12]. Wnt signaling promotes osteogenic differentiation through multiple mechanisms, and it can simultaneously inhibit adipogenic differentiation by repressing the expression and activities of its specific transcription factors, including C/EBPα and PPARγ [13-15]. Furthermore, bone mass and intensity are increased, bone loss is inhibited and fracture wounds are promoted by Wnt signaling. Activation of Wnt signaling inhibits the activity of a critical molecule, GSK3β, allowing β-catenin and Gli to enter the nucleus after escaping from degradation [16-18]. Finally, these two transcription factors promote the expression of target genes downstream of the Wnt pathway. Therefore, GSK3β plays a key role in regulating Wnt signaling.

Clement-Lacorix *et al.* also found that lithium, a known inhibitor of GSK3β, can significantly increase osteogenesis and bone mass in *Lrp5*-/-, SAMP6 and C57BL-6 mice and decrease the amount of adipocytes in SMAP6 mice [19]. The mechanism behind these findings is not completely understood; however, we propose that lithium regulates the osteogenesis and adipogenesis of BMSCs and further increases the bone mass of mice, at least partially, by activating Wnt signaling through the inhibition of GSK3β. The effect of lithium on human BMSC differentiation requires further investigation.

Furthermore, other pathways are also involved in the reverse differentiation of osteogenesis and adipogenesis, and Hedgehog (Hh) signaling is closely associated with these biological processes. Hh is a secreted protein encoded by a single gene. It binds to the transmembrane receptor Ptch resulting in the release of Smo, which phosphorylates Cos2, Fu, Su(fu), and Gli allowing them to enter the nucleus and promote the expression of their target genes [16, 20].

The partial inhibition of Ptch can markedly increase bone mass in Ptch $^{+/-}$ mice [21]. In addition, the activation of Hh signaling can inhibit adipogenic differentiation and promote osteogenic differentiation by repressing C/EBPα and PPARγ2 expression [22]. It was recently found that activation of Hh and Wnt signaling promoted the gene expression of downstream targets of Wnt and Hh signaling, respectively, and that these findings imply the Hh and Wnt pathways are synergistically involved in regulating hBMSC differentiation [23, 24]. Our previous data (unpublished) also showed that Wnt10b activated the Wnt pathway, resulting in the increased expression of Gli-1, a specific transcriptional factor of Hh signaling; however, the mechanism behind this finding is not clear.

The activation of the Wnt and Hh pathways inhibits GSK3β activity and allows β-catenin and GliA to escape degradation and enter the nucleus [23, 25]. Those transcription factors

then trigger the expression of the target genes of the Wnt and Hh pathways. Thus, GSK3β has been deemed a common target of these two signaling pathways.

Lithium can promote Wnt and Hh signaling by inhibiting GSK3β activity. It is important to investigate the role of Hh signaling in regulating bone mass and to determine the interactions between the Hh and Wnt pathways. Knowledge regarding the interactions of these two pathways is crucial for understanding the pathogenesis of osteoporosis and the clinical application of lithium.

Thus, the effect of lithium chloride on the balance of osteogenic-adipogenic differentiation in human BMSCs was explored using molecular biology and biochemistry, and the expression of specific genes was also assessed. Our data suggest that lithium chloride can markedly promote the osteogenesis and inhibit the adipogenesis of human BMSCs by synergistically regulating the Wnt and Hh pathways. These findings indicate that lithium is a novel candidate for the treatment of bone diseases, including osteoporosis and other related diseases.

Materials and Methods

Chemicals and Reagents

FITC anti-human CD34 antibody and PE anti-human CD105 antibody were obtained from BioLegend (San Diego, CA, USA). PE-labeled anti-human CD166 antibody (ALCAM) was purchased from Affymetrix eBioscience (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (NY, USA). Alizarin Red S was obtained from Sigma (St. Louis, MO, USA), and oil red O was purchased from Abcam (Cambridge, MA, USA). High capacity cDNA reverse transcription kit and SYBR green real time PCR master mixes were also purchased from Life Technologies (NY, USA). Beta glycerophosphate, ascorbic acid, insulin, dexamethasone, indomethacin, IBMX and LiCl were purchased from Sigma (St. Louis, MO, USA).

Cell culture

Ilia from patients were cut into small pieces, and bone marrow cells were obtained by washing the ilia with DMEM. The bone marrow cells were harvested, washed twice with phosphate buffered saline (PBS), and pelleted at 13,000 g for 30 min. Then, 2×10^8 cells were placed on 70% percoll. 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. Additionally, the patients indicated they were not taking any treatments that affect hBMSCs, and all patients signed informed consent forms. All experiments were approved by the Ethics Committee of Sichuan University.

