Lithium chloride's inhibition of 3T3-L1 cell differentiation by regulating the Wnt/ β -catenin pathway and enhancing villin 2 expression

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Abstract The aim of this study is to reveal the relation among villin 2, Wnt/ β -catenin, and adipogenesis by adding appropriate lithium chloride (LiCl). The study comprises three parts: the selection of LiCl concentration, the effect of LiCl on adipocyte differentiation during and after differentiation induction. By comprehensively analyzing the results of the experiments, we proved that LiCl can inhibit adipocyte differentiation and enhance villin 2 and β -catenin expressions not only during differentiation induction but also after it. Moreover, villin 2 has a significant impact on β -catenin. We suggest that villin 2 may participate in Wnt/ β -catenin signaling.

Keywords: lithium chloride, villin 2, β -catenin, 3T3-L1, adipocyte differentiation

Introduction

People have a strong interest in the better nutrition. Therefore, high-quality beeves with intramuscular fat, geese fatty liver, and lean meat pigs are gaining popularity. Moreover, people are trying their best to lose weight to avoid obesity. Therefore, knowledge of lipid synthesis and metabolism is particularly important, and we need to further study the signals of adipogenesis. A wide range of research topics on lipid synthesis and metabolism are available to study. Previous proteomics studies indicate that villin 2 protein is down-regulated in adipogenesis of the muscle tissue of Korean native steers (1). Moreover, villin 2 protein is sharply down-regulated in 3T3-L1 mouse embryonic fibroblasts after 2 days of differentiation induction (1). Therefore, we surmise that villin 2 is related to adipogenesis by regulating cytoskeleton associated proteins (2–4). It is generally accepted that the cytoskeleton-associated protein family comprises three proteins and that they are also called the ezrin/radixin/moesin (ERM) family. In recent years, Takahashi et al. found that merlin protein has a similar structure with the ERM family; hence, merlin is classified as a member of the ERM family (5). Villin 2 protein, the earliest purified protein in the family, provides a link between the cell

membrane and cytoskeleton. It has many important physiological functions such as cell growth, differentiation, and signaling (6–9). Villin 2 also regulates cell adhesion by interacting with E-cadherin and β -catenin (10). Wnt signaling participates in regulating adipocyte differentiation in the present study, and β -catenin, as a second messenger, plays an important role in signaling (11). β -Catenin inhibits adipogenesis in cells through Wnt signaling. In a physiological condition, Wnt signaling is not activated. As a member of the degradation complex of β -catenin, GSK-3 β constantly phosphorylates β -catenin (12). LiCl is the agonist of Wnt/ β -catenin signaling (13,14). Li ion can inhibit the activity of GSK-3 β and decreases the phosphorylation level of β -catenin. Accordingly, β -catenin can activate Wnt signaling and start the expression of downstream target genes to regulate adipogenesis (12). Despite some detailed knowledge of the relation between villin 2 protein and β -catenin in cancer (15–19), it is unknown whether villin 2 protein regulates adipogenesis via Wnt/ β -catenin signaling by LiCl.

Hence, the aim of this study is to reveal the relation among villin 2, Wnt/ β -catenin, and adipogenesis by adding appropriate LiCl to increase intramuscular fat and make beef part of a healthy diet.

Materials and Methods

Cell culture 3T3-L1 mouse embryonic fibroblasts were grown in Dulbecco's modified Eagle medium with 4.5 g/L glucose and L-glutamine without sodium pyruvate. The growth medium (Corning Cellgro, Manassas, VA, USA) was supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 1% penicillin-streptomycin (HyClone), and 1% L-glutamine (Sigma-Aldrich, St. Louis, MO, USA). After the cells reached 100% confluency, they were incubated in the differentiation induction medium (growth media containing 1 µg/mL insulin, 1 µM dexamethasone, and 0.5 mM isobutylmethylxanthine) for 2 days. Next, the cells were maintained in a differentiation maintenance medium (growth media supplemented with 1 µg/mL insulin) until they were harvested. In the first experiment, LiCl was ranked to four concentrations: 0, 10, 20, and 30 mM LiCl was added to the differentiation induction medium for 8 days. In the second experiment, 20 mM LiCl was added to the differentiation induction medium, and the cells were harvested for 0, 2, 4, 6, and 8 days. In the third experiment, after 8 days of differentiation induction, 20 mM LiCl was added to the differentiation induction medium, and the cells were harvested for 8, 10, 12, and 14 days.

