Acta Biochim Biophys Sin, 2016, 48(6), 509–519
doi: 10.1093/abbs/gmw032
Advance Access Publication Date: 28 April 2016
Original Article

Original Article

Lipogenesis in myoblasts and its regulation of *CTRP6* by AdipoR1/Erk/PPARγ signaling pathway

Wenjing Wu, Yunmei Sun, Chen Zhao, Cunzhen Zhao, Xiaochang Chen, Guoqiang Wang, Weijun Pang*, and Gongshe Yang*

Laboratory of Animal Fat Deposition & Muscle Development, College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China

*Correspondence address. Tel: +86-29-87091893; Fax: +86-29-87092430; E-mail: pwj1226@nwsuaf.edu.cn (W.P.)/ gsyang999@hotmail.com (G.Y.)

Received 23 November 2015; Accepted 28 March 2016

Abstract

The induced lipogenesis and its regulation in C2C12 myoblasts remain largely unclear. Here, we found that the cocktail method could significantly induce lipogenesis through regulating lipid metabolic genes and Erk1/2 phosphorylation in myoblasts. Meanwhile, the expression and secretion of CTRP6 were increased during ectopic lipogenesis. Moreover, *CTRP6* knockdown down-regulated the levels of lipogenic genes and phosphorylated Erk1/2 (p-Erk1/2) in the early lipogenic stage, whereas up-regulated p-Erk1/2 in the terminal differentiation. Interestingly, the effect of CTRP6 siRNA was attenuated by U0126 (a special p-Erk1/2 inhibitor) in myoblasts. Furthermore, AdipoR1, not AdipoR2, was first identified as a receptor of CTRP6 during the process of mitotic clonal expansion. Collectively, we suggest that *CTRP6* mediates the ectopic lipogenesis through AdipoR1/Erk/PPARγ signaling pathway in myoblasts. Our findings will shed light on the novel biological function of *CTRP6* during myoblast lipogenesis and provide a hopeful direction of improving meat quality of domestic animal by lipogenic regulation in skeletal muscle myoblasts.

Key words: CTRP6, C2C12 myoblast, lipogenesis, AdipoR1/Erk/PPARγ signaling pathway

Introduction

Ectopic lipogenesis commonly occurs in myopathies, as well as in other diseases, such as severe neurogenic atrophy, mitochondrial myopathy, obesity, and ageing-related sarcopenia [1–3]. In the pathogenesis of fatty degeneration, it has been reported that stem cells in the musculature serve as a source of adipocytes. Lipogenesis could be induced in several types of cells isolated from skeletal muscle including satellite cells and side population cells. C2C12 myoblasts, a well-established model for myogenesis study, have been reported to possess this ability [4,5] that could be influenced by several factors including the cytokines, retinoic acid, and transcriptional regulators [6–8]. It is well known that myogenesis is controlled by myogenic regulatory factors (MRFs), while lipogenesis is regulated by the adipogenic transcription factors including peroxisome proliferator activated receptor γ (PPARγ) and the CCAAT enhancer-binding protein α (C/EBPα) [9]. Therefore, the adipogenic marker genes may be involved in the ectopic lipogenesis in skeletal muscle cells. However, the regulatory mechanism of lipogenesis in C2C12 myoblasts is still unclear.

C1q/TNF-related proteins (CTRPs) are a protein family consisting of 15 (CTRP1-CTRP15) newly identified adiponectin paralogs. In addition to sharing structural similarity with adiponectin, CTRPs also regulate similar metabolic processes [10,11]. CTRP6 is a 240-amino acid protein consisting of a signal peptide, a short variable region, a collagen-like region, and a C-terminal globular domain. The C-terminal globular domain is thought to be the functional

domain that may interact with other proteins or receptors. A recent study revealed that the levels of CTRP6 in serum and fat tissues were enhanced in obese, ob/ob, and adiponectin null-mice [12]. As reported, rosiglitazone treatment decreased CTRP6 expression in adipose tissue [12,13]. Our previous study indicated that decrease of CTRP6 expression and secretion inhibited adipogenesis in 3T3-L1 cells [14]. Moreover, in C2C12 cells, CTRP6 could stimulate fatty acid oxidation via the activation of *AMPK* [15]. In order to find a hopeful direction for human myopathies therapy, whether CTRP6 is involved in fat formation needs to be further explored in skeletal muscle cells.

In the present study, we investigated the effects of CTRP6 and U0126 (a special p-Erk inhibitor) on the ectopic lipogenesis in C2C12 myoblasts. Our results suggested that *CTRP6* regulated the ectopic lipogenesis through AdipoR1/Erk/PPARγ signaling pathway in myoblasts. These findings will shed light on the novel biological function of *CTRP6* during lipogenesis in myoblasts.

Materials and Methods

Cell culture and lipogenesis induction

The C2C12 myoblast cells (ATCC, New York, USA) were cultured in growth medium of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics at 37°C in 5% CO_2. At 90%–95% confluence, cells were induced in three different media including the growth medium, the myogenic induction medium (DMEM with 2% horse serum), and the cocktail medium (DMEM with 10% FBS, 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, and 20 μg/ml insulin), respectively. The cells were incubated with the above differentiation media for 2 days and then maintained in DMEM with 10% FBS and 5 mg/ml insulin for another 4–6 days [16]. During the differentiation process, medium was changed every other day.

Oil Red O staining and BODIPY staining

After being fixed in 4% (v/v) paraformaldehyde solution, incubated with 0.5% Oil Red O for 30 min, and washed three times with PBS, the myoblast cells were visualized by phase-contrast microscopy (IS-Elements software, Nikon ECLIPSE, Japan). Oil Red O dissolved in lipid droplets was extracted with 100% isopropanol and its relative concentrations were determined by measuring the absorbance at 510 nm [17].

BODIPY staining was performed according to the previously published method [18].

ELISA

CTRP6 released from myoblast cells was measured using the mouse CTRP6 Quantikine ELISA Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Quantitative real-time reverse-transcriptase polymerase chain reaction (real-time qPCR)

Total RNA was isolated from tissues or cells with TRIZOL® reagent (Takara, Tokyo, Japan). The RNA concentration was determined and reverse transcription was carried out using the First-Strand cDNA Synthesis kit (Takara). Real-time qPCR was carried out on a Bio-Rad iQ5 multicolor Real-Time PCR Detection system (Bio-Rad, Hercules, USA) by using qPCR SYBR Green Master Mix (Vazyme, Piscataway, USA). The functional genes included *PPARγ*, *aP2* (adipocyte fatty acid-binding protein 4), *TGH* (triacylglycerol hydrolase), *ATGL* (adipose triglyceride lipase), *adiponectin*, *AdipoR1* (adiponectin receptor 1), and *CTRP6*, while *β-actin* was used as an internal control. The primer sequences used in this study were listed in **Supplementary Table S1**. Relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Triglyceride (TG) content assay

Treated myoblasts were washed gently with PBS and scraped on ice in 30 μl of 2% Triton X-100. TG contents were measured using TG Assay Kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions [19].

Transfection

CTRP6 siRNA and scrambled oligonucleotides were purchased from the GenePharma Company (Shanghai, China) and listed in **Supplementary Table S2**. Cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) as described in the manufacturer's protocol. At 4 h before transfection, the medium was changed to Opti-MEM®Medium (Gibco, Grand Island, USA). The siRNA (20 μM) was incubated with a 5 μl lipofectamin 2000 transfection reagent in Opti-MEM®Medium for 20 min at room temperature. After 6 h of transfection, the medium was changed into normal cocktail medium and cultured for 6 days. In the later adipogenic stage, myoblasts were treated with U0126 (a special p-Erk1/2 inhibitor) (Beyotime Biotechnology, Shanghai, China) at the indicated concentrations for 60 min and then collected after culturing in DMEM with 10% FBS and 5 mg/ml insulin for another 2 days.

