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Active form Notch4 promotes the proliferation and differentiation of 3T3-L1 preadipocytes

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ABSTRACT

Adipose tissue is composed of adipocytes, which differentiate from precursor cells in a process called adipogenesis. Many signal molecules are involved in the transcriptional control of adipogenesis, including the Notch pathway. Previous adipogenic studies of Notch have focused on Notch1 and HES1; however, the role of other Notch receptors in adipogenesis remains unclear. Q-RT-PCR analyses showed that the augmentation of Notch4 expression during the differentiation of 3T3-L1 preadipocytes was comparable to that of Notch1. To elucidate the role of Notch4 in adipogenesis, the human active form Notch4 (N4IC) was transiently transfected into 3T3-L1 cells. The expression of HES1, Hey1, C/EBPδ and PPARγ was up-regulated, and the expression of Pref-1, an adipogenic inhibitor, was down-regulated. To further characterize the effect of N4IC in adipogenesis, stable cells expressing human N4IC were established. The expression of N4IC promoted proliferation and enhanced differentiation of 3T3-L1 cells compared with those of control cells. These data suggest that N4IC promoted proliferation through modulating the ERK pathway and the cell cycle during the early stage of 3T3-L1 adipogenesis and facilitated differentiation through up-regulating adipogenic genes such as C/EBPα, PPARγ, aP2, LPL and HSL during the middle and late stages of 3T3-L1 adipogenesis.

1. Introduction

Notch is a transmembrane receptor that functions in diverse developmental events to control cell fates [1]. After Notch activation, the Notch intracellular domain (NICD) enters the nucleus, where it interacts with transcription factor CSL to activate the transcription of downstream genes, such as HES and Hey, to regulate cell differentiation [2].

The process of adipogenesis includes five steps: cell proliferation, cell contact inhibition/growth arrest, clonal expansion, permanent growth arrest and lipid accumulation [3]. The transcriptional control of adipogenesis involves the activation of several families of transcription factors, such as CCAAT/enhancer binding protein family proteins (C/EBPs) and peroxisomal proliferator-activated receptor family proteins (PPARs). Adipogenic stimuli induce the expression of C/EBPβ and C/EBPδ, followed by an increase in PPARγ and C/EBPα expression. Subsequently, C/EBPα directly binds to PPARγ promoter and induces its expression [4,5]. Finally, terminal differentiation genes, such as adipocyte protein 2 (aP2), stearoyl-CoA desaturase, glucose transporter isoform 4, phosph*enol*pyruvate carboxykinase, leptin and insulin receptor are up-regulated through C/EBPα and PPARγ. During late stage of adipogenesis, cAMP-dependent protein kinase activates hormone-sensitive lipase (HSL) [6–8].

Previous studies have shown that Notch signaling is involved in adipogenesis. The ectopic overexpression and siRNA knockdown of presenilin enhancer-2 (PSENEN), a component of the γ-secretase complex, inhibited and induced 3T3-L1 adipogenesis, respectively [9]. The overexpression of constitutively active Notch1 also inhibits adipocyte formation and reduces the expression of PPARγ, aP2 and adiponectin genes in human bone marrow-derived stromal cells [10]. However, impaired Notch1 expression by antisense constructs prevented 3T3-L1 adipogenesis and reduced PPARγ and PPARδ expression [11]. The constitutive expression of a Notch downstream target gene, HES1, inhibited adipogenesis at a step prior to the induction of C/EBPα and PPARγ [12,13]. However, the siRNA-mediated reduction of HES1 mRNA in 3T3-L1 cells also inhibited differentiation. This observation might reflect that HES1 promoted adipogenesis through down-regulating Pref-1, an adipogenic inhibitor [13].

Studies concerning the involvement of Notch signaling in adipogenesis have focused on Notch1 and HES1; however, the

Abbreviations: N4IC, active form Notch4; Pref-1, preadipoctye factor 1; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome-proliferator activated receptor; aP2, adipocyte protein 2; LPL, lipoprotein lipase; HSL, hormone-sensitive lipase.

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differential function of other Notch receptors in adipogenesis remains unknown. In this study, we analyzed the transcripts of all Notch receptors on different days during 3T3-L1 differentiation, and observed that the level of increment of Notch4 transcripts was comparable to that of Notch1. To determine the role of Notch4 during the process of 3T3-L1 adipogenesis, we characterized the effects of the transient and stable expression of N4IC. Our data provide further insight into the mechanisms underlying the regulation of adipogenesis.

