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F-box only protein 9 is required for adipocyte differentiation

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ABSTRACT

The objective of this study is to investigate whether F-box only protein 9 (FBXO9), an ubiquitination E3 ligase, has a functional role in adipocyte differentiation. Expression of FBXO9 was compared between obese mice and control lean mice using real-time PCR. Also, expression pattern of FBXO9 was monitored during 3T3-L1 adipocyte differentiation. FBXO9 was highly expressed in obese mice, and increased in the early stages of adipogenesis. To verify a functional role of FBXO9 in adipogenesis, FBXO9 was knocked down using transfection of siRNAs against FBXO9 into 3T3-L1 cells during the induction of adipogenesis. Knockdown of FBXO9 in early stage of adipogenesis almost completely inhibited adipogenesis, and CCAAT/enhancer binding protein β (C/EBPβ) levels were significantly reduced. However, the cells stably expressing C/EBPβ were fairly differentiated into adipocytes in the FBXO9 knockdown condition. These results suggest that FBXO9 is required for adipocyte differentiation, and C/EBPβ plays a role in the effect of FBXO9 on adipogenesis.

1. Introduction

Adipocyte differentiation occurs via a very complex process of gene expression [1–3]. Briefly, C/EBPβ and C/EBPδ are rapidly induced upon treatment with adipogenic inducers, and in particular, the expression and posttranslational modifications of C/EBPβ are key adipogenesis regulation steps [4–6]. C/EBPβ and C/EBPδ induce expression of PPARγ, a key transcription factor that stimulates adipogenic gene expression. PPARγ activates expression of C/EBPα, and C/EBPα also positively regulates PPARγ expression [7]. These two proteins together regulate expression of genes involved in fat accumulation, such as fatty acid binding protein 4 and perilipin, as well as expression of adipokines, such as adiponectin and leptin [8]. In addition to these transcription factors, there are several other factors involved in adipogenesis: signal transducer and activator of transcription 5 (STAT5), Kruppel-like factor (KLF) family members and forkhead box protein C2 (FOXC2) [9–12]. They typically affect adipogenesis by regulating the expression or activity of the central transcription factors mentioned above.

Many reports have demonstrated that Wnt signaling inhibits adipogenesis and that suppression of Wnt signaling is necessary for adipogenesis [13,14]. Wnt10b, an endogenous Wnt, has high expression in preadipocytes and rapidly decreases upon the induction of adipogenesis [13]. Transmembrane frizzled receptors and low-density-lipoprotein-related protein (LRP) corepressors, as well as β-catenin, are also down-regulated during adipogenesis [15,16].

Protein degradation via ubiquitination and the proteasome system plays an important role in various cellular events. SKP1-CUL1-F-Box protein (SCF) complex is an important E3 ligase. F-Box proteins have the F-box motif that associates with SKP1, and an additional domain that interacts with target proteins [17,18]. Therefore, among the proteins consisting of the SCF complex, the F-box protein mediates the target specificity, and there are many F-box proteins identified.

Several transcription factors essential for adipogenesis, including PPARγ and C/EBPs, are targets of ubiquitination: however, only a few reports have shown the involvement of ubiquitination in adipogenesis [19–21]. In the case of C/EBPβ, its E3 ligase, FBXW7, is known to be negatively regulated during adipogenesis [22]. In the process of analyzing proteins that interact with PPARγ, F-box only protein 9 (FBXO9) was identified, which led us to investigate a role of FBXO9 in adipogenesis. Little is known about physiological roles of FBXO9, and recently the function of FBXO9 in promoting cell survival in response to growth factor withdrawal via Tel2/Tti1 degradation in multiple myeloma has been evaluated [23].

2. Materials and methods

2.1. Plasmids, siRNAs, and adenovirus

The Flag-tagged FBXO9 expression vector (pFLAG-FBXO9) was prepared by subcloning the cDNA encoding mouse FBXO9 into

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the pFLAG-CMV vector. The C/EBPβ expression vector (pHA-C/EBPβ) was prepared as previously described [6]. The siRNA against mouse FBXO9 (siFBXO9) was purchased from Invitrogen (Carlsbad, CA). The negative control siRNA (siNS) was purchased from Bioneer (Seoul, South Korea). To generate the adenovirus encoding mouse Flag-FBXO9 (Ad-Flag-FBXO9), the Flag-FBXO9 cDNA was inserted into the pAdTrack-CMV vector, followed by recombination between pAdTrack-CMV-Flag-FBXO9 and pAdEasy adenoviral backbone vectors. Ad-Flag-FBXO9 was generated by transfecting the recombinant adenoviral DNA into 293 cells.