RNA isolation and quantitative real-time PCR (RT-PCR) Total RNA was extracted from 3T3-L1 mouse embryonic fibroblasts using a TRIzol reagent according to the manufacturer's instructions. The concentration of RNA was measured using a NanoDrop 2000 spectrophotometer. In addition, the optical density (OD) A260/A280 and A260/A230 ratios were determined to verify the RNA purity. Reverse transcription was performed using an Oligo(dT)₁₅ primer and a Moloney murine leukemia virus reverse transcriptase. First, a mixture containing 2 µg of RNA, 1 µL of Oligo(dT)₁₅ (Promega Corporation, Madison, WI, USA), and diethyl pyrocarbonate-treated water in a total volume of 13.5 µL was prepared. The tube was

heated at 70°C for 5 min to melt the secondary structure within the template. Further, the tube was cooled immediately on ice to prevent the secondary structure from reforming. Then, a master mix composed of 4 µL of 5X First-Strand buffer (Promega), 1 µL of 10 mM dNTP (Promega), 25 units Recombinant RNasin[®] Ribonuclease Inhibitor, and 200 units M-MLV (Promega) was added to the RNA samples and was gently mixed by flicking the tube and incubated for 60 min at 42°C. Finally, the mixture was incubated for 5 min at 95°C. The genes examined and their specific RT-PCR primers are listed in Table 1; β-actin was used as a control.

RT-PCR was performed in 20 µL reaction mixtures. The mixtures contain FastStart Universal SYBR Green Master (Rox), cDNA, PCR grade water, and forward and reverse primers. The relative quantification of gene expression from different samples was analyzed using the 2^{-ΔΔCT} method.

Western blotting The 3T3-L1 mouse embryonic fibroblasts were homogenized in RIPA lysis buffer (Sigma-Aldrich). The mixtures were vibrated for 30 min at 4°C. Then, the samples were centrifuged at 19,000xg/min for 20 min at 4°C. Protein concentrations were measured using a BCA kit (Thermo Fisher Scientific, Waltham, MA, USA). The proteins (10 µg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (Pall Corp, Port Washington, NY, USA). The membranes were blocked with 5% skim milk in TBST buffer for 3 h. The membranes were incubated overnight at 4°C with primary antibodies: GAPDH (ab181603, 1:10000, 36 kDa; Abcam, Cambridge, UK), mouse monoclonal antibody to ezrin (ab4069, 1:500, 81 kDa; Abcam), and rabbit polyclonal antibody to β-catenin (ab6302, 1:4000, 94 kDa; Abcam). Then, the membranes were washed 3 times with 1X TBST. Next, they were incubated with secondary antibodies: rabbit polyclonal secondary antibody to mouse IgG-H&L (ab6728, 1:2000; Abcam) and goat anti-rabbit IgG-H&L (ab6721, 1:3000; Abcam) for 3 h. Antibody bindings were quantified using Tanon Gis