Vector construction

Interference vectors were constructed with lentiviral plasmid pLentiHI (Invitrogen, Carlsbad, USA) and the inserted shRNAs were designed by online Invitrogen RNAi Designer (https://rnaidesigner.invitrogen.com/ rnaiexpress/). Three *AdipoR1* and *AdipoR2* shRNA vectors were constructed (**Supplementary Table S3**). They were annealed and inserted into pLentiHI plasmids at *Bam*HI and *Xho*I sites, and confirmed by sequencing.

Lentivirus package and infection

The pLentiHI-*AdpoR1* shRNAs, *AdpoR2* shRNAs or scrambled shRNA (9 μg), combined with 6 μg Δ8.9 packaging plasmid and 9 μg VSVG envelope protein plasmid, were co-transfected into HEK293T packaging cells (2×10^5 cells per well) with the calcium phosphate method. Forty-eight hours after transfection, the supernatant containing virus particles was collected and passed through a 0.45 μm filter to remove cellular debris. When myoblasts reached 70%–80% confluence, the viral suspension of pLentiHI-*AdipoR1* shRNAs, pLentiHI-*AdipoR2* shRNAs or scrambled shRNA was added respectively, and incubated for 6–8 h, and then the medium was replaced by adipogenic induction medium that includes the recombinant complement CTRP6 protein (Prospec, East Brunswick, USA).

Western blotting

Cells were lysed in lysis buffer (Beyotime Biotechnology) supplemented with 1 mM PMSF. Each protein sample was separated by 12% SDS-PAGE and electro-transferred to PVDF membrane (Millipore, Billerica, USA) for immunoblot analysis [16]. The primary antibodies against the following proteins were used: PPARγ (Abcam,

Cambridge, UK), aP2 (Santa Cruz Biothech, Santa Cruz, USA), ATGL (Cell Signaling, Beverly, USA), CTRP6 (Abcam), Erk1/2 (Cell Signaling), p-Erk1/2 (Cell Signaling), adiponectin (Cell Signaling), and AdipoR1 (Santa Cruz). β-Actin (Santa Cruz) was used as the loading control. After incubation with the appropriate HRP-conjugated secondary antibody, proteins were detected using a ChemiDoc XRS imaging system (Bio-Rad) and analyzed using software Quantity One (Bio-Rad).

Co-immunoprecipitation

When myoblasts reached 90% confluence, medium was changed to adipogenic induction medium that includes the recombinant complement adiponectin protein (Sino Biological Inc, Beijing, China). Lysates were extracted from the cells with RIPA buffer. Then, equivalent amounts of proteins were incubated with antibodies against CTRP6, AdipoR1, adiponectin or IgG separately overnight at 4°C, followed by immunoprecipitation using protein A/G-conjugated agarose beads (Beyotime Biotechnology). Samples were washed with RIPA buffer supplemented with protease inhibitor, boiled at 100°C for 10 min with equivalent amounts of 5 × loading buffer, and then analyzed by western blotting using antibodies against CTRP6, adiponectin or AdipoR1.

Statistical analysis

All results were obtained from at least three independent experiments, presented as mean ± SEM and analyzed using SPSS 19.0 statistical software (SPSS, Inc.). Statistical significance was determined using Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Ectopic lipogenesis in C2C12 myoblasts

To investigate lipogenesis in myoblasts, three induction methods were used to culture the cells for 8 days. The results showed that the cocktail method was the best in myoblast ectopic lipid accumulation as revealed by staining with Oil Red O and BODIPY (**Fig. 1A,B**). Compared with the other methods, the OD values at day 4 and 6, and the TG contents at day 2, 4 and 6 were significantly increased ($P < 0.05$) in C2C12 cells induced with the cocktail method (**Fig. 1C,D**).

Furthermore, compared with the control groups, the levels of *PPARγ* and *aP2* mRNA at day 2, 4 and 6 were significantly up-regulated (**Fig. 2A,B**), whereas the levels of key lipolytic gene *TGH* at day 2, 4 and 6 ($P < 0.05$), and the levels of *ATGL* at day 2 and 4 ($P < 0.05$) were markedly down-regulated (**Fig. 2C,D**) during lipogenic induction with the cocktail method in C2C12 myoblasts. The trend of ATGL protein levels was the same as their mRNA levels (**Fig. 2E,F,H**). The protein levels of PPARγ and aP2 in cocktail medium-treated cells were also significantly up-regulated, compared with the other methods (**Fig. 2G**). In order to avoid errors, the adipocyte lysate control was also detected and results were shown in **Supplementary Fig. S1**.

Erk signaling pathway is implicated in C2C12 myoblast lipogenesis

To determine whether Erk signaling pathway is implicated in myoblast lipogenesis, the levels of Erk1/2 and p-Erk1/2 were detected during this process. The results indicated that the level of p-Erk1/2

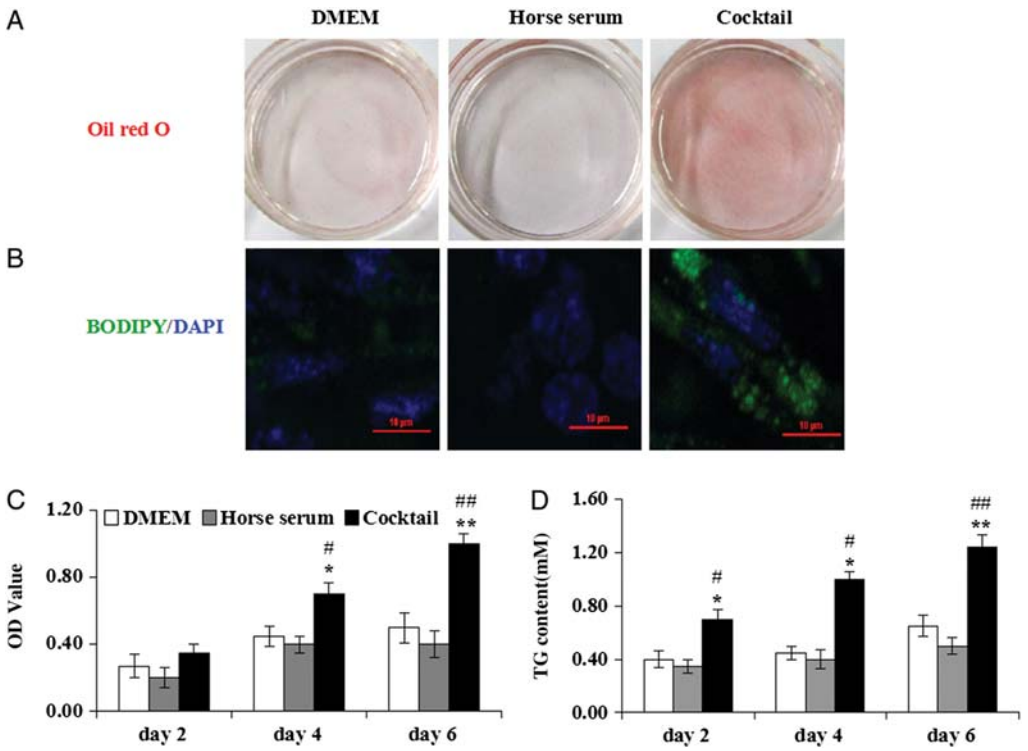

Figure 1. Lipogenesis in C2C12 cells (A) C2C12 cells were stained with Oil Red O. C2C12 cells treated with DMEM (growth medium), horse serum (myogenic induction medium) or the cocktail medium (adipogenic induction medium) and induced for 8 days. (B) BODIPY staining. (C) Oil Red O relative absorbance. OD (optical density) value. (D) Triglyceride content analysis. Data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with DMEM; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with horse serum.