2.2. Cell culture

COS-7 cells were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 incubator. 3T3-L1 cells were maintained in DMEM containing 10% calf serum. Adipogenesis was stimulated by the addition of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM dexamethasone and 5 μg/ml insulin in DMEM supplemented with 10% FBS for 2 days. Cells were maintained in DMEM with 10% FBS and 1 μg/ml insulin for the following 2 days, followed by maintenance in DMEM with 10% FBS for another 4 days. Lipid droplets were stained using 1.5% Oil red O in 60% isopropanol 8 days after the induction of differentiation. Preparation of 3T3-L1 cells stably expressing C/EBPβ (pC/EBP) and control cells (pBabe) was previously described [6].

2.3. Animals

C57BL/6 mice were purchased from Orient Bio (Orient Bio Inc, Gyungi-do, Korea). Eight-week-old mice were fed a 60% high fat ($n = 4$) or normal chow ($n = 4$) diet for 12 weeks, and then sacrificed. C57BLKSJ/*db*/*db* (*db*/*db*) and lean control (*db*/*m*) mice were purchased from SLC (SLC, Japan). The mice were sacrificed at 13 weeks of age and white adipose tissues were isolated. All animal studies were conducted in compliance with the Guidelines for Experimental Animal Research from the Laboratory for Experimental Animal Research, Biomedical Research Institute, Seoul National University Hospital.

2.4. Transient transfection of plasmids or siRNA

COS-7 cells in 12 well plates were transfected with pHA-C/EBPβ (50 ng) and pFlag-FBXO9 (50, 100, or 200 ng) using Lipofectamine plus (Invitrogen) for 24 h. To transfect the same amount of plasmids in each well, pcDNA was added. For FBXO9 knockdown, 3T3-L1 cells were transfected with siFBXO9 (50 nM) using Lipofectamine RNAiMAX (Invitrogen).

2.5. Real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNAs were prepared by reverse transcription with 1 μg of total RNA, and subjected to real-time polymerase chain reaction (PCR) with specific primers for each gene. Primer sets were as follows: mouse FBXO9 sense primer, 5′-GGAGCGGCTACATCGAAGAG-3′; antisense primer, 5′-TGAGTCTGACTGGTCTCAAGC-3′; mouse C/EBPβ sense primer, 5′-ACCTGGAGACGCAGCACAAG-3′, antisense primer, 5′-CTGCTTGAACAAGTTCCGCAG-3′; mouse 18S rRNA sense primer, 5′-CGCGGTTCTATTTTGTTGGT-3′, antisense primer, 5′-AGTCGGCATCGTTTATGGTC-3′. Real-time PCR was performed using SYBR Premix Ex Taq reagents (TaKaRa) and a 7500 real-time PCR system (Applied Biosystems, CA). As an endogenous control, 18S rRNA was used. Experiments were performed in duplicate for each sample.

2.6. Western blot analyses

Cell lysates were prepared in the lysis buffer containing 20 mM Tris–HCl (pH 7.4), 1% NP-40, 10 mM $Na_4P_2O_7$, 5 mM EDTA, 100 mM NaF, 2 mM Na_3VO_4, 7 μg/ml leupeptin, 7 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Whole cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted with specific antibodies. Antibodies against PPARγ, C/EBPβ, C/EBPα, LRP6, green fluorescent protein (GFP), and hemagglutinin (HA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Flag, γ-tubulin, and β-actin were purchased from Sigma–Aldrich (St. Louis, MO). The antibody against β-catenin was purchased from BD Biosciences (San Jose, CA). All blots were developed using the enhanced chemiluminescence kit (Thermo, Rockford, IL).

2.7. Statistics

SPSS version 12.0 (SPSS Inc., Chicago, IL) was used for statistical analyses. Statistical significance was assessed using the Mann–Whitney U test. A P value less than 0.05 denoted a statistically significant difference.

