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Knocking down clock control gene CRY1 decreases adipogenesis via canonical Wnt/β-catenin signaling pathway

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ABSTRACT

Cryptochrome gene 1(CRY1) is a member of circadian clock genes, which play an important role in adipocyte biology. CRY1 was reported to be related with the lipid metabolism, but the molecule mechanism of CRY1 in regulating the adipogenesis remains unclear. Here we report that CRY1 is a key regulator in adipogenic differentiation. We found that the expression levels of CRY1 in 3T3-L1 cells and C3H10T1/2 cells gradually increased during the process of adipogenic differentiation. Knockdown of endogenous CRY1 significantly inhibited the expression of adipogenic markers and lipid droplet formation in cells under adipogenic induction. In addition, knockdown of endogenous CRY1 promoted the expression and nuclear accumulation of β-catenin, the critical signal molecular in the canonical canonical Wnt signaling pathway, suggesting the regulation effect of CRY1 in adipogenesis was mediated by canonical Wnt/β-catenin signaling. Taken together, our study suggests that CRY1 regulates adipogenic differentiation through modulating the canonical Wnt/β-catenin signaling pathway.

1. Introduction

Steroid-induced osteonecrosis of the femoral head (SONFH) is a common bone disease with high disability rate [1]. Due to excessive use of glucocorticoid, trabecular bone and bone marrow necrosis occur in the femoral head, and the femoral head collapses and deforms, eventually leading to hip dysfunction [2]. SONFH is characterized by lipid metabolism disorders such as increased adipogenesis and fat cell hypertrophy in the bone marrow which cause increased intraosseous pressure, ultimately leading to avascular necrosis of the bone [3]. At present, the treatment of femoral head necrosis is hip replacement, which has many problems such as high cost, great pain and need for renovation. So, a better treatment is urgently needed. Improving the lipid metabolism disorder in the femoral head is of great importance to control the necrosis of the femoral head from the root cause, such as inhibiting the excessive adipogenic differentiation of stem cells, which may be achieved through gene therapy.

Adipogenic differentiation is a complicated process involves the determination phase and the terminal differentiation phase [4]. During the former phase multipotent mesenchymal stem cells(MSCs)commit to the preadipocyte, which is not morphologically distinguishable from MSCs. During the latter phase, preadipocytes differentiate into adipocytes, which can synthesize and store lipid. Throughout the adipogenesis, multiple signaling cascades is involved such as Wnt Signaling, Hedgehog Signaling, and BMP Signaling [5–7], converging at the level of peroxisome proliferator-activated receptor-γ (PPARγ) transcriptional activity, which is a master adipogenic transcription factor and play important roles in adipogenic differentiation [4].

The circadian clock plays an important role in regulating the coordinated and orderly progress of various complex life activities in the body and maintaining normal life activities, such as circadian rhythm, immunity, and lipid metabolism [8–10]. Abnormal expression of the circadian clock genes due to circadian rhythm disorders result in many diseases such as cancer, endocrine diseases, cardiovascular diseases and lipid metabolism disorder [11–14]. Lipid metabolism disorder has been reported to be significantly associated with circadian disruption, and components of the molecular clock network could regulate adipogenic differentiation. As reported, BMAL1 can inhibits adipogenesis through the TGF-β pathway together with BMP signaling [15]. Rev-erbα can promote brown adipogenesis [16]. Deletion of Per3 promotes adipogenesis by a clock output pathway [17]. These studies highlight the connection between circadian gene and adipogenic

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differentiation.

Up to now, 14 circadian clock genes have been discovered. These clock genes are present in almost all cells in the human body, which interact to form a network of multiple positive and negative feedback loops at the transcriptional and translational levels [18]. Different clock genes affect the life activities of cells by regulating different downstream clock controlled genes [19]. CRY1, one of the core clock genes, plays an important role in lipid metabolism. Toledo M et al. reported that high-fat-fed mice have reduced levels of CRY1 protein, leading to obesity-associated hyperglycemia [20]. Griebel et al. knocked out the mouse CRY1 gene to study the relationship between CRY1 and fat metabolism, and found that CRY1 deficient mice are less likely to gain weight on a high-lipid diet [21]. These studies indicate that CRY1 is closely related to fat metabolism. However, the effect and exact mechanism of CRY1 on adipogenic differentiation of MSCs are still unclear.