Flow cytometry

The second generation BMSCs were harvested by digesting with trypsin and were fixed with 1% paraformaldehyde after washing with PBS twice. Then, 5×10^5 cells were re-suspended in PBS (including 0.1% NaN_3+0.5% BSA) after centrifugation at 1000 rpm for 5 min. The cells were then incubated with 10 μL of anti-CD34-FITC, anti-CD105-PE and anti-CD166-PE at 4°C for 2 hours. Then, the fluorescence of the labeled cells was detected by flow cytometry to determine the proportion of positive cells (labeled).

Osteogenic induction

BMSCs were cultured with DMEM and passaged twice. Then, cells were seeded into 6-well plates, and osteogenic induction was performed using DMEM medium supplemented with 50 μM ascorbic acid, 10 mM beta glycerophosphate and 100 nM dexamethasone. A calcium node was typically observed after 9-10 days of induction.

Adipogenic induction

BMSCs were seeded into 6-well plates, and adipogenic induction was performed using DMEM medium supplemented with 1 μM dexamethasone, 10 μg/mL insulin, 200 μM indomethacin and 0.5 mM IBMX. A lipid droplet in the adipose cells can be observed after approximately two weeks of adipogenic induction.

Oil red O staining

The cells were fixed with 10% formalin-calcium at 4°C overnight. Then, the cells were rinsed with 60% isopropanol. Subsequently, the cells were stained with freshly prepared Oil Red O for 30 min at RT. Furthermore, the cells were washed with distilled water three times for 15 min before the slides were mounted with VECTASHIELD mounting medium from Vector Lab. (H-1000, Burlingame, CA, USA). Finally, the cells were mounted on slides with a specific buffer. Positive cells were observed using a common inverted microscope.

Alizarin red S staining

The culture medium was discarded, and the cells were gently rinsed with PBS twice. Then, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature (RT). The cells were washed with distilled water three times and stained with 2% Alizarin red S for 30 min at RT. The cells were washed again with distilled water three times. Finally, the cells were rinsed with water, and the Alizarin red stain was observed using a microscope. Furthermore, the intensity of calcium nodules was calculated using Image J.

RT-qPCR

Total RNA was extracted using TRIzol reagent from Invitrogen (Grand Island, NY, USA) [26]. For reverse transcription, 1 μg of total RNA and a kit from ABI was used (Grand Island, NY, USA). The primers of the target genes were as follows: RUNX2, CCG CCT CAG TGA TTT AGGGC (sense) and GGG TCT GTA ATC TGA CTC TGTCC (antisense); PPARG, GGG ATC AGC TCC GTG GATCT (sense) and TGC ACT TTG GTA CTC TTG AAGTT (antisense); PTCH1, GAA GAA GGT GCT AAT GTC CTGAC (sense) and GTC CCA GAC TGT AAT TTCGCC (antisense); AXIN2, CAA CAC CAG GCG GAA CGAA (sense) and GCC CAA TAA GGA GTG TAA GGACT (antisense); and GLI1, AGC GTG AGC CTG AAT CTGTG (sense) and CAG CAT GTA CTG GGC TTT GAA (antisense). The parameters of the real time quantitative polymerase chain reaction (real time qPCR) were set as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Beta-actin was used as an internal control, and all experiments were performed in triplicate.

Statistical analysis

All data are presented as means ± standard deviations (SD) or means ± standard errors of the mean (SEM). Statistical significance for comparisons between groups was determined using Wilcoxon test in Prism 5.0, and $P < 0.05$ was designated as the level of significance.

Results

Isolation and verification of BMSCs from human ilium

To study the role of LiCl in the differentiation of human BMSCs, hBMSCs were isolated from human ilia by rinsing the ilia with DMEM medium 2-3 times. The cells were cultured in a 10-cm culture dish, and the hBMSCs adhered to the culture dish (Fig. 1A). To determine the identity of these cells, three antigens on the cell surface, CD34, CD166 and CD105, were detected with flow cytometry using the corresponding antibodies (Fig. 1B, C and D). Our data showed that these cells were positive for the markers of hBMSCs, suggesting that the hBMSCs were successfully isolated from human ilium using percoll centrifugation.