3. Results

3.1. Expression of FBXO9 is related with obesity

We examined FBXO9 expression levels in white adipose tissues of mice fed the control or high fat diet. FBXO9 expression was significantly increased (5-fold) when the mice were fed the high fat-diet for 12 weeks (Fig. 1A). Expression of FBXO9 in the obese mice, *db*/*db* mice, was also higher than in the *db*/*m* mice (Fig. 1B). These results imply that FBXO9 expression is related with obesity.

3.2. FBXO9 expression is increased during adipogenesis

To determine whether FBXO9 expression is regulated during adipogenesis, 3T3-L1 preadipocytes were treated with differentiation inducers (dexamethasone, IBMX, and insulin, also called DMI) and total mRNA was collected several times during adipogenesis. FBXO9 expression was increased more than 2-fold 6 h after the induction of differentiation and the expression level was sustained until day 6 (Fig. 1C), suggesting a role for FBXO9 in adipogenesis.

3.3. Knockdown of FBXO9 inhibits adipogenesis

To determine whether FBXO9 is required for adipogenesis, siRNAs against FBXO9 (siFBXO9) were added to the cells at different time points of differentiation. First, FBXO9 knockdown by siFBXO9 was evaluated and we found that FBXO9 mRNA levels were effectively down-regulated by the FBXO9 siRNAs (Fig. 2A). When siRNAs against FBXO9 were transfected 1 day before or simultaneously with the induction of differentiation, adipogenesis was significantly inhibited as shown by Oil red O staining (Fig. 2B). In contrast, treatment with FBXO9 siRNAs 1 day after the induction of differentiation did not affect adipocyte differentiation. Therefore, FBXO9 seems to play an important role in the early stages of adipogenesis. Next, we compared the expression levels of several proteins involved in adipogenesis using immunoblotting in cells treated with siRNAs at day -1. C/EBPβ, which is induced in the very early stages of adipogenesis and predominantly involved in PPARγ expression, was significantly reduced after FBXO9 knockdown (Fig. 2C and D). When FBXO9 was knocked down, PPARγ was hardly detected, which may be caused by the reduction of C/EBPβ. We also examined the protein levels of LRP6 and β-catenin which