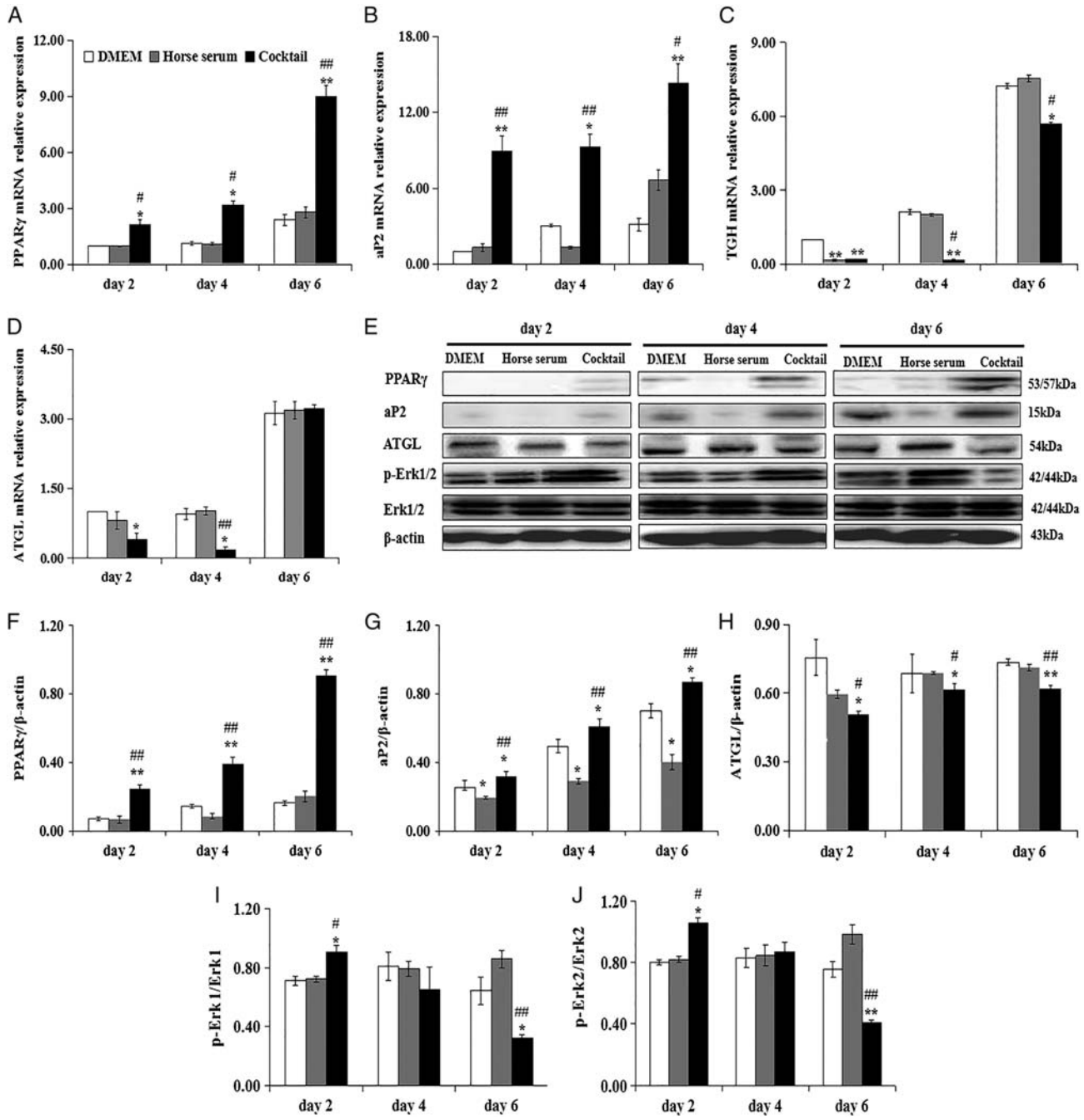

Figure 2. The adipogenic regulation in C2C12 cells The cells were respectively treated with DMEM, horse serum or the cocktail medium for 2, 4 and 6 days. The mRNA expressions of adipogenic marker genes were analyzed by real-time qPCR at day 2, 4 and 6: (A) PPARγ, (B) aP2, (C) HSL, (D) ATGL. (E) The protein levels of PPARγ, aP2, ATGL, Erk and p-Erk. (F–J) The levels of PPARγ, aP2, ATGL, Erk and p-Erk were analyzed using Image J. Data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with DMEM; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with horse serum.

was visibly up-regulated in myoblasts treated with the cocktail method at day 2, but it was opposite at day 6 (**Fig. 2I,J**).

Expression and secretion of CTRP6 during C2C12 myoblast lipogenesis

At day 2, 4 and 6 of lipogenesis in myoblasts, the levels of *CTRP6* mRNA were increased by approximately 2.5 folds, 2.2 folds and 2 folds compared with the controls, respectively (**Fig. 3A**). Meanwhile, the secretion of CTRP6 in the culture medium and the levels of CTRP6 protein in the C2C12 myoblasts were significantly induced and up-regulated at day 2, 4 and 6 (**Fig. 3B–D**, $P < 0.05$). Therefore, CTRP6 may be involved in lipogenesis of myoblasts.

CTRP6 knockdown inhibits lipogenesis in C2C12 myoblasts

To explore the effects of *CTRP6* on the ectopic lipogenesis in myoblasts, the experiment of *CTRP6* knockdown was performed (**Supplementary Fig. S2**). The results showed that the knockdown

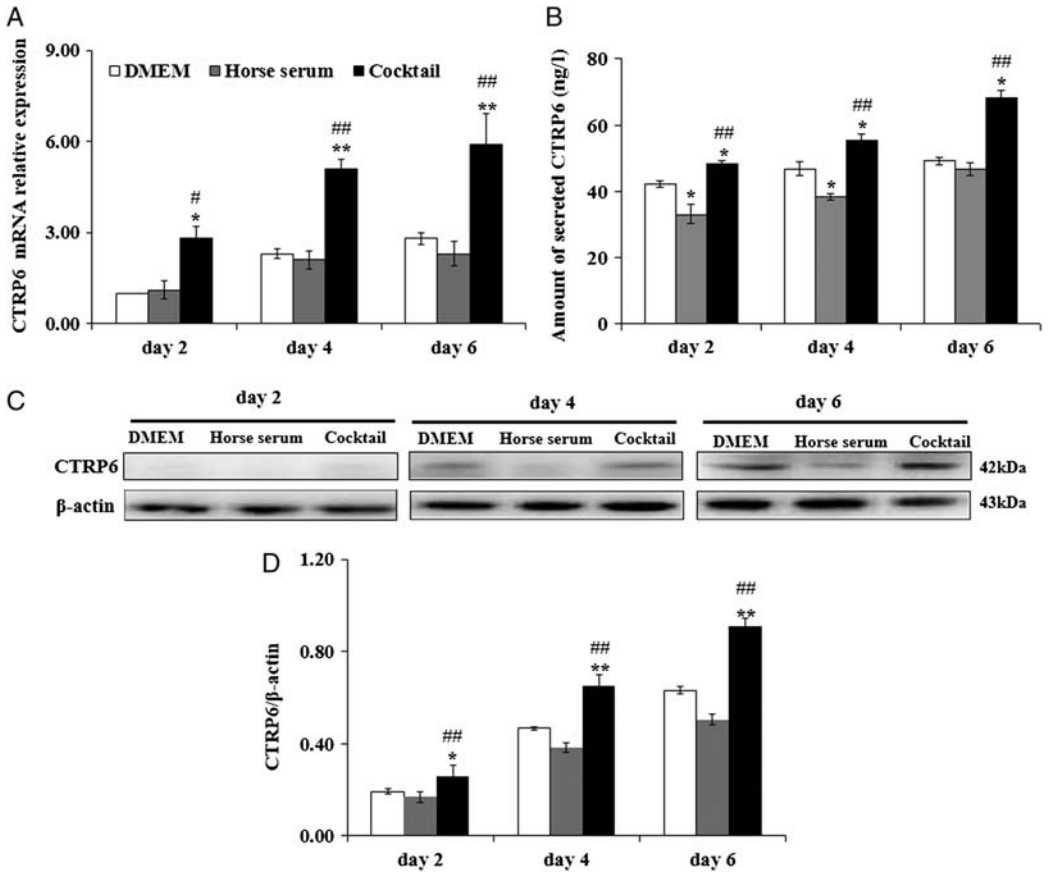

Figure 3. The expression and secretion of *CTRP6* (A) The mRNA levels of *CTRP6* at day 2, 4 and 6. (B) Secretions of CTRP6. (C) The levels of intracellular CTRP6 protein. (D) The protein levels of CTRP6 were analyzed using Image J. Data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with DMEM; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with horse serum.