LiCl promotes osteogenesis of hBMSCs in a Wnt signaling-dependent manner

*T*o demonstrate the effect of LiCl on the osteogenesis of hBMSCs, hBMSCs were cultured in medium containing ascorbic acid, beta glycerophosphate and dexamethasone. As shown in Fig. 2A, calcium nodules in the cells were markedly stained with alizarin red S, suggesting that hBMSCs cultured with inductive medium can be effectively differentiated into mature bone cells. LiCl (20 mM) can significantly increase the number of calcium nodules after 14 days of induction (Fig. 2C and E). In contrast, as a canonical inhibitor of Wnt signaling, DKK1 markedly inhibited the formation of calcium nodules (Fig. 2B and E). Interestingly, for the cells treated with DKK1, calcium nodules again increased after the addition of LiCl to the culture medium (Fig. 2D and E). In addition, the mRNA expression of RUNX2, a marker gene for osteogenesis, was detected. Our data showed that RUNX2 expression was increased by

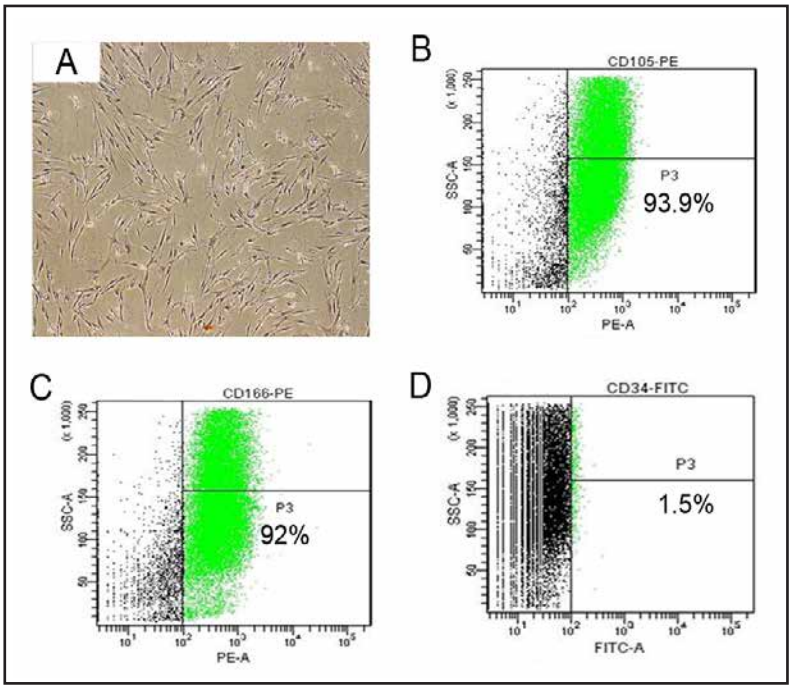


Fig. 1. Culture and identification of hBMSCs. (A) Morphology of adhered hBMSCs: elongated and spindle-shaped cells were observed. (B) Cells were detected with CD105 antibodies using flow cytometry, and 93.9% of cells were positive. (C). Cells were detected with CD166 antibodies using flow cytometry, and 92% of cells were positive. (D) Cells were detected with CD34 antibodies using flow cytometry, and 98.5% of cells were negative.