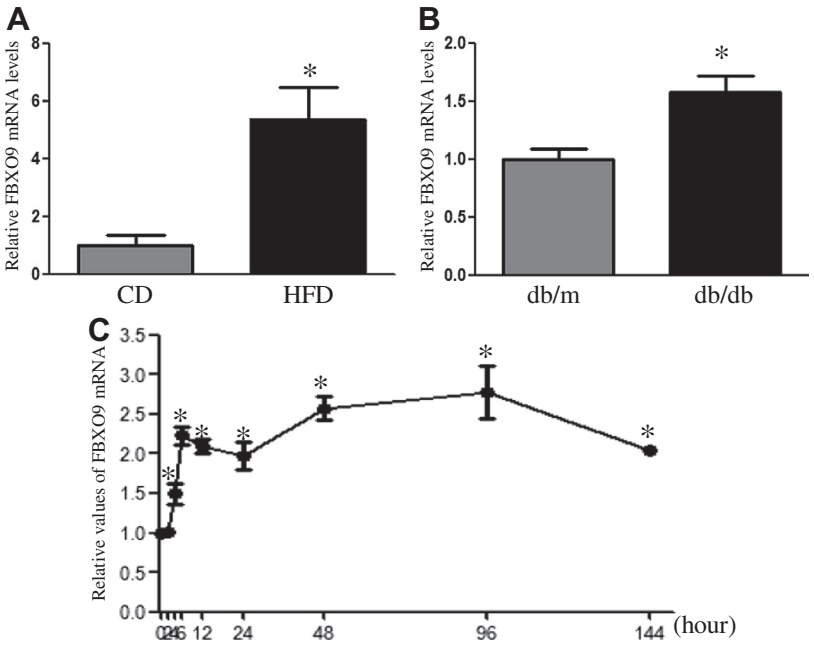

Fig. 1. FBXO9 expression was increased in obese animal models and during adipocyte differentiation. Total RNAs were prepared from white adipose tissues of (A) mice fed with control diet (CD, $n = 4$) or high fat diet (HFD, $n = 4$) or (B) *db/m* ($n = 5$) or *db/db* mice ($n = 5$). FBXO9 mRNA levels were quantitated using real-time PCR and normalized to the level of 18s rRNA of each sample. The values obtained from the control diet mice or *db/m* mice were set to 1 and the other values are presented as relative values (mean ± SEM). $^*P < 0.05$ vs. the value of control diet (A) or *db/m* (B). (C) Total RNAs were prepared at the indicated time points after the induction of adipogenesis in 3T3-L1 cells. FBXO9 mRNA levels were evaluated by real time PCR and normalized to the levels of 18S rRNA. The value obtained from the cells harvested just before the induction of adipogenesis (0 h) was set to 1 and the other values are presented as relative values (mean ± SEM, $n = 5$). $^*P < 0.05$ vs. the value at 0 h.