efficiency of CTRP6 siRNA3 was the best. At day 2, 4 and 6 after transfection and lipogenic induction, the levels of *CTRP6* mRNA were significantly down-regulated in the treatment group (**Fig. 4A**, $P < 0.05$). Furthermore, not only the secretion of CTRP6 in the culture medium was significantly reduced (**Fig. 4B**, $P < 0.05$), but also the levels of the CTRP6 protein in the myoblasts were visibly down-regulated in the treatment group at day 2, 4 and 6 (**Fig. 4G**). Oil Red O staining showed that knockdown of *CTRP6* inhibited lipogenesis in myoblasts at day 6 (**Fig. 4C** and **Supplementary Fig. S3**). The analysis of Oil Red O extraction indicated that the suppression of *CTRP6* expression resulted in approximately 40% and 50% decrease in intracellular lipid content in comparison with the control at day 4 and 6, respectively (**Fig. 4C**, $P < 0.05$). In addition, at day 2, 4 and 6 of lipogenesis induction, the TG contents were also decreased by approximately 50%, 54% and 53%, respectively, as revealed by TG content analysis (**Fig. 4D**). Furthermore, the levels of *PPARγ* and *aP2* mRNA were reduced by approximately 40%, 37%, and 29%, and 60%, 41%, and 32% respectively, in the treatment group at day 2, 4 and 6 (**Fig. 4E,F**). Knockdown of *CTRP6* could also visibly down-regulate the levels of *PPARγ* and *aP2* proteins (**Fig. 4G**).

CTRP6 regulates the ectopic lipogenesis by Erk signaling pathway in C2C12 myoblasts

To determine whether CTRP6 regulates the ectopic lipogenesis through Erk signaling pathway, the levels of Erk1/2 and p-Erk1/2 were examined. The results showed that CTRP6 knockdown inhibited Erk1/2 activation in the early adipogenic stage in myoblasts, but improved the level of p-Erk1/2 in the terminal stage (**Fig. 4G–I**).

To further confirm this finding, myoblasts were treated with U0126 (a special p-Erk1/2 inhibitor) in the later adipogenic induction stage. The level of p-Erk1/2 induced by *CTRP6* siRNA was gradually suppressed with the increase of U0126 concentrations (**Fig. 5A**), whereas there was no reduction in the amount of total Erk1/2 protein. The effect of U0126 on adipogenesis in myoblasts was also examined and shown in **Supplementary Fig. S4**. U0126 could significantly promote lipogenesis in myoblasts. At day 6 of the adipogenic induction, Oil Red O staining showed that U0126 recovered lipogenesis against the inhibition caused by *CTRP6* knockdown (**Fig. 5B**). Meanwhile, there was no effect on the expression of *CTRP6* (**Fig. 5C**). Interestingly, the mRNA and protein levels of *PPARγ* and *aP2* were not only rescued but also significantly up-regulated in myoblasts treated with *CTRP6* siRNA plus U0126 (**Fig. 5D–F**). Based on above results, we suggest that *CTRP6* may regulate the ectopic lipogenesis by Erk signaling pathway in C2C12 myoblasts.

AdipoR1 rather than AdipoR2 may be a receptor of CTRP6

The sequence and structure of CTRPs are similar to those of adiponectin, however, whether CTRPs interact with any of the APN