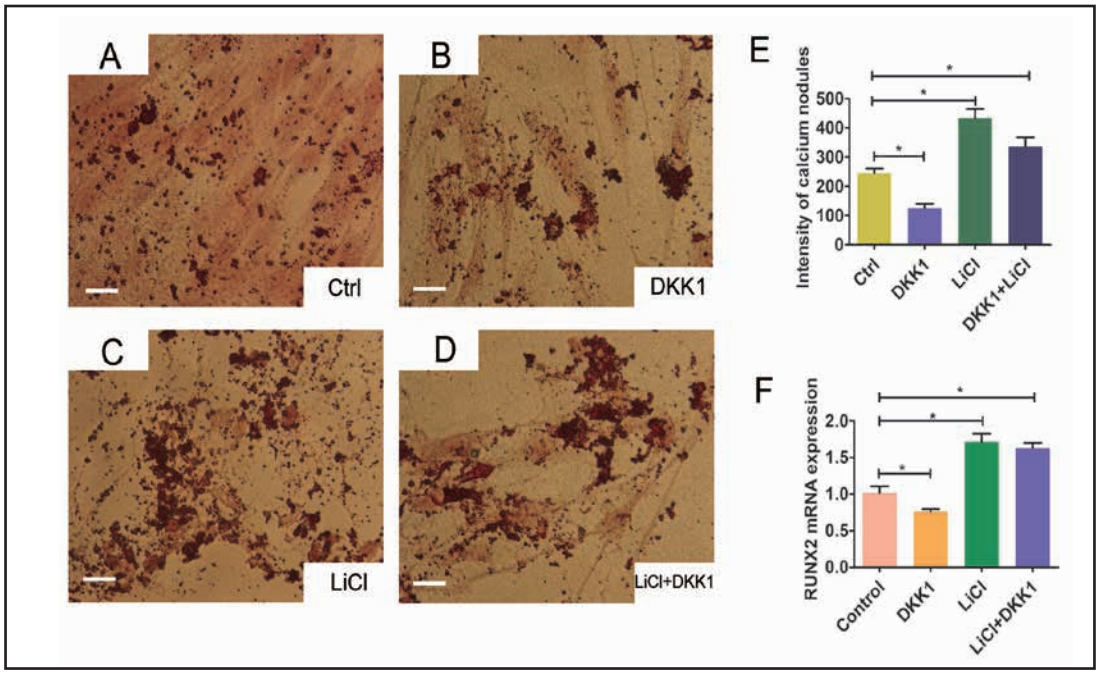


Fig. 2. LiCl promotes osteogenesis in a Wnt signaling-dependent manner. (A) hBMSCs were induced to form osteocytes using osteogenic induction medium. (B) DKK1 significantly inhibited the number of calcium nodules, suggesting the inhibition of osteogenesis. (C) The treatment of 20 mM LiCl markedly increased the osteogenesis of hBMSCs, as indicated by the increase in calcium nodules. (D) LiCl counteracted the inhibitory effect of DKK1 on osteogenesis. (E) Statistical analysis of the number of calcium nodules in these four groups. (F) RUNX2 served as a marker gene and downstream gene of the Wnt pathway; its expression was detected with RT-qPCR. RUNX2 gene expression was strongly associated with osteogenesis. The level of significance was set at $P<0.05$.

LiCl and decreased by DKK1 (Fig. 2F). These findings indicate that LiCl effectively induced osteogenesis in a Wnt signaling-dependent manner.

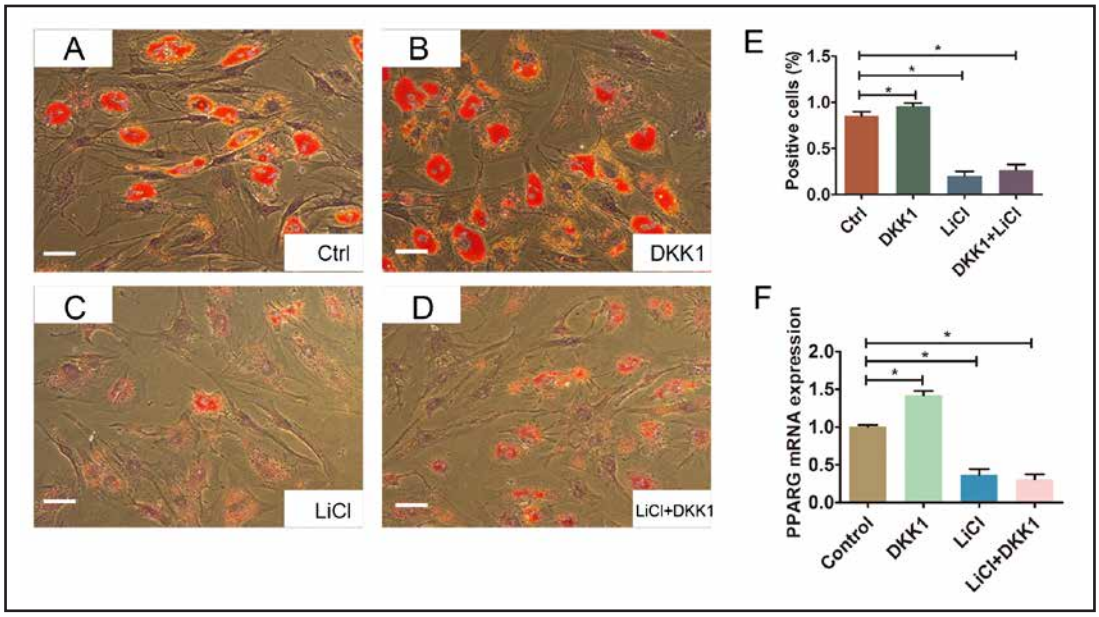
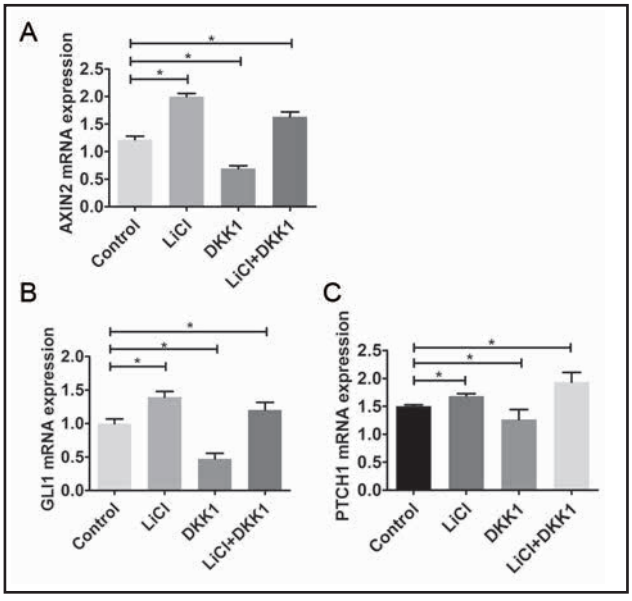