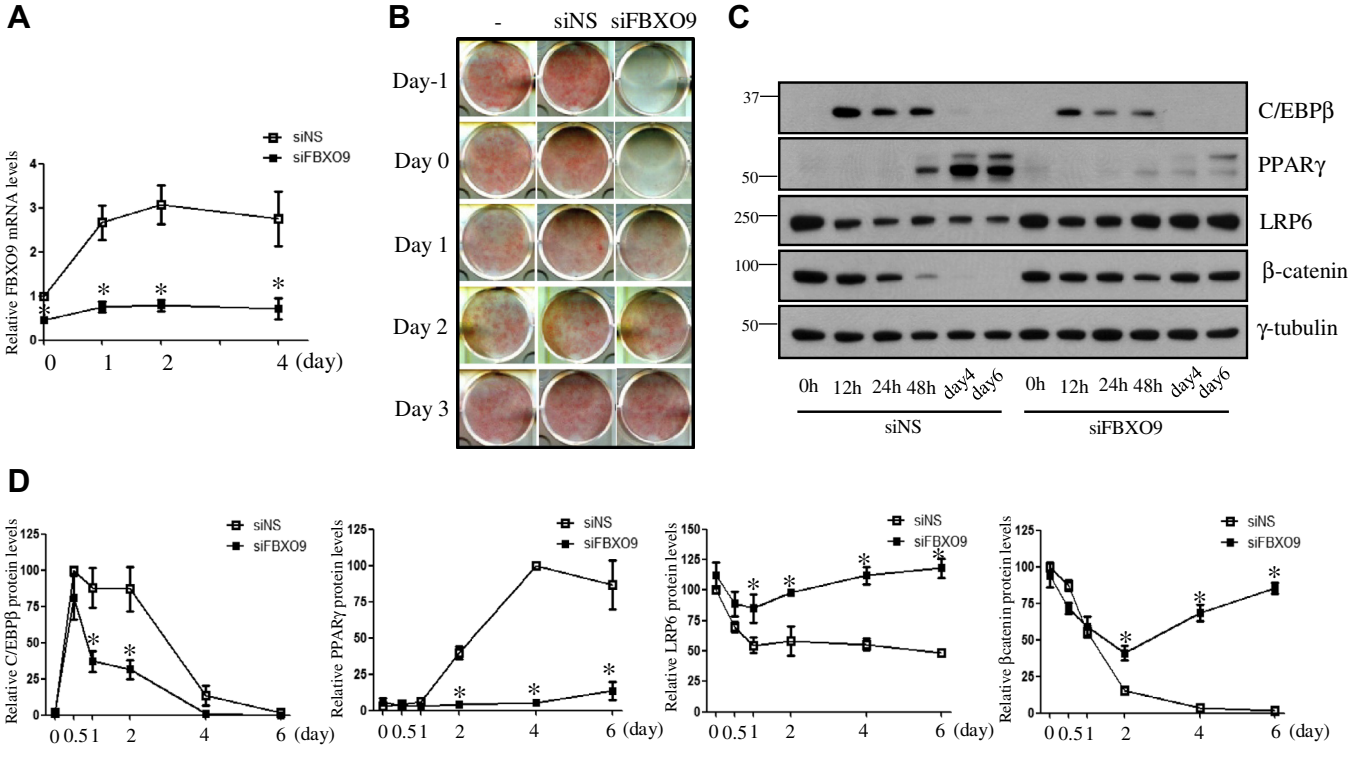

Fig. 2. FBXO9 knockdown inhibits adipogenesis. (A) Specific siRNAs against FBXO9 (siFBXO9) were transfected into 3T3-L1 cells 1 day before the induction of adipogenesis. FBXO9 mRNA levels were measured at the indicated times (days). The value obtained from the cells transfected with non-specific siRNAs (siNS) at day 0 was set to 1 and the others were expressed as relative values (mean ± SEM, $n = 4$). (B) The siNS or siFBXO9 were treated at the indicated days (adipocyte differentiation inducers were treated at day 0). Cells were stained with Oil red O 8 days after the induction of adipogenesis. (C) Cells were treated with siRNAs (siNS or siFBXO9) 1 day before adipogenesis induction, and protein lysates were prepared at the indicated times after the induction. Western blot analyses were performed with antibodies against C/EBPβ, PPARγ, LRP6, β-catenin, and γ-tubulin. (D) The intensity of each band in (C) is normalized to γ-tubulin. The maximum values of siNS treated cells were set to 100 and the other values are expressed as relative values. The mean value was calculated from four independent experiments (mean ± SEM). $^*P < 0.05$ vs. the value of siNS at the same time point.

are involved in the Wnt signaling pathway and known to decrease during adipogenesis. When FBXO9 was knocked down, LRP6 and β-catenin were slightly reduced until 24 or 48 h after the induction of adipogenesis, respectively, but thereafter the levels increased to the levels observed in preadipocytes (Fig. 2C and D). As a result, the protein expression pattern 6 days after the cells were treated with FBXO9 siRNAs was similar to that of preadipocytes (0 day).