In this study, we examined the role of CRY1 in the 3T3-L1 cells and C3H10T1/2 cells, which well proved for the natural manner of adipogenic differentiation. Our results show that knockdown of CRY1 inhibited adipogenic differentiation, as well as the expression of adipogenesis related genes. Besides, canonical Wnt/β-catenin signaling pathway was activated in the CRY1 knockdown cells. All the findings suggest that CRY1 is an important regulator of adipogenic differentiation.

2. Materials and methods

2.1. Reagents and antibodies

Dexamethasone (DM), Recombinant human Insulin (IS), Isobutylmethylxanthine (IBMX), Indomethacin (ID), Oil Red O were purchased from Sigma Aldrich. Antibody to CRY1 was from Abcam. Antibody to β-catenin was from Santa Cruz Biotech. Antibodies to GSK-3β, Histone and β-Actin were purchased from Cell Signaling Inc.

2.2. Plasmids

Three plasmids including pLKO.1-EGFP- puromycin, psPAX2, and pMD2.G were purchased from GeneChem, CHN. They are involved in the lentivirus system for short hairpin RNA (shRNA) expression. we have designed two shRNAs (Table 1) to knockdown CRY1, which were annealed and inserted into the lentiviral vector pLKO.1-EGFP-puromycin.

2.3. Lentivirus production

To product lentiviruses, lentiviral vector (pLKO.1-puro or pCDH-CMV-MCS-EF1-Puro), psPAX2, pMD2.G and Lipofectamine2000 (Invitrogen) were mixed and added into HEK293T cells which were planted in high glucose DMEM without FBS. The cell density is about 80%. 12 h after transfection, the medium was changed to high glucose DMEM with 10% FBS. Two days after changing the medium, the supernatants were collected and filtered through a 0.45-μm membrane (Millipore).

2.4. Cell transfection

The 3T3-L1 cells and C3H10T1/2 cells were cultured to a density of 80% in 6-well plates. 6 μg/ml polybrene (Sigma, USA) and lentiviruses were added to the medium to get CRY1 knockdown cells. After 24h, the medium was changed to DMEM with 4 μg/mL of puromycin (Sigma, USA) for 3 days. In the next week, the medium was changed to DMEM with 1 μg/mL of puromycin. Then the stable CRY1 knockdown cells were gotten and cultured in high glucose DMEM with 10% FBS.

2.5. Cell culture and adipogenic induction

The 3T3-L1 and C3H10T1/2 cell lines were planted into growth media (10% FBS in High Glucose DMEM) and changed with growth media every three days. These cell lines were induced to differentiate when they reached 100% confluence by replacing differentiation medium, which was compounded with 500 μM IBMX, 200 μM Indomethacin, 1uM Dexamethasone and 10uM insulin in High Glucose DMEM media containing with 10% FBS. Then the differentiation media was renewed every 2 days until day 7.

2.6. Oil Red O staining and imaging

Oil red O Staining was taken to measure the accumulation of liquid. The differentiated cells were rinsed with PBS and fixed in 4% formaldehyde for half an hour in 25 °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.

2.7. Quantitative real-time PCR

Total RNA was obtained from cells by using Trizol method according to manufacturers' instructions. 500 ng of total RNA was reverse transcribed to cDNA using a cDNA synthesis kit. The 10uL reaction volume was diluted with 90uL water that was treated by DEPC. The diluted cDNAs were combined with SYBR Green, Dye II, forward and reversed primer in 96-well-plates. The real-time PCRs were run on an ABI 7500 Real-time PCR system using the following thermocycling conditions: (1) 95 °C 10 min, 1× (2) 95 °C 15s, 57 °C 15s, 72 °C 15s, 40×. The qRT-PCR primers for CRY1, PPARγ, CEBPα, SREBP1and β-actin were searched from PrimerBank. Every qRT-PCR was performed three times. All of primer sequences can be found in Table 2. The data was analyzed by using the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blot

The medium was removed and the adherent cells was washed with cold PBS twice. 60 μL modified RIPA buffer with PMSF (1:200) was added to the 60 mm dishes and the cells were scraped on the ice. The cell lysate was collected into EP tubes and ice bathed for 30 min with a vortex for 10 s every 3 min. Then the cell lysate is centrifuged (12000 rpm, 4 °C, 10min) and supernatant is taken and the protein concentration was measured by the BCA Protein Assay Kit (Beyotime, China) according to the manufacturer. The protein sample was subjected to gel electrophoresis and transferred to a

Table 1
Sequences of CRY1-shRNA.