Fig. 3. LiCl inhibits adipogenesis in a Wnt signaling-dependent manner. (A) hBMSCs were induced to form adipocytes using adipogenic induction medium. (B) DKK1 significantly increased the number of oil droplets in cells, suggesting that adipogenesis was markedly promoted by DKK1. (C) After the treatment with 20 mM LiCl, the adipogenesis of hBMSCs was markedly decreased. (D) LiCl counteracted the promoting effect of DKK1 on adipogenesis. (E) Statistical analysis of cells with positive staining of oil droplets. (F) As a marker gene, PPARG expression was detected using RT-qPCR, and PPARG gene expression was closely correlated with adipogenesis. The level of significance was set at $P<0.05$.

Fig. 4. Expression of genes downstream of the Wnt and Hh pathways. (A) AXIN2 is a downstream gene of the Wnt pathway, and its expression was markedly increased and decreased after LiCl and DKK1 treatment, respectively. (B) GLI1 is a key member of the Hh signaling pathway and is also a target gene of the Wnt pathway. GLI expression was significantly regulated by LiCl and DKK1. (C) PTCH1 is a target gene of Hh signaling, and LiCl greatly increased its expression. The level of significance was set at $P<0.05$.



LiCl inhibits adipogenesis of hBMSCs in a Wnt signaling-dependent manner

As reported previously, hBMSCs primarily differentiate into bone cells and adipocytes. Here, LiCl significantly increased osteogenesis; thus, adipogenesis was thought to have decreased. To verify this hypothesis, the role of LiCl in hBMSC adipogenesis was examined using Oil Red O staining. First, hBMSCs were induced with adipogenic medium containing

dexamethasone, insulin, indomethacin and IBMX. A majority of the cells successfully differentiated into adipocytes (Fig. 3A). As expected, LiCl significantly decreased the number of lipid droplets (Fig. 3C and E). As shown in Fig. 3B, DKK1 can effectively increase adipogenesis (Fig. 3E,). Additionally, only a few oil droplets were observed in the group of DKK1 + LiCl, suggesting that the target of LiCl was located downstream of DKK1 (Fig. 3D and E). These findings indicate that LiCl markedly inhibited adipogenesis in a Wnt signaling-dependent manner. To confirm the inhibition of adipogenesis by LiCl, the expression of a marker gene, PPARG, was simultaneously detected using RT-qPCR. The results suggest that LiCl and DKK1 significantly downregulated and upregulated PPARG mRNA expression, respectively (Fig. 3F). Taken together, our data indicate that LiCl inhibited adipogenesis of hBMSCs in a Wnt signaling-dependent manner.

LiCl promotes the expression of downstream genes of the Wnt and Hh pathways

As shown in Fig. 2 and 3, LiCl can significantly promote and inhibit osteogenesis and adipogenesis, respectively, by acting on the Wnt signaling pathway. The expressions of two target genes of Wnt signaling, RUNX2 and AXIN2, were detected at the mRNA level. Fig. 4A shows that LiCl markedly increased AXIN2 expression. However, Hh signaling was also found to be involved in the regulation of hBMSC differentiation. A core factor of Hh signaling, GLI1, is a downstream target gene of Wnt signaling. Hence, GLI1 expression was examined, and the data suggest that LiCl can also significantly increase GLI1 expression. The expression of PTCH1, a downstream gene of Hh signaling, was detected and shown to be significantly increased by LiCl. Our findings indicated that LiCl can simultaneously promote the expression of the downstream genes of the Wnt and Hh pathways and that Hh signaling may be partially regulated by the Wnt pathway.