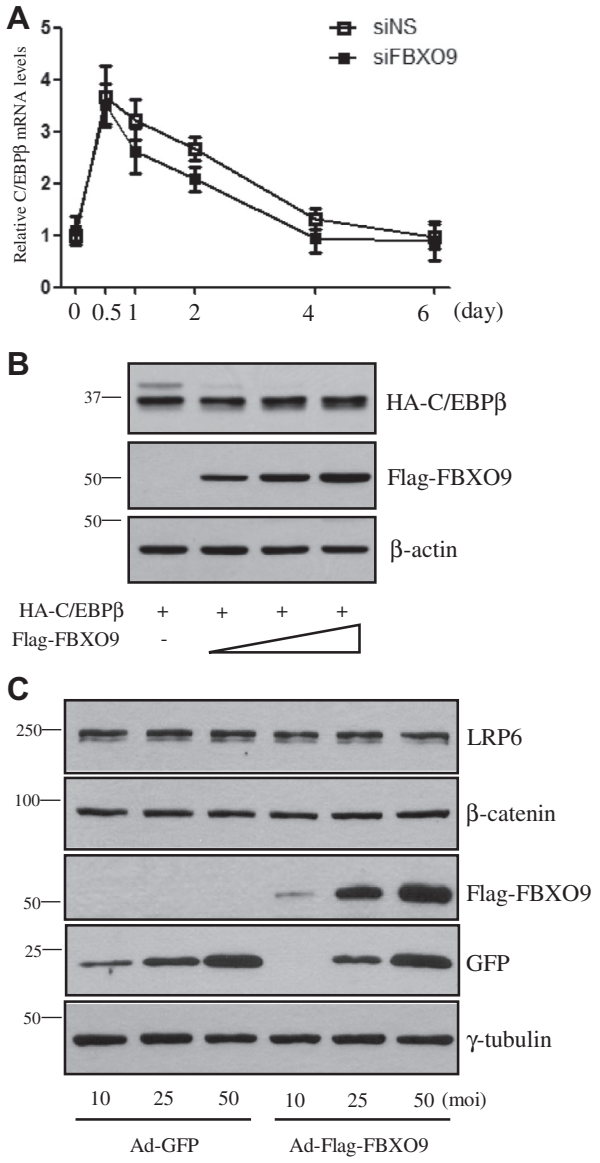

Fig. 3. FBXO9 did not affect C/EBPβ or LRP6 protein stability in Cos 7 cells. (A) Total mRNAs were prepared from the cells treated with siNS or siFBXO9 as described in Fig 2C at the indicated times after the induction. C/EBPβ mRNA levels were measured using real-time PCR, and normalized to the levels of 18S rRNA. The value obtained from the cells harvested just before the induction of adipogenesis (0 h) was set to 1 and the other values are presented as relative values (mean ± SEM, $n = 6$). (B) Cos-7 cells were transfected with expression vectors for C/EBPβ (HA-C/EBPβ) and/or FBXO9 (Flag-FBXO9). Twenty-four hours after the transfection, proteins were prepared for immunoblot analyses. Immunoblot was performed with antibodies against HA, Flag, or β-actin. (C) Cos-7 cells were infected with control adenovirus (Ad-GFP) or adenovirus expressing Flag-FBXO9 (Ad-Flag-FBXO9). Lysates were prepared 24 h after infection. Endogenous LRP6 and β-catenin were detected by specific antibodies. GFP blots represent the efficiency of virus infection.

3.4. FBXO9 does not directly regulate C/EBPβ or LRP6 protein level

Since the reduction of C/EBPβ protein level is the most notable phenomenon by FBXO9 knockdown in the early stages of adipogenesis (day 0–2) (Fig. 2D), we tested whether FBXO9 knockdown affected transcriptional levels of C/EBPβ. When FBXO9 was knocked down, the mRNA levels of C/EBPβ was not significantly changed (Fig. 3A). Therefore, the reduction of C/EBPβ protein levels by FBXO9 knockdown was not caused by decrease of C/EBPβ transcription but might be mediated by a change in the C/EBPβ stability. Next, we tested whether FBXO9 could directly regulate the stability of C/EBPβ. When C/EBPβ was exogenously expressed in COS-7 cells using transient transfection, C/EBPβ protein levels were not altered by overexpression of FBXO9 (Fig. 3B). We also tested whether FBXO9 could directly regulate LRP6. Endogenous LRP6 levels were not affected by adenovirus-mediated FBXO9 overexpression (Fig. 3C). In addition, knockdown of FBXO9 did not affect LRP6 protein levels in 3T3-L1 preadipocytes (data not shown). From these results, we did not find any evidence that C/EBPβ or LRP6 was directly regulated by FBXO9.

3.5. Overexpression of C/EBPβ alleviates suppression of adipogenesis by FBXO9 knockdown

Although C/EBPβ protein stability was not directly regulated by FBXO9, the reduction in C/EBPβ levels seems to be a major cause of adipogenesis inhibition by FBXO9 knockdown. Next, we tested whether overexpression of C/EBPβ could rescue adipogenesis in the FBXO9 knockdown condition. We used 3T3-L1 preadipocytes stably expressing C/EBPβ (pC/EBPβ) which had been prepared by infection of a retrovirus-mediated C/EBPβ expression construct. The preadipocytes continuously expressing C/EBPβ (pC/EBPβ) fairly differentiated to adipocytes when siFBXO9 was added, whereas