2. Materials and methods

2.1. Cell lines and culture

3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% calf serum and penicillin- streptomycin-amphotericin. To establish stable 3T3-L1 cells expressing human c-myc-N4IC (3T3-L1 N4IC), the cells were transfected with pcDNA3-N4IC using Turbofect™ reagent (Fermentas). After 48 h, the cells were replated in selection medium containing 1000 μg/ml G418. Stable clones were screened for the human Notch4 transcripts using RT-PCR. Control stable cells (3T3-L1 GFP) were also established through transfection with pcDNA3-GFP.

2.2. Adipogenesis assay

The differentiation of 3T3-L1 preadipocytes was induced by growing the cells to confluence (Day -2), the medium was replaced 48 h later with differentiation medium (DMI) (Day 0). The DMI contained 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 10 μg/ml of insulin in DMEM supplemented with 10% fetal bovine serum (FBS). After DMI treatment for 48 h (Day 2), the medium was replaced with DMEM supplemented with 10% FBS and 10 μg/ml of insulin. The medium was replenished every day for 6 days (Day 8).

2.3. RT-PCR and Q-RT-PCR

Total RNA was isolated from cells using REzol C&T reagent (PROTECH) and the RNA was reverse transcribed into cDNA using MMLV High Performance Reverse Transcriptase (Epicentre). The obtained cDNA was used directly as a template for PCR or diluted 30-fold for Q-PCR. The Q-PCR reactions were performed using the KAPA SYBR® FAST qPCR kit (KAPA Biosystems). Each reaction was performed in duplicate and run in an ABI StepOne detection system using analysis software (Applied Biosystems). All samples were run in triplicate for each PCR reaction. The levels of the test genes were normalized to β-actin mRNA level using a comparative threshold cycle ($2^{-[\mathrm{delta}]\ Ct}$) method which converts the differences in the cycle numbers to test gene/β-actin ratios. The primer sequences are listed in Supplementary Table 1.

2.4. Cell proliferation assay

The cells (1.5×10^3) were plated in triplicate into each well of 96-well plates and grew for 24, 48, 72 or 96 h. For drug treatment, the cells were treated with DMSO or U0126 (1 or 5 μM) and the effect of compounds on cell growth was measured at 3 days after treatment. The MTT solution (2 mg/ml) was added at the indicated times for 3 h at 37 °C. The reactions were terminated upon the addition of solubilization solution (DMSO) and the absorbance was measured at 490 and 595 nm.

2.5. Oil Red O stain

At the indicated times, the cells were washed with PBS and fixed with 3.7% formaldehyde for 1 h at room temperature followed by staining with 0.3% Oil Red O in isopropanol for 3 min. The images of Oil Red O staining cells were acquired using a Nikon microscope. Subsequently, the stained oil droplets were treated with isopropanol to elute Oil Red O dye and the absorbance was quantified at 490 nm.

2.6. Immunofluorescence staining

3T3-L1 cells grew on cover slips and were transfected with pcDNA3-N4IC or pcDNA3-GFP for 2 days. Cells were fixed in ice-cold methanol for 5 min at −20 °C. After blocking with 1% BSA and 0.0375% saponin in PBS, the cover slips were incubated with mouse anti-c-myc antibody (1:200, Santa Cruz) for 1 h at room temperature, followed by incubation with Alexa Fluor® 555 goat anti-mouse antibody (1:400, Molecular Probes) for 30 min at room temperature. The cells were subsequently counterstained with DAPI, and the cover slips were mounted onto microscope slides. The expression of N4IC protein in 3T3-L1 cells was examined using a confocal microscope (Olympus).

2.7. Immunoblot

To prepare whole cell lysates, the cells were lysed with lysis buffer containing 20 mM HEPES pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche). Added Laemmli's sample buffer to the cell lysates (50 μg) and boiled for 5 min. The samples were subsequently resolved using SDS–PAGE and electro-transferred onto a PVDF membrane. After blocking with skim milk, membranes were incubated with primary antibodies (1:1000) overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. The immunoreactive proteins were visualized using an enhanced chemiluminescent reagent (PerkinElmer).

2.8. Cell cycle analysis

The cells (1.5×10^5) were cultured in medium containing 10% calf serum for overnight followed by serum starvation treatment for 36 h. Subsequently, the serum was restored and cultured the cells for 0, 4, 8, 12 or 24 h. To perform cell cycle analyses during cell differentiation, the cells grew to confluence followed by DMI treatment for 0, 4, 8, 12, 16 and 24 h. The treated cells were fixed with ice-cold 70% ethanol overnight at −20 °C. The fixed cells were centrifuged and resuspended the cell pellets in 4 μg/ml propidium iodine solution (containing 100 μg/ml RNase A) for 30 min at room temperature. The populations of treated cell at G1, S and G2/M stages were analyzed using a flow cytometer (Becton Dickinson) and the distribution was analyzed using the ModFit LT software (Becton Dickinson).