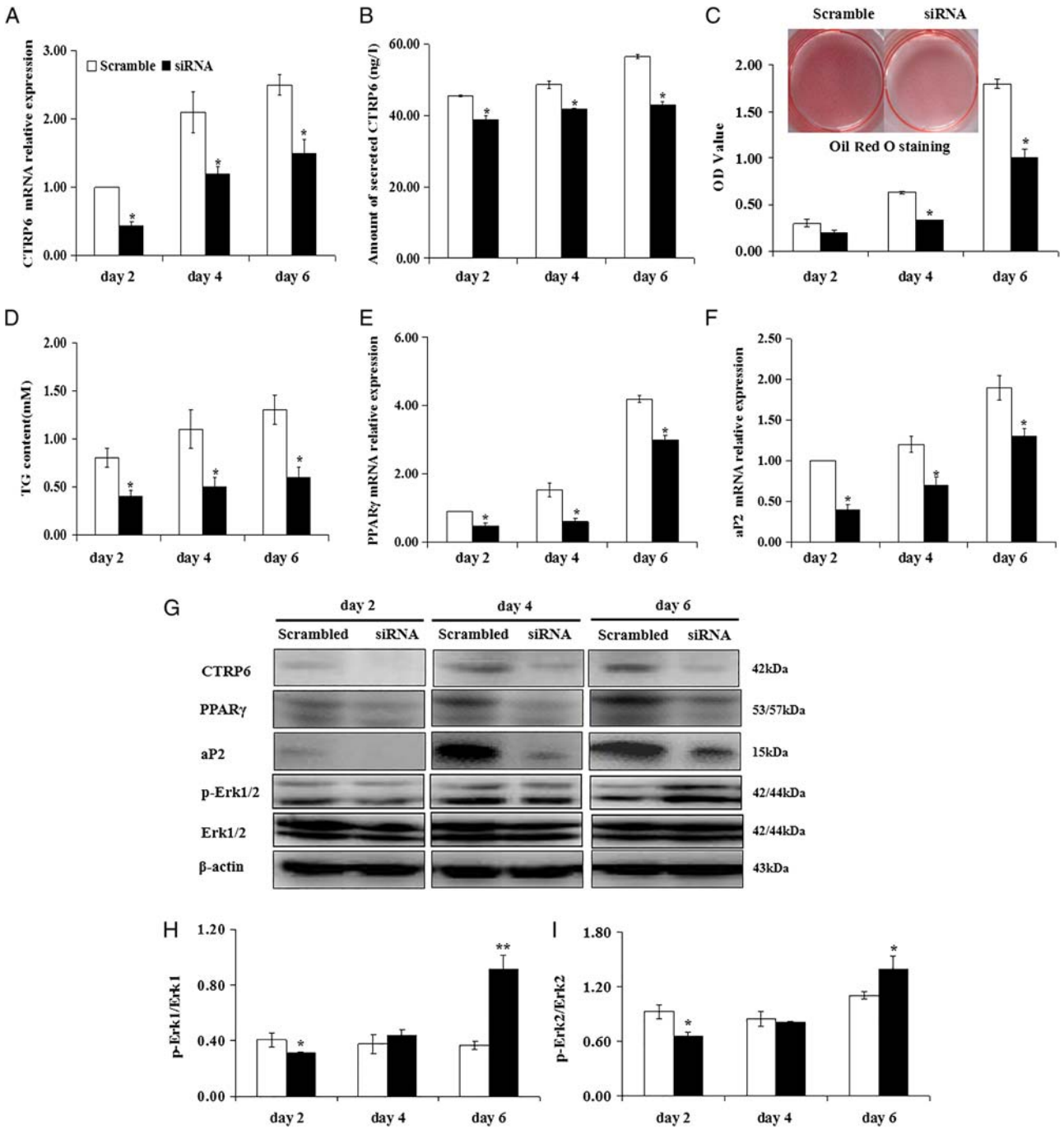

Figure 4. *CTRP6* knockdown inhibits lipogenesis via Erk signaling in C2C12 cells For transfection followed by adipogenic differentiation, cell density must reach 80%–85% to ensure that the cells can grow to confluency in two days after transfection. (A) The efficiency of CTRP6 knockdown by siRNA at day 2, 4 and 6. (B) The secretion of CTRP6 in the culture medium. (C) Oil Red O staining and Oil Red O relative absorbance. (D) TG content. (E,F) Real-time qPCR analysis of PPARγ (E) and aP2 (F). (G) The protein levels of CTRP6, PPARγ, aP2, ATGL, Erk and p-Erk. (H,I) The levels of p-Erk1 (H) and p-Erk2 (I) were normalized vs. total Erk1 and Erk2, respectively. Data were expressed as mean ± SEM, $*P < 0.05$, $**P < 0.01$, compared with the scramble.

receptors/binding proteins and what their role is in mediating subsequent biological functions remain largely unknown. Currently, CTRP5 and CTRP9 are the CTRP members whose receptor dependencies have been investigated. Experimental results suggested that CTRP5 and CTRP9 exerted their metabolic effect in an AdipoR1-independent manner [20,21]. To evaluate the hypothesis that AdipoR1 and/or AdipoR2 are the receptors of CTRP6, the two genes were knocked down in myoblasts, respectively (**Supplementary Fig. S5**). AdipoR1 shRNA1 and AdipoR2 shRNA3 were selected for the subsequent experiments. The activities of CTRP6 and adiponectin protein were shown in the **Supplementary Figs. S6** and **S7**. The most effective concentration of CTRP6 and adiponectin protein was 0.5 μg/ml. The results showed that only the addition of CTRP6 could increase the expression of PPARγ and aP2 and change the levels of p-Erk1/2 in myoblasts (**Supplementary Fig. S8**). The *AdipoR1* knockdown in combination with CTRP6

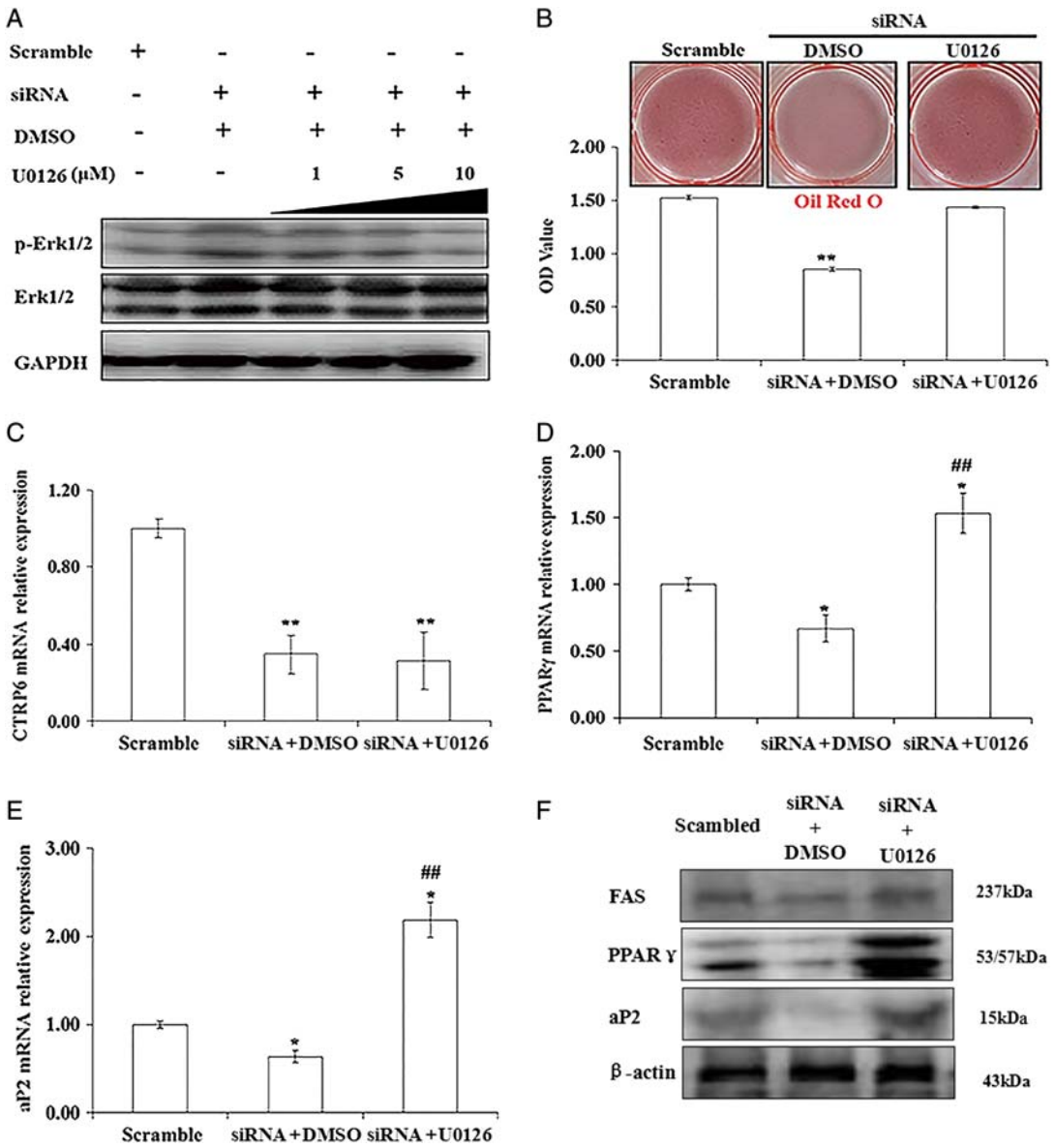

Figure 5. The inhibition of lipogenesis by CTRP6 knockdown was attenuated by U0126 through the Erk signaling pathway C2C12 cells were treated with U1026 for 60 min and then collected after culturing in DMEM with 10% FBS and 5 mg/ml insulin for another 2 days. (A) The levels of Erk1/2 and p-Erk1/2. (B) Oil Red O staining and Oil Red O relative absorbance. (C–E) The mRNA levels of CTRP6 (C), PPARγ (D), and aP2 (E), respectively. (F) The levels of PPARγ, aP2 and FAS proteins. Data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with scramble; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with treatment with CTRP6 siRNA and DMSO.

protein addition did not change the level of p-Erk1/2 or the levels of PPARγ and aP2 in C2C12 myoblasts induced with the cocktail method at day 2 and 4 (**Fig. 6A,C**). However, CTRP6 protein could down-regulate the level of p-Erk1/2 and up-regulate the levels of PPARγ and aP2 even when AdipoR1 was knocked down at day 6. Interestingly, *AdipoR2* knockdown did not affect the function of CTRP6 (**Fig. 6B,D**). Therefore, we think that AdipoR1 rather than AdipoR2 may be a receptor of CTRP6 in the early adipogenic stage.