Group	Sense strand	Antisense strand
CRY1-shRNA1	5'-CAAGTGTTTGATAGGAGTT-3′	5′- AACTCCTATCAAACACTTGGC -3′
CRY1-shRNA2	5′-GCCACCTCTAACATATAAA-3′	5′- TTTATATGTTAGAGGTGGCTG -3′
CRY1-shRNA3	5′-ATCAGTGTTTGATCTAATT-3′	5′-AATTAGATCAAACACTGATGT-3′
Control-shRNA	5′-TTCTCCGAACGTGTCACGT-3′	

Table 2
Primer sequences.

Gene	Forward	Reverse
CRY1	5′-CACTGGTTCCGAAAGGGACTC-3′	5′-CTGAAGCAAAAATCGCCACCT-3′
CEBPα	5′- GCGGGAACGCAACAACATC -3′	5′- GTCACTGGTCAACTCCAGCAC -3′
PPARγ	5′- GGAAGACCACTCGCATTCCTT -3′	5′- GTAATCAGCAACCATTGGGTCA -3′
SREBP1	5′- TGACCCGGCTATTCCGTGA -3′	5′- CTGGGCTGAGCAATACAGTTC -3′
β-actin	5′- GGGACCTGACTGACTACCTC-3′	5′- TCATACTCCTGCTTGCTGAT-3′

PVDF membrane (Millipore, USA). The PVDF membrane is then blocked with 5% skimmed milk. These membranes were incubated overnight at 4 °C with primary antibody including CRY1,β-catenin,gsk-3β and β-actin. The bands were visualized with a Fluor Chem E system using the ECL Detection kit (Share-bio, China). Every western blot was performed three times.

2.9. Statistic analysis

Each experiment was repeated 3 times independently, and statistical analysis was performed using Statistical Package for Social Sciences (SPSS) 21.0 software. The data were expressed as mean ± standard deviation. Statistical analysis of the differences between the groups was performed by analysis of variance (one-way ANOVA). $P < 0.05$ indicates that the difference was statistically significant.

3. Results

3.1. Adipogenesis induces CRY1 expression

Before we evaluated the effect of CRY1 on adipogenic differentiation, we first examined the expression of CRY1 in 3T3-L1 cells and C3H10T1/2 cells by using Western Blotting. We found that CRY1 is expressed in both 3T3-L1 cells and C3H10T1/2 cells. (Fig. 1A 1B). We then measured the expression of CRY1 in 3T3-L1 cells and C3H10T1/2 cells treated with adipogenic differentiation medium(500 μM IBMX, 200 μM Indomethacin, 1uM Dexamethasone and 10uM insulin)for adipogenic differentiation. The expression level of CRY1 gradually increased with time during the adipogenic differentiation (Fig. 1C 1D 1E). During this period, the mRNA expression of classic adipogenic marker genes such as CEBPα, PPARγ and SREBP1 were increased (Fig. 1F–H) and the fat droplets in the cells have also increased as shown in Fig. 1I. All of these findings suggest that CRY1 may be essential for adipogenic differentiation.

3.2. Establishment of CRY1 silencing cell lines

To investigate the potential role of CRY1 in adipogenic differentiation, we knockdown the expression of endogenous CRY1 by lentivirus-mediated infection with three specific shRNAs (shRNA1-3). The expression protein level of CRY1 was measured using Western Blot (Fig. 2A–D) and the mRNA level of CRY1 was examined by qRT-PCR (Fig. 2E–F). Both methods show that the CRY1 expression level was significantly reduced in 3T3-L1 cells and C3H10T1/2 cells expressing CRY1 shRNA-3.

3.3. Knockdown of CRY1 inhibits adipogenic differentiation and adipogenesis-specific gene expression

We next examined the effect of CRY1 on adipogenic differentiation. The control shRNA and CRY1 shRNA treated 3T3-L1 cells and C3H10T1/2 cells were cultured in the adipogenic differentiation medium. The Oil red O staining and its quantitative assay clearly showed that CRY1 knockdown attenuated differentiation of 3T3-L1 cells and C3H10T1/2 cells from day 4 onward, as indicated by the presence of fewer lipid accumulating cells (Fig. 3A–C). During the adipogenic differentiation, the mRNA expression levels of classic adipogenic marker genes such as CEBPα, PPARγ and SREBP1 decreased compared with the control cells (Fig. 3D–I), which is consistent with results of oil red O staining. Collectively, these findings suggest that knock down CRY1 inhibits adipogenic differentiation.