Table 1. Gene information and PCR primer sequences

Gene	Forward primer	Reverse primer	GenBank accession No.	Product size (bp)
LPL	5'-TGTCATCTCATTCTGGATTAGC-3'	5'-TCTCATACATTCCCGTTACCGTC-3'	NM_008509.2	109
FAS	5'-CCTCGTGATGAACGTGTACC-3'	5'-TGAGGACGTTTACAAAGGCA-3'	NM_007988.3	110
ACC	5'-GCCAGTGCTATGCTGAGATTGAG-3'	5'-CTATCACACAGCCAGGGTCAAGT-3'	NM_133360.2	120
PPAR _γ	5'-CCTGGCAAAGCATTGTATG-3'	5'-TGGTGATTGTCCGTTGTCT-3'	NM_001127330.1	100
c/EBP _α	5'-GAAGTCGGTGACAGAAGACAGC-3'	5'-GGTCATTGCTACTGGTCAACTCC-3'	NM_001287514.1	143
c/EBP _β	5'-CAAGCTGAGCGACGAGTACA-3'	5'-AGCTGCTCCACCTTCTCTG-3'	NM_001287738.1	156
SREBP1c	5'-AGAGCGAGCGTTGAACTGTATT-3'	5'-ATCCAAGGCGATCTGAGAACTC-3'	NM_011480.3	81
SCD1	5'-CTACACCTGCCTCTTCGGGATT-3'	5'-CCGTGCCTTGTAAGTTCTGTG-3'	NM_009127.4	100
AP2	5'-TGATCATCAGCGTAAATGGG-3'	5'-GCCAGTTTGAAGGAAATCT-3'	NM_024406.2	83
GLUT4	5'-CTGCCCCGAAAGAGTCTAAAGC-3'	5'-CTCCAACCTCCGTTTCTCATC-3'	NM_009204.2	89
Villin2	5'-GGATGAGAACAAGAGGACCCA-3'	5'-TGCCGCAGCGTCTTATACTT-3'	NM_009510.2	84
β-catenin	5'-GTTGTACTGCTGGGACTCTGC-3'	5'-GTGTCGTGATGGCGTAGAAC-3'	NM_007614.3	141
Radixin	5'-CAGGCATTGAGTTCAGAAATAGC-3'	5'-GAAGGGTCTTGACTTATCACGG-3'	NM_009041.3	112
Merlin	5'-GCTCTAGAAATCGCCACCA-3'	5'-CGCTTCATGTCCGTATCCTT-3'	NM_001252250.1	135
Moesin	5'-CAATGCCCGAGATGAGTCC-3'	5'-AATGCGTTGTTTGGTGTGC-3'	NM_010833.2	124
β-actin	5'-CTTGGGTATGGAATCCTGTGG-3'	5'-GAGGCTTTACGGATGTCAACG-3'	NM_007393.3	82

software (Tanon-Tanon Science & Technology Co., Ltd., Shanghai, China). Protein levels were normalized via comparison with GAPDH signals on the same membrane.

Oil Red O staining for cultured cells The 3T3-L1 mouse embryonic fibroblasts were washed with PBS. First, 2 mL of 10% formalin was added and incubated for 10 min at room temperature. Then, 2 mL of fresh formalin was added and incubated for at least 1 h. Next, the cells were washed with 2 mL of 60% isopropanol for 5 min and were completely dried at room temperature. Further, 1 mL of Oil Red O (Sigma-Aldrich) working solution was added and incubated at room temperature for 10 min. Finally, 1 mL of 100% isopropanol was added and incubated for 10 min with gentle shaking. OD was measured at 510 nm using 100% isopropanol as control.

Statistical analysis The data represents the mean±standard error. Data were analyzed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Two treatment effects were evaluated using an independent sample *t*-test, and various treatment groups were evaluated using one-way ANOVA. **p*<0.05 was statistically significant.

Results and Discussion

Oil Red O staining, mRNA, and protein expressions after differentiation induction in 3T3-L1 mouse embryonic fibroblasts

The aim of the experiment is to determine whether LiCl has an inhibitory effect in adipocyte differentiation. Moreover, we want to select the optimal concentration of LiCl.

Generally, 3T3-L1 is differentiated into mature adipocytes after differentiation induction for 8 days. A most of studies has found that lithium can modulate various biological processes such as mobility, apoptosis, glycogen synthesis, gene expression, and inflammation (20). Recently, LiCl, as the agonist of Wnt/ β -catenin signaling, has been widely applied. In particular, 20 mM treatment concentration is considered to be the optimal concentration (21). In this experiment, we set three treatment concentrations to verify the feasibility of 20 mM LiCl. Oil Red O stain indicates that OD values of the LiCl treatment group were significantly lower than those of the control group (Fig. 1A) (**p*<0.05). In particular, OD values were the lowest in the 20 mM LiCl treatment group (Fig. 1A) (**p*<0.05). Thus, 20 mM LiCl successfully inhibits adipocyte differentiation in this experiment, the results of which are similar to the findings of Du's study (22).