We then explored whether there was a direct interaction between AdipoR1 and CTRP6. Indeed, AdipoR1 was co-immunoprecipitated in complex with CTRP6 at day 2 (**Fig. 6E**), suggesting a direct interaction involving CTRP6 binding to AdipoR1 during the process of mitotic clonal expansion. But in the late stage of lipogenesis, CTRP6 could not interact with AdipoR1 (**Fig. 6E**). This was likely because that CTRP6 could compete with adiponectin in binding to AdipoR1. The adiponectin expression in the later stage of lipogenesis was increased by approximately 12 folds when compared with that in the early stage, but the expression of AdipoR1 mRNA was only increased by 2 folds (**Supplementary Fig. S9**). When myoblasts were treated with adiponectin protein in the early adipogenic stage, AdipoR1 was found to bind to adiponectin rather than CTRP6 (**Fig. 6F**). Taken together, a model for the regulatory lipogenic mechanism of *CTRP6* in myoblasts was proposed and shown in **Fig. 7**.

Discussion

It is a quite interesting issue how to activate the ectopic lipogenesis in myoblasts. Several reports have indicated that hypoxia

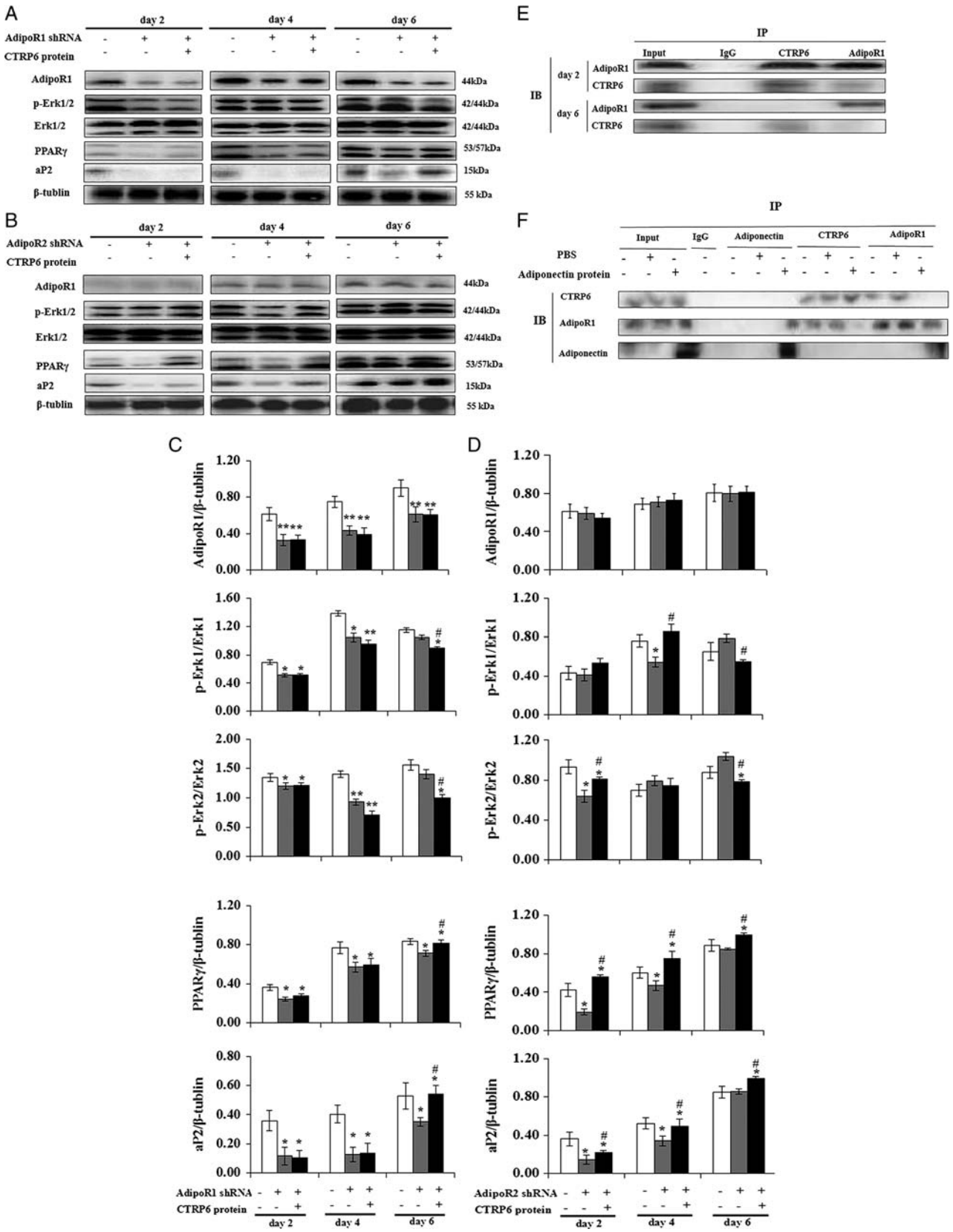

Figure 6. AdipoR1 rather than AdipoR2 may be a receptor of CTRP6 The cells were treated with CTRP6 protein for 4 days after infection with pLentiHI-AdpoR1 or -AdpoR2 shRNA. (A) The effect of AdipoR1 knockdown plus CTRP6 protein Erk/PPARγ signaling. (B) The effect of AdipoR2 knockdown plus CTRP6 protein Erk/PPARγ signaling. (C,D) The levels of proteins were analyzed using Image J. Data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with treatment with AdipoR1 shRNA or AdipoR2 shRNA. (E) Representative co-immunoprecipitation results showing that CTRP6 binds to AdipoR1 in mitotic clonal expansion not during terminal adipogenic differentiation. The cells were inducted by the cocktail method for 2 days or 6 days and collected. Samples were immunoprecipitated with CTRP6- or AdipoR1-specific antibody followed by immunoblotting with antibodies against AdipoR1 or CTRP6. (F) CTRP6 could compete the binding of adiponectin to AdipoR1. The experiments were repeated three to four times/condition.

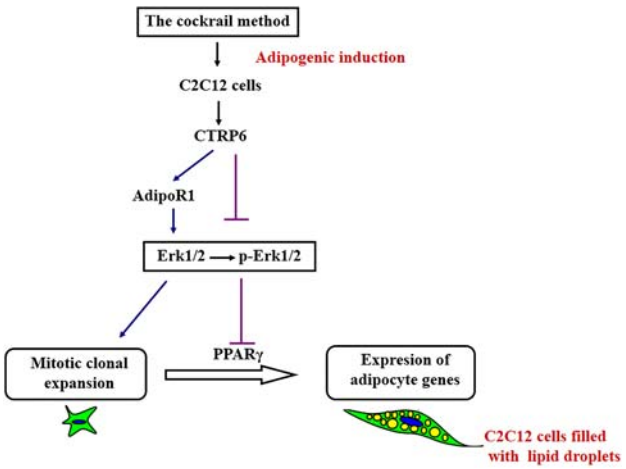

Figure 7. Model for the regulatory mechanism of CTRP6 on the ectopic lipogenesis in C2C12 cells

and cyclic mechanical stretch stimulate or inhibit lipogenesis of myoblastic cells through Wnt signaling [22–24]. Moreover, the medium with dexamethasone and insulin (MDI) treatment also leads to a significant decrease in *Wnt10b* mRNA expression and enhancement of myogenic and adipogenic differentiations in C2C12 cells [23], but it is not clear whether other signaling pathways were also involved. We found that the level of p-Erk1/2 was significantly increased at day 2 of lipogenesis induction in myoblasts, whereas its level was decreased at day 6. The Erk signaling pathway plays a pivotal role in many vital cellular processes including proliferation and differentiation [25]. These results suggest that Erk signaling pathway may be implicated in the ectopic lipogenesis in myoblasts.