3.4. Knockdown of CRY1 activates canonical Wnt/β-catenin signaling pathway

To further explore the mechanism involved in the regulation of adipogenic differentiation of CRY1, we examined signaling pathways which can regulate adipogenic differentiation such as Wnt Signaling, Hedgehog Signaling, and BMP Signaling in control shRNA and CRY1 silencing 3T3-L1 cells and C3H10T1/2 cells. We found that canonical Wnt/β-catenin signaling pathway was significantly activated. As shown in Fig. 4A 4C 4D, CRY1 knockdown upregulated the protein level of both total β-catenin and the β-catenin in nuclear, as well as decreased the protein level of GSK-3β. We also examined the canonical Wnt/β-catenin signaling pathway in response to adipogenic stimulation. We found that as the time of adipogenic stimulation increased, the protein levels of CRY1 in both the control and shRNA cells increased, and the levels of β-catenin decreased. At each time point, the protein levels of β-catenin in the shRNA cells were always higher than the control cells. Thus, we considered that the canonical Wnt/β-catenin signaling pathway may mainly participate in the CRY1-regulated adipogenic differentiation.

4. Discussion

The importance of the present findings is that CRY1 is functionally expressed in multipotent 3T3-L1 and C3H10T1/2 cell lines and appears to regulate adipogenic differentiation and maturation. Although C3H10T1/2 cells are more primitive than 3T3-L1 cells, as a mesenchymal stem cell, C3H10T1/2 cells have strong differentiation ability, especially the adipogenic differentiation model of this cell has been widely used by researchers [22–24]. We examined dynamic changes in CRY1 expression during adipogenic differentiation and found that the levels of CRY1 were increased during adipogenic differentiation in both cell lines, which indicated that CRY1 may contribute to the differentiation of these cell lines.

The canonical Wnt/β-catenin signaling pathway plays an important role in regulating adipogenic differentiation [25]. As reported, multiple members of the canonical Wnt/β-catenin signaling family have been verified to inhibit the early stages of adipogenic differentiation. For example, overexpression of Wnt1 suppresses PPARγ and inhibits adipogenic differentiation of 3T3-L1 cells [26]. In addition, Wnt10B has been reported to inhibit the expression of PPARγ and C/EBP-α to prevent 3T3-L1 cells adipogenic differentiation [26]. Inhibition of GSK-3β resulted in general in the suppression of adipogenesis [27,28]. Other canonical Wnt