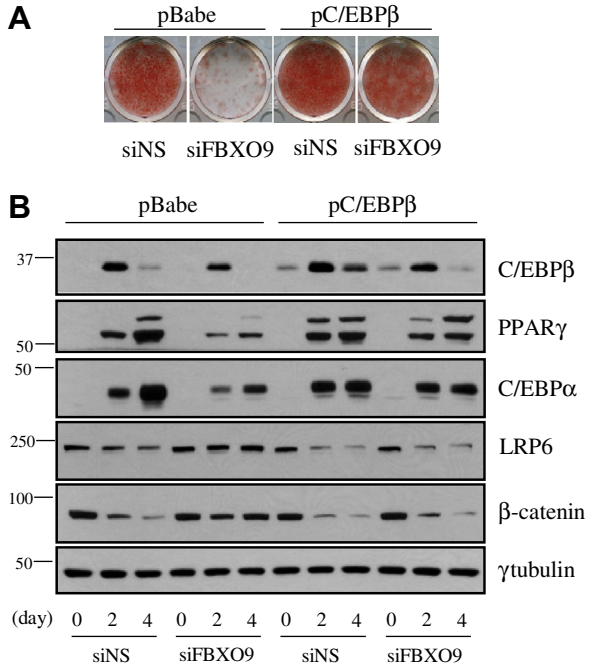

Fig. 4. C/EBPβ overexpression remarkably alleviates the inhibition of adipogenesis by FBXO9 knockdown. (A) 3T3-L1 preadipocytes were infected with retrovirus harboring a C/EBPβ expression construct (pC/EBPβ) or an empty construct (pBabe). Cells resistant to puromycin were selected. Cells were transfected with 25 nM of siNS or siFBXO9 1 day before the induction of adipogenesis. Cells were stained with Oil red O at day 8 of adipogenesis. (B) Protein samples were prepared at day 0, 2, and 4 of adipogenesis and subjected to immunoblotting.

adipogenesis of the control cells (pBabe) was completely inhibited (Fig. 4A). In the FBXO9 knockdown condition, C/EBPβ protein levels maintained high and expression of PPARγ and C/EBPα, two target genes of C/EBPβ during adipogenesis, seemed to be normally induced in the pC/EBPβ cells (Fig. 4B). In contrast, LRP6 and β-catenin were negatively regulated during differentiation of the pC/EBPβ cells. These results suggest that FBXO9 knockdown inhibits adipogenesis predominantly by affecting C/EBPβ protein levels through an unknown, indirect mechanism.

4. Discussion

When FBXO9 was knocked down only in the early stages of adipogenesis, for example, at day 0 of differentiation, adipogenesis was inhibited. FBXO9 knockdown at the later stages did not affect adipogenesis (Fig. 2B). The most important and distinct phenomena observed on the first day of differentiation are the induction of C/EBPβ and C/EBPδ expression, and C/EBPβ is known to play a major role whereas C/EBPδ cooperates with C/EBPβ. C/EBPβ protein levels were significantly reduced by FBXO9 knockdown (Fig. 2C and D), suggesting that FBXO9 knockdown suppresses adipogenesis by affecting C/EBPβ protein levels. This possibility was strongly supported by the result that adipogenesis was largely not affected by FBXO9 knockdown in cells stably expressing C/EBPβ (Fig. 4). Currently, we do not know how FBXO9 regulates C/EBPβ protein levels. It is possible that FBXO9 indirectly regulates C/EBPβ protein stability by stimulating ubiquitination of another protein that is involved in C/EBPβ stability, such as a C/EBPβ-specific E3 ligase. In addition, exogenously expressed C/EBPβ was not regulated by FBXO9 in Cos-7 cells (Fig. 3B). Therefore, the regulation of C/EBPβ by FBXO9 might be cell-type specific. It has been reported that regulation of C/EBPβ stability is important to maintain its high levels for 2 days after the induction of differentiation [6]. Further studies will be aimed at elucidating the regulation mechanism of FBXO9.

In addition to the induction of specific transcription factors such as C/EBPβ, C/EBPδ, C/EBPα, and PPARγ, the suppression of Wnt signaling, characterized by reduction of Wnt10b, LRP6, and β-catenin, is an important phenomenon of adipogenesis. The protein levels of LRP6 and β-catenin were slightly decreased by differentiation inducers, and then increased after 1 or 2 days in the FBXO9-knocked down cells (Fig. 2C and D). In addition, overexpression or knockdown of FBXO9 did not alter LRP6 in Cos-7 cells (Fig. 3C) and preadipocytes. These results indicate that the effect of FBXO9 knockdown on LRP6 and β-catenin protein levels after the addition of differentiation inducers is indirect. The mechanism by which Wnt signaling is suppressed during adipogenesis is not well elucidated, and it has been reported that Wnt10b, an endogenous Wnt, is negatively regulated by cAMP elevation [15]. In addition, our previous data showed that C/EBPβ negatively regulates Wnt10b expression [24]. It seems that there is a cross talk between adipogenesis-specific transcription factors and Wnt signaling proteins [25], which likely makes the mechanism involved in the regulation of adipogenesis by FBXO9 more complex.

In our study, FBXO9 expression increased in high fat diet-fed or genetically obese mice. In addition, FBXO9 expression increased during adipogenesis, and FBXO9 knockdown almost completely inhibited adipogenesis. In summary, these data strongly suggest that FBXO9 is an important factor for adipogenesis, although the mechanism involved in this process has not been elucidated.

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