In addition, we measured the relative mRNA expression of fat synthesis and adipocyte differentiation correlation factors. As shown in Fig. 1B relative mRNA expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) were significantly higher than those of the control group (**p*<0.05) compared with groups treated with 20 mM LiCl. Relative mRNA expression of lipoprotein lipase (LPL) at 20 mM 20 mM LiCl was significantly lower than that of the control group (Fig. 1B) (**p*<0.05). These results suggest that mRNA expression

of ACC, FAS, and LPL may be affected by LiCl via negative feedback regulation. Of course, another explanation rests in the fact that LPL acts synergistically with PPAR γ . LPL can hydrolyse triglycerides to fatty acids, and fatty acids are activators of PPAR γ . Therefore, LPL may regulate adipocyte differentiation by regulating PPAR γ expression. As a transcriptional regulation factor, PPAR γ relative mRNA expression of 20 mM LiCl was significantly lower than that of the control group (Fig. 1B) (**p*<0.05). The other three transcriptional regulation factors of the 20 mM LiCl treatment group including C/EBP α , C/EBP β , and SREBP1c, did not significantly changed (Fig. 1B) (**p*>0.05). The results also confirmed that Wnt/ β -catenin signaling can inhibit adipocyte differentiation, perhaps by decreasing PPAR γ relative mRNA expression. This is similar to the result of Kang's study, which reported that over-expressed β -catenin can rapidly inhibit PPAR γ relative mRNA expression (23). In addition, Kawai's study proposed that C/EBP α and PPAR γ were the mechanisms of Wnt/ β -catenin signaling, regulating adipogenic differentiation (24). Although, in this experiment, C/EBP α mRNA expression at 20 mM LiCl was not significantly lower than that of the control group, it had a relatively obvious downside. These results partly confirm the findings of Chen's study that miR-709 inhibits 3T3-L1 cell differentiation leading to a reduction in the mRNA relative expression of PPAR γ 2, CEBP α , FABP2, and adiponectin (25). Stearoyl-CoA desaturase-1 (SCD1) mRNA expression at 20 mM LiCl was higher than that of the control group but not significantly (Fig. 1B) (**p*>0.05). SCD1 can catalyze saturated fat acids to mono-unsaturated fat acids, and monounsaturated fat acids are easier to use as the substrate of diacylglycerol acyltransferase to synthesize triglycerides. As important indicators of adipocyte differentiation, adipocyte protein 2 (AP2) and glucose transporter 4 (GLUT4) mRNA expression at 20 mM LiCl were significantly lower than those of the control group (Fig. 1B) (**p*<0.05). Thus, 20 mM of LiCl successfully inhibits adipocyte differentiation.

LiCl can activate Wnt/ β -catenin signaling and enhances β -catenin expression (26); thus, β -catenin mRNA expression at 20 mM LiCl was significantly higher than that of the control group (Fig. 1C) (**p*<0.05). This suggest that Wnt/ β -catenin signaling was opened. Simultaneously, villin 2 mRNA expression at 20 mM LiCl treatment group was significantly higher than that of the control group (Fig. 1C) (**p*<0.05). Therefore, we can conclude that LiCl can affect the mRNA expression of villin 2.

Generally, villin 2, radixin, moesin, and merlin belong to the ERM cytoskeleton-associated protein family. The other three members of the ERM cytoskeleton-associated protein family did not show any significant difference from those of the control group on the relative mRNA expression (Fig. 1C) (**p*>0.05). The results reveal that the cell structure may not be affected; thus, villin 2 is related with adipocyte differentiation. The result also confirmed that villin 2 is the down-regulated during adipocyte differentiation (1).