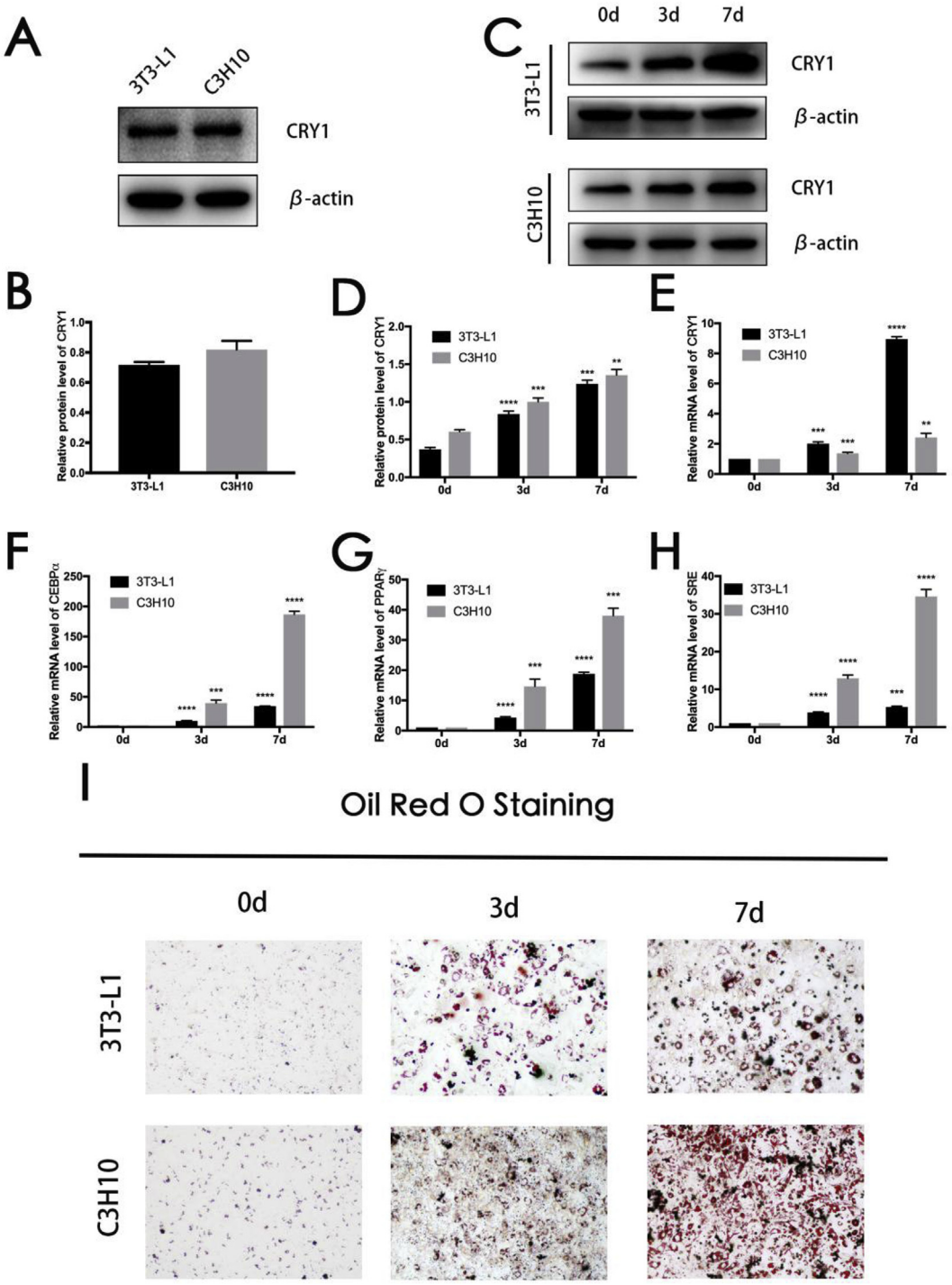

Fig. 1. Expression of CRY1 was increased during 3T3-L1 cells and C3H10T1/2 cells adipogenesis. A. The expression of CRY1 in 3T3-L1 and C3H10T1/2 cells using Western Blot. **B.** Quantitative data of immunoblots of fig A. **C.** The expression level of CRY1 gradually increased with time during the adipogenic differentiation in 3T3-L1 and C3H10T1/2 cells. **D-E.** Measurement of CRY1 in protein levels in the 3T3-L1 and C3H10T1/2 cells. **F-H.** mRNA levels at time points of differentiation of 3T3-L1 cells and C3H10T1/2 cells. **I.** Microscopic images (200X) of 3T3-L1 cells and C3H10T1/2 cells stained with Oil Red O during the adipogenic differentiation. (0d, 3d, and 7d represented 0 days, 3 days, and 7 days after induction of differentiation, respectively. Values are expressed as the mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with 0d.). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ligands, such as Wnt6 and Wnt10a, exhibit similar effects in inhibiting adipogenesis [29]. In our current study, we found that as the time of adipogenic stimulation increased, the protein levels of GSK-3β increased, and the levels of β-catenin decreased, suggesting the inhibition of canonical Wnt/β-catenin signaling pathway, which is consistent with the literature report. Besides, CRY1 silencing resulted in the up-regulation of the protein level of both total β-catenin and the β-catenin in nuclear and declined GSK-3β in adipogenic stimulation. These results demonstrate that down-regulation of CRY1 may activate canonical Wnt/β-catenin signaling pathway.