2.9. Statistical analysis

Samples were analyzed in duplicate and the data of three independent experiments were presented as the means ± standard deviation (SD). The statistical analyses were performed using GraphPad Prism 5 software. Student's *t* test was used to compare quantitative data among the groups.

3. Results

3.1. Expression of Notch receptors during 3T3-L1 adipogenesis

To elucidate the role of Notch in 3T3-L1 adipogenesis, the transcript levels of four Notch receptors on different differentiation days were analyzed using Q-RT-PCR. The expression levels of all Notch receptors fluctuated during the adipocyte differentiation. However, the expression of them on different differentiation days was higher than that observed before DMI addition (Day -2) (Fig. 1A). The increment of Notch4 mRNA was comparable to that of Notch1; this observation promoted further investigation of the role of Notch4 in 3T3-L1 adipogenesis.

3.2. Overexpression of N4IC in 3T3-L1 cells up-regulated the expression of HES1, Hey1, C/EBPδ and PPARγ and down-regulated the expression of Pref-1

3T3-L1 cells were transiently transfected with pcDNA3-N4IC, and the transcript levels of Notch downstream and early adipogenic genes were analyzed using Q-RT-PCR. The expression of N4IC protein was confirmed through immunofluorescence staining (Fig. 1B) and immunoblot analysis (Fig. 2C) using c-myc antibody. Immunocytochemistry showed that N4IC protein expressed throughout the entire cell, particularly in the perinuclear region (Fig. 1B). In addition, transcript levels of the Notch downstream genes, HES1 and Hey1, were up-regulated in the N4IC-transfected cells (Fig. 1C), confirming the activation of Notch signaling. The transcript levels of the pro-adipogenic genes, C/EBPδ, C/EBPβ, C/EBPα and PPARγ, were also analyzed, and C/EBPδ and PPARγ mRNAs were up-regulated in the N4IC-transfected cells (Fig. 1C). On the contrary, the expression level of Pref-1 was down-regulated in the N4IC-transfected cells. These data suggested that N4IC might facilitate the differentiation of 3T3-L1 cells through the up-regulation of C/EBPδ and PPARγ and down-regulation of Pref-1.

3.3. Establishment of 3T3-L1 cells stably expressing N4IC

To further investigate the role of N4IC signaling in adipogenesis, stable 3T3-L1 cells expressing N4IC were established (3T3-L1 N4IC cells). RT-PCR with designed primers specific for human Notch4 showed that only N4IC stable cells expressed human Notch4 transcripts (Fig. 2A) and the sequence of this PCR product was identical to human Notch4. However, the N4IC protein was not detectable using immunoblot analysis and immunoprecipitation with anti-myc antibody, potentially reflecting the short half-life of N4IC protein in stable cells. We surmised that NICD proteins were short-lived and readily degraded [14,15]. Therefore, the cells were treated with the proteosome inhibitor, MG132, and the autophagy inhibitor, chloroquine, and the expression of myc-N4IC protein was determined through immunofluorescence staining. Immunocytochemistry showed that different levels of N4IC proteins, particularly in the nucleus, were detected in 3T3-L1 N4IC cells after co-treatment with 10 μM MG132 and 100 μM chloroquine for 12 h (Supplementary Fig. 1).

3.4. N4IC affected proliferation of 3T3-L1 cells through modulating the ERK pathway and the cell cycle

Because cell proliferation is the first step of adipogenesis [3], we analyzed the proliferation rate of N4IC cells using MTT assay. The 3T3-L1 N4IC cells showed faster growth rate than the control cells (Fig. 2B). To elucidate the mechanism of N4IC in the proliferation of 3T3-L1 cells, the extracellular signal-regulated kinase (ERK) pathway and cell cycle distribution were analyzed. After transfecting 3T3-L1 cells with pcDNA3-N4IC for 48 h, immunoblot analysis showed that the activation of ERK (phosphorylated-ERK-1/2) was higher in N4IC-expressing cells than in control cells (Fig. 2C). To further confirm the effect of N4IC on ERK activity, the stable cells were treated with a MEK inhibitor, U0126, and the proliferation rates were measured. At a lower concentration (1 μM) of U0126, the growth of the control cells was more suppressed than that of N4IC cells (Fig. 2D). This result was consistent with the increased level of activated ERK (pERK-1/2) in N4IC-transfected cells, which might lead to higher resistance to MEK inhibitor than observed in the control cells. After synchronizing cells with serum-free media, the distribution of the cell populations at cell cycle stages was analyzed at the indicated times after re-culturing cells in serum-restored media. The result showed that the population in S phase was higher in N4IC cells (39.6 ± 4.1%) than in control cells (31.6 ± 0.8%) at 24 h. In parallel, the population in G1 phase was lower in N4IC cells (41.6 ± 6.0%) than in control cells (53.3 ± 1.9%) (Fig. 2E).