Therefore, we suggest that villin 2 may regulate adipocyte differentiation via Wnt/ β -catenin signaling by LiCl. Previous research has shown that Wnt5a/Ror2 signaling can regulate villin expression

Fig. 1. Oil Red O staining, mRNA, and protein expressions after differentiation induction for 8 days in 3T3-L1 mouse embryonic fibroblasts. (A) Oil Red O staining analysis of OD values in different treatment groups. (B) RT-PCR analysis of ACC, FAS, LPL, PPAR γ , c/EBP α , c/EBP β , SREBP1c, SCD1, AP2, and GLUT4 mRNA expressions in different treatment groups. (C) RT-PCR analysis of villin 2, β -catenin, radixin, moesin, and merlin mRNA expressions in different treatment groups. (D) Western blot analysis of villin 2 protein expression in different treatment groups. (E) Western blot analysis of β -catenin protein relative expression in different treatment groups. * p <0.05; letters (a, b, c, and d or A and B), p <0.05 or p <0.01.

in human intestinal cells (27). The correlation between villin 2 and β -catenin on the relative protein expression further strengthens this possibility (Fig. 1D and 1E) (* p <0.05).

mRNA and protein expressions during differentiation induction in 3T3-L1 mouse embryonic fibroblasts From the first experiment, we have confirmed that 20 mM LiCl can inhibit adipocyte differentiation after differentiation induction for 8 days in 3T3-L1 mouse embryonic fibroblasts. Therefore, we want to know the mRNA and protein expressions during differentiation induction in depth.

As shown in Fig. 2A and 2B, from the next day, GLUT4 and AP2 mRNA expression of 20 mM LiCl were significantly lower than those of the control group. Moreover, this declining tendency continued throughout differentiation induction in 3T3-L1 mouse embryonic fibroblasts (* p <0.05).

PPAR γ and SCD1 mRNA expression at 20 mM LiCl were significantly lower than those of the control group for 2 days (Fig. 2C and 2D) (* p <0.05). However, the two genes appeared to have rebounded

after 4 days; the latter was more serious (Fig. 2C and 2D) (* p >0.05). PPAR γ mRNA expression at 20 mM LiCl treatment group tends to drop off over time (Fig. 2C) (* p <0.05). SCD1 mRNA expression at 20 mM LiCl treatment group dropped significantly after 6 days but rebounded after 8 days (Fig. 2D). This may be because of the half-life of mRNA (28–30). Several studies have proven that this protein has a half-life of 3–4 h and generates a 20-kDa C-terminal SCD fragment (28–30).

Western blotting showed that villin 2 protein relative expression tended to increase at first and subsequently decreased during differentiation induction in the control and treatment groups (Fig. 2E). Villin 2 protein relative expression at 20 mM LiCl was higher than that of the control group after 2 and 4 days but the difference was not significant (Fig. 2E) (* p >0.05). Until differentiation induction for 6 days, villin 2 protein relative expression at 20 mM LiCl was significantly higher than that of the control group (Fig. 2E) (* p <0.05). It remained significant and was rising after 8 days (Fig. 2E) (* p <0.05).

Similarly, β -catenin protein relative expression tended to increase at first and subsequently decreased during differentiation induction

Fig. 2. mRNA and protein expressions during differentiation induction in 3T3-L1 mouse embryonic fibroblasts. (A) RT-PCR analysis of GLUT4 mRNA expression in different treatment groups. (B) RT-PCR analysis of AP2 mRNA expression in different treatment groups. (C) RT-PCR analysis of PPAR γ mRNA expression in different treatment groups. (D) RT-PCR analysis of SCD1 mRNA expression in different treatment groups. (E) Western blot analysis of villin 2 protein expression in different treatment groups. (F) Western blot analysis of β -catenin protein expression in different treatment groups. * $p < 0.05$; letters (a, b, and c), $p < 0.05$.