CRY1 was also reported to be related with PPAR and SREBP

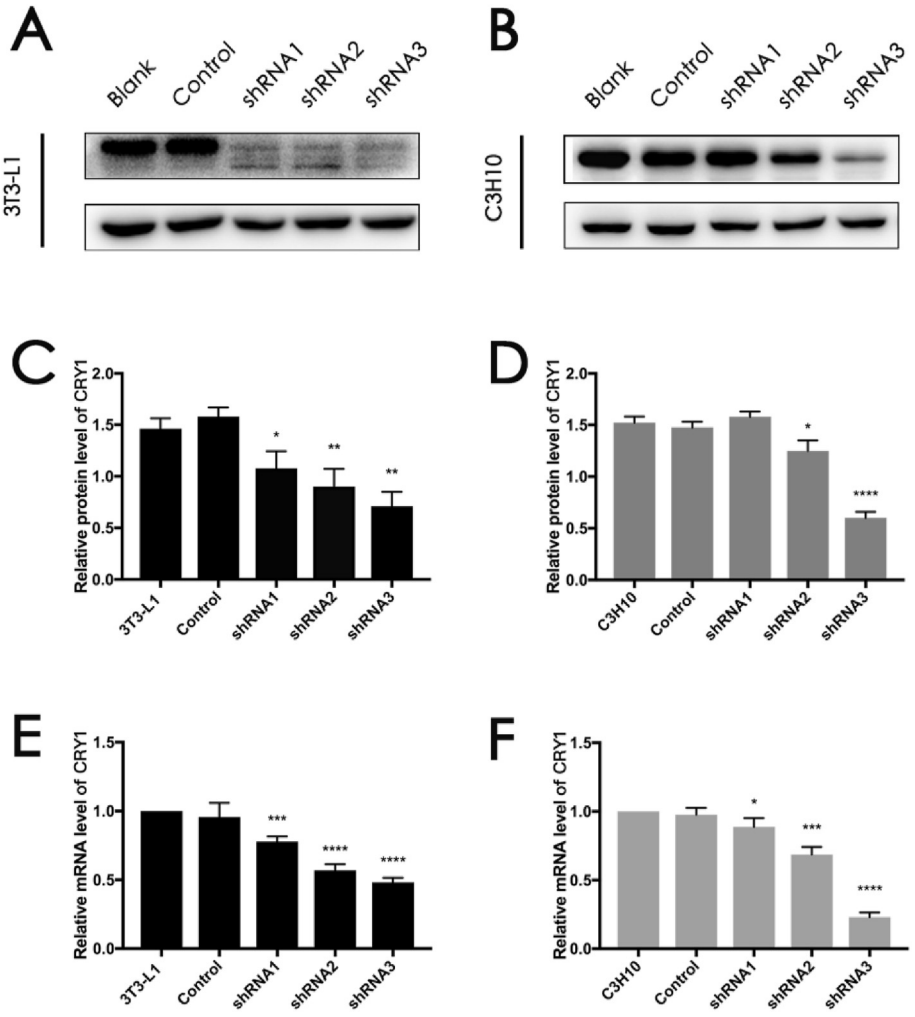

Fig. 2. The establishment of CRY1 knockdown 3T3-L1 cells and C3H10T1/2 cells. A. Western Blot results of CRY1 silencing in the 5 groups of 3T3-L1 cells. **B.** Western Blot results of CRY1 silencing in the 5 groups of C3H10T1/2 cells. **C-D.** Measurement of CRY1 silencing efficiency in protein in the 3T3-L1 cells and C3H10T1/2 cells. **E-F.** Measurement of CRY1 silencing efficiency in mRNA in the 3T3-L1 cells and C3H10T1/2 cells. (0d, 3d, and 7d represented 0 days, 3 days, and 7 days after induction of differentiation, respectively. Values are expressed as the mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with Control.)

[30,31], which can be considered as adipogenic marker genes. In our study, during the adipogenic differentiation, the levels of CRY1 were increased and the expression of adipogenic marker genes such as CEBPα, PPARγ and SREBP1 are promoted, which is consistent with the literature. When the CRY1 was knocked down, the adipogenic gene marker was downregulated. Simultaneously, the Oil Red O Staining showed that the accumulation of lipid has been decreased, which is consistent with the adipogenic marker genes. These results could demonstrate that CRY1 knockdown inhibit adipogenic differentiation of 3T3-L1 and C3H10T1/2 cells.

Studies have shown that the transcriptional and translational levels between the circadian clock genes in normal cells mainly include three network structures of feedback loops [32,33]. In the three feedback loops, CLOCK/BMAL1 heterodimers act as transcription factors to activate the transcription of PERs, CRYs, DECs, REV-ERBA and RORA genes, and initiate the expression of these genes. PERs and CRYs combine as a heterodimer to undergo nuclear transfer and inhibits CLCOK/BMAL1 transcriptional activation, forming the canonical negative feedback pathway, which is also the main pathway. Secondly, DEC1 and DEC2 combine to form a dimer or heterodimer, which acts as a transcription inhibitor to transport to the nucleus and compete with CLCOK/BMAL1 to reduce the transcriptional activation of CLOCK/BMAL1 and form the second negative feedback pathway. REV-ERBA and RORA are transported into the nucleus to inhibit the expression of BMAL1, respectively, forming a third negative feedback pathway. As reported, BMAL1 activates canonical Wnt/β-catenin signaling pathway [34]. In our study, we found that CRY1 knockdown by shRNA activate canonical Wnt/β-catenin signaling pathway. This may be caused by CRY1 silencing directly, or negative feedback effect of the clock gene network. Knocking down CRY1 may cause the canonical negative feedback pathway, leading to overexpression of BMAL1 which activates the canonical Wnt/β-catenin signaling pathway. But the specific mechanism of this still requires further research.