After treating confluent 3T3-L1 cells with DMI, the cells underwent approximately two cycles of synchronized cell division, a process known as mitotic clonal expansion [16,17]. To investigate whether N4IC modulates the cell cycle during the clonal expansion step of 3T3-L1 cells, cell cycle stages were analyzed at the indicated times after DMI treatment. The data showed that the population in the S phase was significantly higher in N4IC cells (36.4 ± 4.4%) than in control cells (16.2 ± 3.2%) after DMI treatment for 12 h (Fig. 2F). Taken together, our observations suggest that N4IC affects the cell proliferation of 3T3-L1 cells through modulating the ERK pathway and the cell cycle.

3.5. N4IC facilitated 3T3-L1 differentiation

3T3-L1 N4IC cells proliferated significantly faster than the control cells during the early stage of adipogenesis (Fig. 2). To investigate whether there is any difference during differentiation; the cells were induced by a standard DMI protocol. The Oil Red O staining showed that N4IC significantly facilitated 3T3-L1 adipogenesis, particularly on differentiation Day 8 (Fig. 3A). The quantization of solubilized Oil Red O dye also showed that the differentiation was more enhanced for 3T3-L1 N4IC cells than for control cells (Fig. 3B). The differentiation analyses were also used to examine the effects of omitting one of three components in DMI. The adipogenesis of control and N4IC cells was defective under these one-reagent dropout conditions, as shown using Oil Red O dye staining on differentiation Day 12 (Fig. 3C). However, the N4IC cells still demonstrated better differentiation than the control cells, regardless of which component was omitted (Fig. 3C). This result suggests that N4IC might slightly compensate for the hormonal activation or generation of cAMP under these one-reagent-dropout conditions.

3.6. N4IC up-regulated adipogenic signals

To determine how N4IC facilitates 3T3-L1 adipogenesis, the expression levels of adipogenic genes, C/EBPα, PPARγ and aP2, were analyzed during the differentiation of both control and N4IC cells. The expression of these genes was up-regulated at both mRNA and protein levels on differentiation Days 6 and 8 in N4IC cells compared with control cells (Fig. 4A and B). Specifically, the PPARγ mRNA level was much higher in N4IC cells than in control cells, even on differentiation Day 2. The transcript levels of LPL and HSL adipogenic genes were also up-regulated on differentiation Days 6 and 8 in N4IC cells compared with those in control cells (Fig. 4A). Recent studies have shown that the activation of signal transducer and activator of transcription 3 (STAT3) involved in the early stage of adipogenesis through regulating C/EBPβ and