in the control and treatment groups (Fig. 2F). Relative to villin 2, β -catenin protein relative expression at 20 mM LiCl was significantly higher than that of the control group earlier (Fig. 2F) (* $p < 0.05$). GLUT4, AP2, PPAR γ , and SCD mRNA expression at 20 mM LiCl treatment group were significantly lower than those of the control group after 2 days (Fig. 2A, 2B, 2C, and 2D). Particularly, β -catenin protein relative expression at 20 mM LiCl treatment group was significantly higher than that of the control group after 2 days (Fig. 2F). Moreover, in Jin's study, villin 2 protein is sharply down-regulated in 3T3-L1 mouse embryonic fibroblasts after 2 days of differentiation induction (1). Therefore, 20 mM LiCl may open Wnt/ β -catenin signaling after 2 days. However, β -catenin protein expression of the control group significantly increased after 4 days (Fig. 2F) (* $p < 0.05$). Thus, β -catenin protein expression at 20 mM LiCl was not significantly different from that of the control group after 4 and 6 days (Fig. 2F) (* $p > 0.05$). This may be because villin 2 protein expression of 20 mM LiCl relatively increased after 2 days, which

rapidly opened Wnt/ β -catenin signaling. At this point, because of negative physiological feedback, β -catenin protein expression of the treatment group was not significantly different from that of the control group after 4 and 6 days. Villin 2 protein expression of the treatment group continued to increase; therefore, negative physiological feedback did not resist the increasing of villin 2 protein expression. Therefore, β -catenin protein expression at 20 mM LiCl was again significantly higher than that of the control group after 8 days (Fig. 2F) (* $p < 0.05$).

mRNA and protein expressions after differentiation induction in 3T3-L1 mouse embryonic fibroblasts From the above experiments, we confirmed that LiCl can inhibit adipocyte differentiation and enhances villin 2 and β -catenin expressions during differentiation induction. Thus, we want to know the mRNA and protein expressions after differentiation induction in 3T3-L1. In the third experiment, after 8 days of differentiation induction, 20 mM LiCl was added to the

Fig. 3. mRNA and protein expressions after differentiation induction in 3T3-L1 mouse embryonic fibroblasts. (A) RT-PCR analysis of PPAR γ , SREBP1c, SCD1, AP2 mRNA expressions in different treatment groups. (B) Western blot analysis of villin 2 protein expression in different treatment groups. (C) Western blot analysis of β -catenin protein expression in different treatment groups. (D) Correlation analysis of villin 2 and β -catenin protein expressions not only during differentiation induction but also after differentiation induction. * $p < 0.05$ or ** $p < 0.01$; letters (a, b, and c), $p < 0.05$.

differentiation induction medium and the cells were harvested for 8, 10, 12, and 14 days. PPAR γ , SREBP1c, SCD1, and AP2 mRNA expression of 20 mM LiCl were significantly lower than those of the control group after 14 days (Fig. 3A) (* $p < 0.05$). This result suggested that 20 mM LiCl not only inhibits cell differentiation during differentiation induction but also after differentiation induction.

Western blotting revealed that villin 2 protein expression tended to be significantly decreased in the control group (Fig. 3C) (* $p < 0.05$). This finding is similar to the finding of Jin's study, which reported that villin 2 is the down-regulated gene of adipocyte differentiation (1). When LiCl was added, the downtrend was alleviated (Fig. 3C) (* $p > 0.05$). Villin 2 protein expression of 20 mM LiCl was significantly higher than that of the control group after 12 and 14 days (Fig. 3C) (* $p < 0.05$).

The level of β -catenin protein expression was similar to villin 2 protein expression (Fig. 3D). This again suggest that villin 2 has a close relation with β -catenin. Moreover, LiCl can inhibit adipocyte differentiation and enhances villin 2 and β -catenin expressions not

only during differentiation induction but also after differentiation induction. As further analysis, we examined the correlation between villin 2 and β -catenin protein expression not only during differentiation induction but also after differentiation induction. Figure 3E displays significant correlation between villin 2 and β -catenin (* $p < 0.01$). Moreover, we obtained regression equations ($y = 0.122x + 0.182$, $r = 0.691$).

From the above three experiments, we can conclude that LiCl inhibits 3T3-L1 cell differentiation via regulating the Wnt/ β -catenin pathway and enhancing villin 2 expression. We suggest that villin 2 may participate in Wnt/ β -catenin signaling by LiCl, but we are still unable to confirm whether villin 2 regulates Wnt/ β -catenin signaling or Wnt/ β -catenin signaling regulates villin 2. Therefore, we need more specific research to prove our points.

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Disclosure The authors declare no conflict of interest.

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