In conclusion, a major finding of our study is that CRY1, as a novel regulator of adipogenic differentiation in vitro, inhibits adipogenic differentiation after knocked down, at least in partial, through the canonical Wnt/β-catenin signaling pathway. It is conceivable that CRY1 knockdown may help to develop new therapeutic strategies through inhibiting adipogenic differentiation of MSCs in the femoral head of patients who have taken glucocorticoid for a long time. Therefore, CRY1 may be a useful target to develop a gene therapeutics to prevent steroid-induced osteonecrosis of the femoral head.

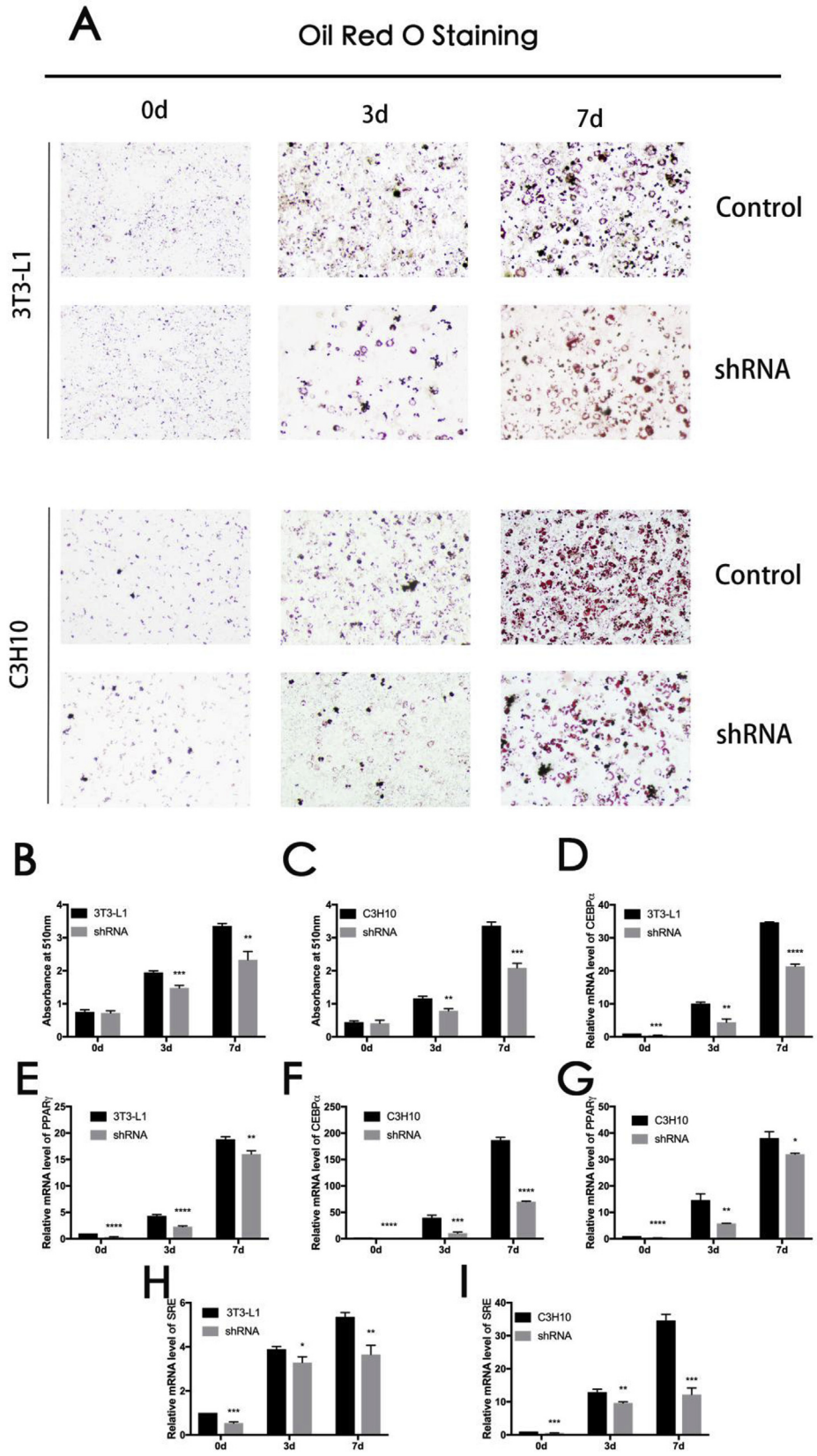

Fig. 3. Stable CRY1 knockdown inhibited adipogenic differentiation of 3T3-L1 and C3H10T1/2 cells. A. Representative microscopic images of Oil-Red-O staining of 3T3-L1 and C3H10T1/2 cells during adipogenic differentiation. **B-C.** Quantitative data of lipid accumulation by measuring the absorbance of extracted Oil-Red-O at 510 nm. **D-I.** In shRNA-CRY1 cells, mRNA expression levels of classic adipogenic marker genes decreased compared with the control cells. (0d, 3d, and 7d represented 0 days, 3 days, and 7 days after induction of differentiation, respectively. Values are expressed as the mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with control cells.). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