Discussion

Osteoporosis is a common disease in aging persons and often causes bone fractures. Therefore, osteoporosis significantly decreases the quality of life of aging patients. There is a strict balance between bone loss and bone formation that is regulated by osteoclasts and osteoblasts, respectively [27]. Importantly, hBMSCs are one of the main sources of mature bone cells. As reported previously, hBMSCs mainly differentiate into adipocytes and osteocytes [28]. Although a great deal of basic research on BMSC differentiation and cell fate has been performed, few translational studies (from bench to bedside) have been performed.

Lithium compounds were first used to safely treat mental disorders, including mania and depression, for several decades [29]. Recently, lithium was found to play an important role in the differentiation of BMSCs; however, the mechanism of this role remains unknown [30]. To determine the exact role of LiCl in BMSC differentiation, this study treated human BMSCs with LiCl, and osteogenic and adipogenic differentiation was observed using alizarin red S and oil red O staining.

First, adherent cells were separated from human ilia and were examined by flow cytometry using three different markers. CD166 and CD105 were used as BMSC markers, and CD34 was mainly used to exclude hematopoietic cells [31]. After screening the cells, the isolated cells were confirmed as primarily BMSCs. These cells were used in the subsequent studies.

Osteogenesis inductive medium can efficiently induce hBMSCs to form mature osteocytes characterized by calcium nodules. As shown in Fig. 2C, LiCl significantly increased the number of calcium nodules. Interestingly, the cell number in the LiCl group was less than that in the control group. This is probably because LiCl strongly inhibited cell proliferation and promoted hBMSC differentiation. As expected, RUNX2 gene expression concurrently increased with the osteogenesis induced by LiCl.

Previous studies have proposed that BMSC differentiation involves a competition between adipogenesis and osteogenesis and that the cell fate of BMSCs is predominantly

dependent on the inductive condition [32]. Our study found that LiCl increased osteogenesis and markedly decreased adipogenesis. With adipogenic induction, oil droplets can be clearly observed after 7-9 days, and the size of the droplets increased with the induction time. Additionally, genes related to adipogenesis were expressed under adipogenic induction.

In addition to observing the phenotype of the induced cells, the potential mechanism of induction was examined by detecting the expression of associated genes. Here, the expression of the downstream genes of the Wnt and Hh pathways were detected after lithium induction. Intriguingly, the expression of the downstream genes of Wnt and Hh signaling was significantly influenced by the induction of LiCl. AXIN2 acted as a negative regulator of Wnt signaling, and GLI1 and PTCH1 were the main members of Hh signaling. GLI1 was also a target gene of the Wnt pathway.

On the one hand, LiCl promoted Wnt signaling by inhibiting the activity of GSK3β; therefore, the gene expressions of downstream genes of Wnt signaling, including RUNX2 and AXIN2, increased and osteogenesis was markedly promoted. On the other hand, LiCl increased PTCH1 expression by augmenting GLI activity through the inhibition of GSK3β. Furthermore, GLI1 expression also increased after the activation of Wnt signaling by LiCl. These data clearly show that LiCl was involved in BMSC differentiation by simultaneously regulating the Hh and Wnt pathways.

Furthermore, DKK1 is a canonical upstream inhibitor of Wnt signaling [33, 34]; this study showed that LiCl can counteract the DKK1 inhibition of Wnt signaling. These findings indicate that LiCl regulated BMSC differentiation in a Wnt signaling-dependent manner.

Hh signaling has been reported to be significantly involved in BMSC differentiation; therefore, to a limited extent, this paper described the role of Hh signaling in this biological process[35]. However, subsequent studies should be performed to uncover the exact relationship between Wnt and Hh signaling pathways and to especially determine the effect of the interaction of these pathways on BMSC differentiation.

In summary, our findings suggest that LiCl significantly promotes the osteogenesis of hBMSCs by inhibiting adipogenesis through synergistically regulating Wnt and Hh pathways. LiCl is a candidate treatment option for patients with osteoporosis, and the results of this study indicate that it has good potential for clinical applications.

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Disclosure Statement

None conflict of interest was declared.

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