Our previous studies revealed that *CTRP6* could regulate adipogenesis via lipogenic marker genes in 3T3-L1 adipocytes [14], implying that *CTRP6* was potentially involved in the ectopic lipogenesis in myoblasts. Expectedly, this hypothesis was confirmed by the results of expression and secretion of CTRP6 during the ectopic lipogenesis in myoblasts. Although CTRP6 was found to stimulate fatty acid oxidation via the activation of AMPK in C2C12 myoblasts [15], our research focused on adipogenic differentiation. The process of adipogenic differentiation includes growth arrest, clonal expansion, early changes in gene expression and terminal differentiation [26]. It is interesting to explore when and how CTRP6 regulates adipogenic differentiation.

It is well known that *aP2* and *PPARγ* play major roles in adipocyte adipogenesis and lipogenesis. *aP2* has been previously reported to directly interact with *PPARγ*, and the hundreds of genes were activated by PPARγ, which are responsible for terminal adipogenic differentiation [27–29]. In addition, recent studies provided the evidence that the promoter region of *CTRP3* carries a functional *PPRE* (*PPARγ* response element) that promotes adipogenesis through directly up-regulating *PPARγ* level in adipocytes [30–32]. In the present study, CTRP6 knockdown was found to inhibit the ectopic lipogenesis and down-regulate the levels of *PPARγ* and *aP2* in myoblasts. Therefore, our experiment results demonstrate that *CTRP6* regulates the ectopic lipogenesis by lipogenic marker genes. Additionally, our results also indicate that *CTRP6* knockdown inhibits Erk signaling at the early stage of adipogenic induction, but improves p-Erk1/2 in the terminal. Those findings suggest that *CTRP6* mediates the ectopic lipogenesis through Erk signaling pathway in myoblasts.

To further confirm the above point, experiments were carried out in myoblasts treated with *CTRP6* siRNA plus U0126. We found that the suppression of lipogenesis by *CTRP6* siRNA occurred in the whole adipogenic process, but in the later adipogenic stage the effect of *CTRP6* siRNA was attenuated by U0126 in myoblasts. Several studies reported that ERK signaling pathway could promote or inhibit lipogenesis depending on its time of activation during the process [25]. Erk1/2 needs to be activated during early lipogenesis for normal clonal expansion, while p-Erk1/2 stimulates p-PPARγ that leads to the inhibition of lipogenesis in the later adipogenic stage. Therefore, our results indicate that the inhibition of lipogenesis by *CTRP6* knockdown with down-regulation of *PPARγ* and *aP2* is through the induction of p-Erk1/2 in the later adipogenic stage.

Not only adipocyte secretes CTRP6 [12], but in our study the myoblasts induced with the cocktail method also secrete CTRP6. In addition, mtDNA depletion induces an increase in CTRP6 expression by increasing mRNA stability in skeletal muscle [13]. However, the receptor of CTRP6 is still unknown. Adiponectin is produced exclusively in animal adipose tissue and considered as a 'true' adipokine [33,34]. The biological properties of adiponectin are mediated via two receptors: AdipoR1 and AdipoR2 [35,36]. Both AdipoR1 and AdipoR2 are surface membrane proteins with seven transmembrane domains, sharing similar molecular structure with each other, with expression detectable in most tissues and cell types [37]. While AdipoR1 is prevalent in the skeletal muscle and heart, AdipoR2 is the chief hepatic subtype [38]. AdipoR1 binds to gADN with high affinity and fADN with low affinity, whereas AdipoR2 binds to both gADN and fADN with medium affinity. Adiponectin, via AdipoR1/R2, rapidly and robustly activates the Erk1/2 pathway in primary cardiac fibroblasts [39], vascular endothelial cells, vascular smooth muscle [40], and hepatocytes [41]. Because the globular domain of CTRP6 shares a high degree of amino acid identity (33%) with adiponectin [12], we hypothesize that AdipoR1 and/or AdipoR2 are also the receptors of CTRP6. We showed that AdipoR1 knockdown in combination with CTRP6 protein did not change the levels of p-Erk1/2, or the levels of PPARγ and aP2 within the first 2 days of myoblast differentiation. These results indicate that CTRP6 may activate Erk1/2 phosphorylation and regulate the mitotic clonal expansion of differentiating myoblasts via *AdipoR1*. The mitotic clonal expansion process is essential for the induction of adipogenesis and lipogenesis [42]. Failure to undergo mitotic clonal expansion prevents the implementation of adipogenic program [43–44]. Our results also revealed that CTRP6 could bind to AdipoR1 in the early adipogenic stage but not in the later stage. This was because that CTRP6 and adiponectin competitively bound to AdipoR1. The adiponectin expression in the later stage of lipogenesis was greater than in the early stage (**Supplementary Fig. S9**). Further studies are needed to elucidate how CTRP6 regulates the activation of Erk signaling pathway in the later adipogenic stage.

In summary, the present study not only provides the evidence that Erk signaling pathway is involved in the ectopic lipogenesis, but also shows that *CTRP6* regulates the process via AdipoR1/Erk/PPARγ pathway. These findings will shed light on the novel biological function of CTRP6 in myoblast lipogenesis and provide a hopeful direction for human myopathies therapy by lipogenic regulation in skeletal muscle myoblasts.

Supplementary Data

Supplementary data is available at *ABBS* online.

Funding

This work was supported by the grants from the National Key Basic Research Program of China (Nos. 2015CB943102 and 2012CB124705) and the National Natural Science Foundation of China (Nos. U1201213 and 31572366).