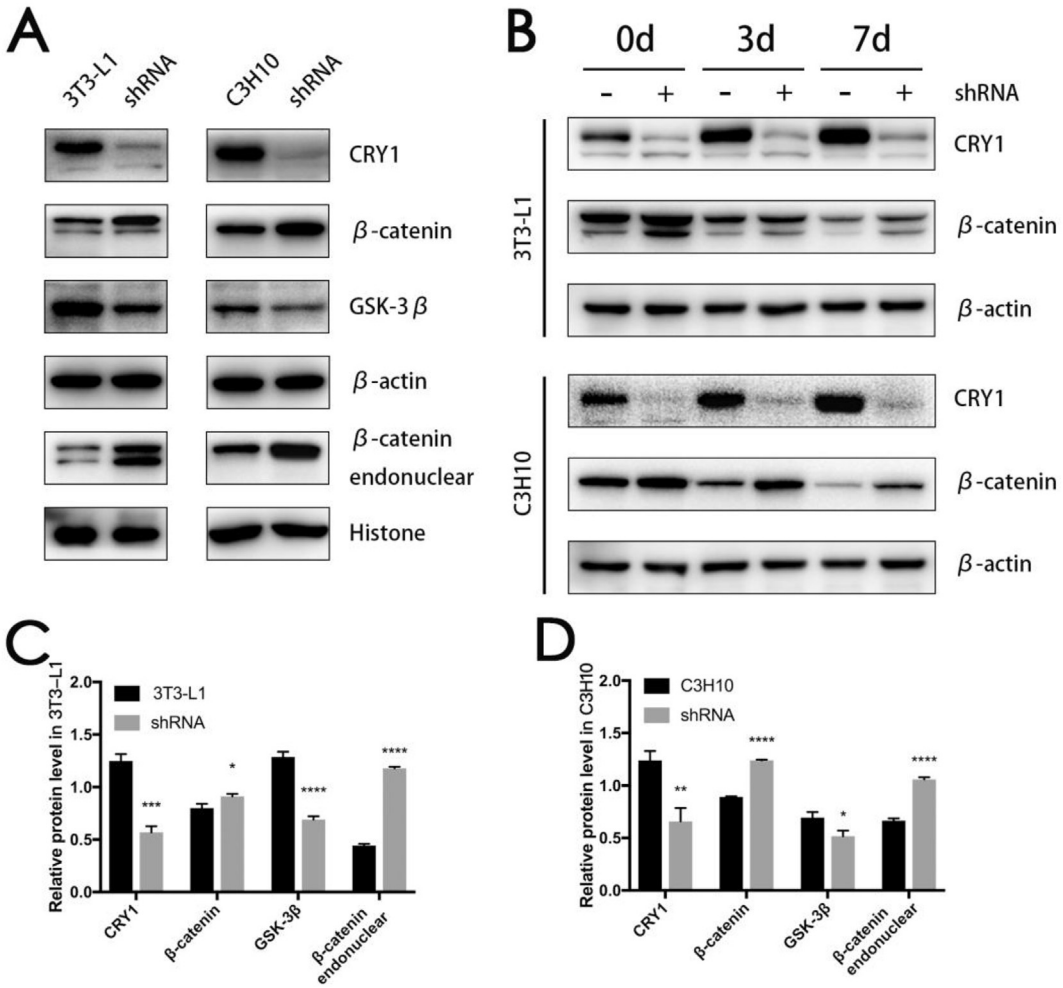

Fig. 4. Knockdown of CRY1 activates canonical Wnt/β-catenin signaling pathway. A. Western Blot results of critical signal molecular of canonical Wnt/β-catenin signaling pathway in 3T3-L1 and C3H10T1/2 cells after CRY1 knockdown. **B.** Critical signal molecular of canonical Wnt/β-catenin signaling pathway in control and shRNA cells changed during adipogenic differentiation. **C-D.** Quantitative data of immunoblots of fig A.

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