References

1. Greco AV, Mingrone G, Giancaterini A, Manco M, Morroni M, Cinti S, Granzotto M, *et al.* Insulin resistance in morbid obesity: reversal with intramyocellular fat depletion. *Diabetes* 2002, 51: 144–151.
2. Visser M, Goodpaster BH, Kritchevsky SB, Newman AB, Nevitt M, Rubin SM, Simonsick EM, *et al.* Muscle mass, muscle strength, and muscle fat infiltration as predictors of incident mobility limitations in well-functioning older persons. *J Gerontol A Biol Sci Med Sci* 2005, 60: 324–333.
3. Goodpaster BH, Wolf D. Skeletal muscle lipid accumulation in obesity, insulin resistance, and type 2 diabetes. *Diabetes* 2004, 5: 219–226.
4. Lee EJ, Kamli MR, Bhat AR, Pokharel S, Lee DM, Kim SH, Kim TI, *et al.* Effect of porcine placenta steroid extract on myogenic satellite cell proliferation, transdifferentiation, and lipid accumulation. *In Vitro Cell Dev Biol Anim* 2012, 48: 326–333.
5. Yeow K, Phillips B, Dani C, Cabane C, Amri EZ, Dérijard B. Inhibition of myogenesis enables adipogenic trans-differentiation in the C2C12 myogenic cell line. *FEBS Lett* 2001, 506: 157–162.
6. Asakura A, Komaki M, Rudnicki M. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. *Differentiation* 2001, 68: 245–253.
7. Fux C, Mitta B, Kramer BP, Fussenegger M. Dual-regulated expression of C/EBP-alpha and BMP-2 enables differential differentiation of C2C12 cells into adipocytes and osteoblasts. *Nucleic Acids Res* 2004, 32: e1.
8. Lee SJ, Lee EJ, Kim SH, Choi I, Lee DM, Lee HJ, Yoon D, *et al.* IL-17A promotes transdifferentiation of mouse myoblast cells (C2C12) into adipocytes by increasing the expression of peroxisome proliferator-activated receptor γ through CAAT/enhancer binding protein β signaling. *Biotechnol Lett* 2011, 33: 229–235.
9. Hu E, Tontonoz P, Spiegelman BM. Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proc Natl Acad Sci* 1995, 92: 9856–9860.
10. Andreas S, Christa B. CTRP family: linking immunity to metabolism. *Trends Endocrinol Metab* 2012, 23: 194–201.
11. Seldin MM, Tan SY, Wong GW. Metabolic function of the CTRP family of hormones. *Rev Endocr Metab Disord* 2014, 15: 111–123.
12. Wong GW, Krawczyk SA, Kitidis-Mitrokostas C, Revett T, Gimeno R, Lodish HF. Molecular, biochemical and functional characterizations of C1q/TNF family members: adipose-tissue-selective expression patterns, regulation by PPAR-gamma agonist, cysteine-mediated oligomerizations, combinatorial associations and metabolic functions. *Biochem J* 2008, 416: 161–177.
13. Kim MJ, Lee W, Park EJ, Park SY. Depletion of mitochondrial DNA stabilizes C1qTNF6-related protein 6. mRNA in muscle cells. *J Korean Med Sci* 2012, 27: 465–470.
14. Wu WJ, Mo DL, Zhao CZ, Zhao C, Chen YS, Pang WJ, Yang GS. Knockdown of CTRP6 inhibits adipogenesis via lipogenic marker genes and Erk1/2 signalling pathway. *Cell Biol Int* 2015, 39: 554–562.
15. Lee W, Kim MJ, Park EJ, Choi YJ, Park SY. C1qTNF-related protein-6 mediates fatty acid oxidation via the activation of the AMP-activated protein kinase. *FEBS Lett* 2010, 584: 968–972.
16. Xiong Y, Pang WJ, Wei N, Wang Y, Ren WK, Yang GS. Knockdown of both FoxO1 and C/EBPb promotes adipogenesis in porcine preadipocytes through feedback regulation. *Cell Biol Int* 2013, 37: 905–916.
17. Schweiger M, Eichmann TO, Taschler U, Zimmermann R, Zechner R, Lass A. Measurement of lipolysis. *Methods Enzymol* 2014, 538: 171–193.
18. Pang W, Wang Y, Wei N, Xu R, Xiong Y, Wang P, Shen Q, *et al.* Sirt1 inhibits akt2-mediated porcine adipogenesis potentially by directprotein-protein interaction. *PLoS One* 2013, 8: e71576.
19. Xiao Y, Cui J, Li YX, Shi YH, Wang B, Le GW, Wang ZP. Dyslipidemic high-fat diet affects adversely bone metabolism in mice associated with impaired antioxidant capacity. *Nutrition* 2011, 27: 214–220.
20. Park SY, Choi JH, Ryu HS, Pak YK, Park KS, Lee HK, Lee W. C1q tumor necrosis factor alpha-related protein isoform 5 is increased in mitochondrial DNA-depleted myocytes and activates AMP-activated protein kinase. *J Biol Chem* 2009, 284: 27780–27789.
21. Zheng Q, Yuan Y, Yi W, Lau WB, Wang Y, Wang X, Sun Y, *et al.* C1q/TNF-related proteins, a family of novel adipokines, induce vascular relaxation through the adiponectin receptor-1/AMPK/eNOS/nitric oxide signaling pathway. *Arterioscler Thromb Vasc Biol* 2011, 31: 2616–2623.
22. Akimoto T, Ushida T, Miyaki S, Akaogi H, Tsuchiya K, Yan Z, Williams RS, *et al.* Mechanical stretch inhibits myoblast-to-adipocyte differentiation through Wnt signaling. *Biochem Biophys Res Commun* 2005, 329: 381–385.
23. Itoigawa Y, Kishimoto KN, Okuno H, Sano H, Kaneko K, Itoi E. Hypoxia induces adipogenic differentitation of myoblastic cell lines. *Biochem Biophys Res Commun* 2010, 399: 721–726.
24. Li HX, Chen KL, Wang HY, Tang CB, Xu XL, Zhou GH. Chemerin inhibition of myogenesis and induction of adipogenesis in C2C12 myoblasts. *Mol Cell Endocrinol* 2015, 414: 216–223.
25. Bost F, Aouadi M, Caron L, Binétruy B. The role of MAPKs in adipocyte differentiation and obesity. *Biochimie* 2005, 87: 51–56.
26. Ma X, Lee P, Chisholm DJ, James DE. Control of adipocyte differentiation in different fat depots; implications for pathophysiology or therapy. *Front Endocrinol* 2015, 6: 1.
27. Farmer SR. Regulation of PPARγ activity during adipogenesis. *Int J Obes* 2005, 29: S13–S16.
28. Farmer SR. Transcriptional control of adipocyte formation. *Cell Metab* 2006, 4: 263–273.
29. Rajan S, Gupta A, Beg M, Shankar K, Srivastava A, Varshney S, Kumar D, *et al.* Adipocyte transdifferentiation and its molecular targets. *Differentiation* 2014, 87: 183–192.
30. Peterson JM, Wei Z, Wong GW. C1q/TNF-related protein-3 (CTRP3), a novel adipokine that regulates hepatic glucose output. *J Biol Chem* 2010, 285: 39691–39701.
31. Hofmann C, Chen N, Obermeier F, Paul G, Büchler C, Kopp A, Falk W, *et al.* C1q/TNF-related protein-3 (CTRP-3) is secreted by visceral adipose tissue and exerts inflammatory and antifibrotic effects in primary human colonic fibroblasts. *Inflamm Bowel Dis* 2011, 17: 2462–2471.
32. Kopp A, Bala M, Weigert J, Büchler C, Neumeier M, Aslanidis C, Schölmerich J, *et al.* Effects of the new adiponectin paralogous protein CTRP-3 and of LPS on cytokine release from monocytes of patients with type 2 diabetes mellitus. *Cytokine* 2010, 49: 51–57.
33. Hu E, Liang P, Spiegelman BM. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 1996, 271: 10697–10703.
34. Scherer PE, Williams S, Fogliano M, Baldini G., Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 1995, 270: 26746–26749.
35. Heiker JT, Kosel D, Beck-Sickinger AG. Molecular mechanisms of signal transduction via adiponectin and adiponectin receptors. *J Biol Chem* 2010, 39: 1005–1018.
36. Buechler C, Wanninger J, Neumeier M. Adiponectin receptor binding proteins–recent advances in elucidating adiponectin signalling pathways. *FEBS Lett* 2010, 584: 4280–4286.
37. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, *et al.* Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 2003, 423: 7629.
38. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev* 2005, 26: 439–451.
39. Hattori Y, Hattori S, Akimoto K, Nishikimi T, Suzuki K, Matsuoka H, Kasai K. Globular adiponectin activates nuclear factor-kappaB and activating protein-1 and enhances angiotensin II-induced proliferation in cardiac fibroblasts. *Diabetes* 2007, 56: 804–808.

40. Lee MH, Klein RL, El-Shewy HM, Luttrell DK, Luttrell LM. The adiponectin receptors AdipoR1 and AdipoR2 activate ERK1/2 through a Src/Ras-dependent pathway and stimulate cell growth. *Biochemistry* 2008, 47: 11682–11692.
41. Wanninger J, Neumeier M, Weigert J, Bauer S, Weiss TS, Schäffler A, Krempl C, *et al*. Adiponectin-stimulated CXCL8 release in primary human hepatocytes is regulated by ERK1/ERK2, p38 MAPK, NF-kappaB, and STAT3 signaling pathways. *Am J Physiol Gastrointest Liver Physiol* 2009, 297: G611–618.
42. Tang QQ, Otto TC, Lane MD. CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. *Proc Natl Acad Sci USA* 2003, 100: 850–855.
43. Kawaji A, Nishizuka M, Osada S, Imagawa M. TC10-like/TC10betaLong regulates adipogenesis by controlling mitotic clonal expansion. *Biol Pharm Bull* 2010, 33: 404–409.
44. Tang QQ, Otto TC, Lane MD. Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc Natl Acad Sci USA* 2003, 100: 44–49.