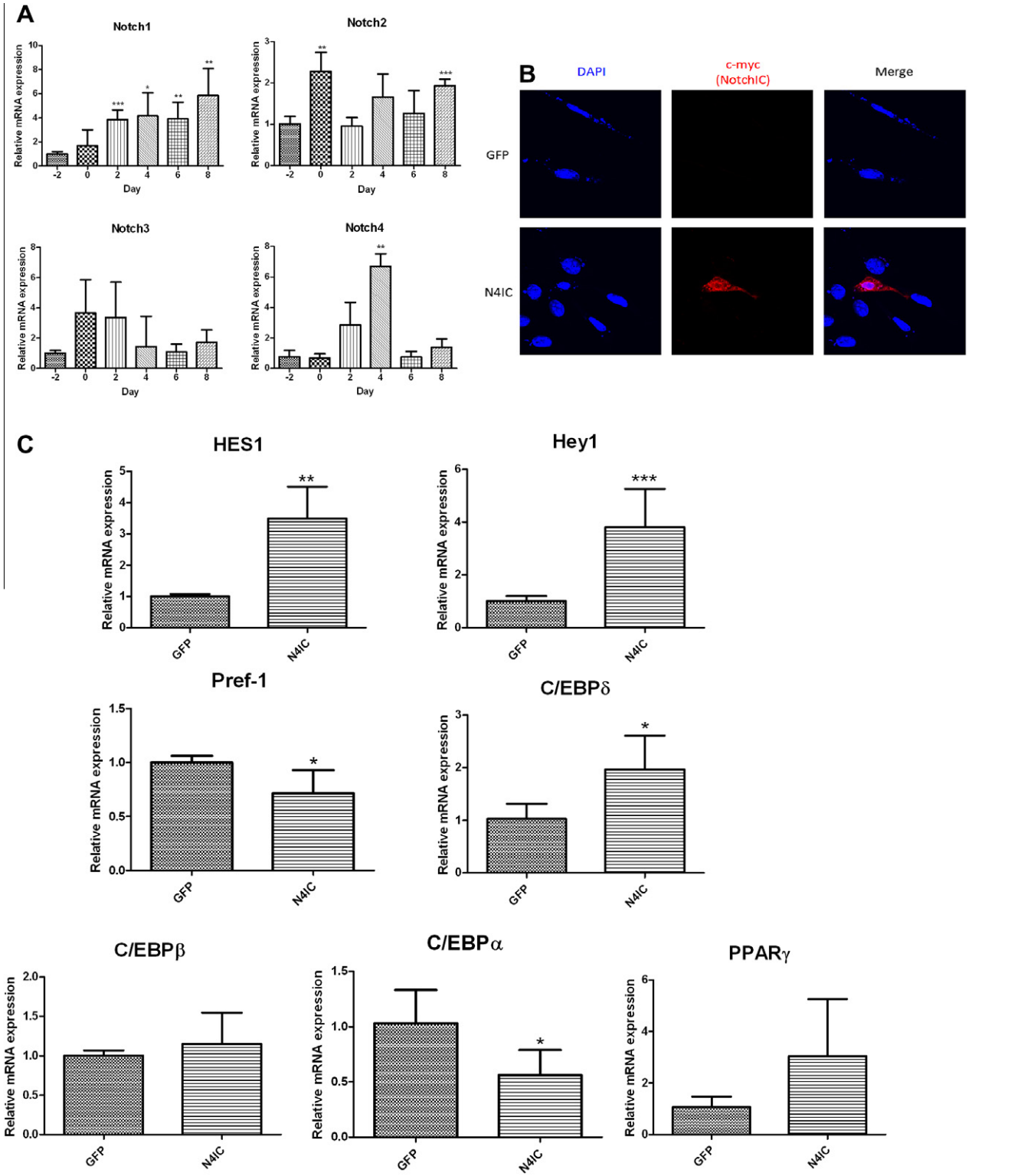
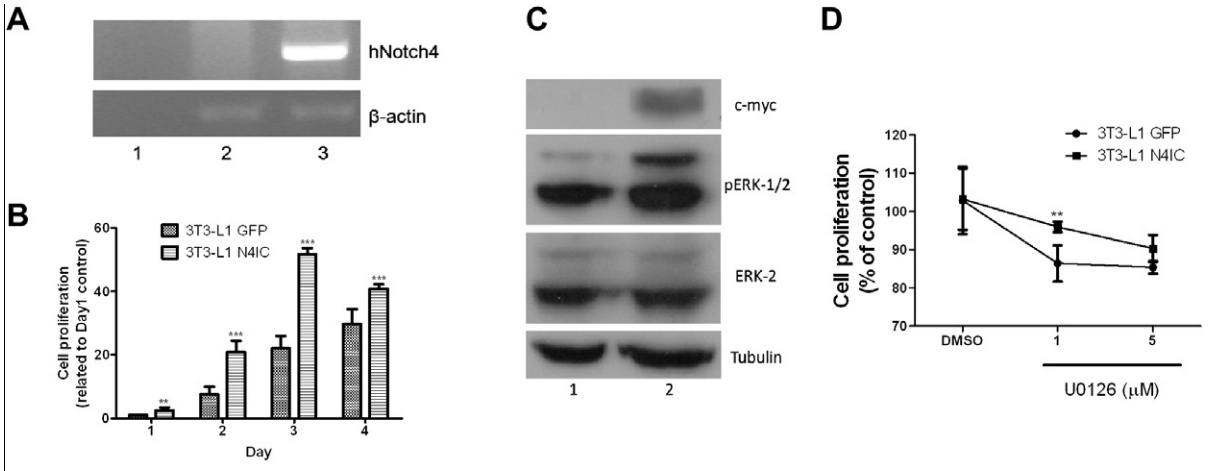
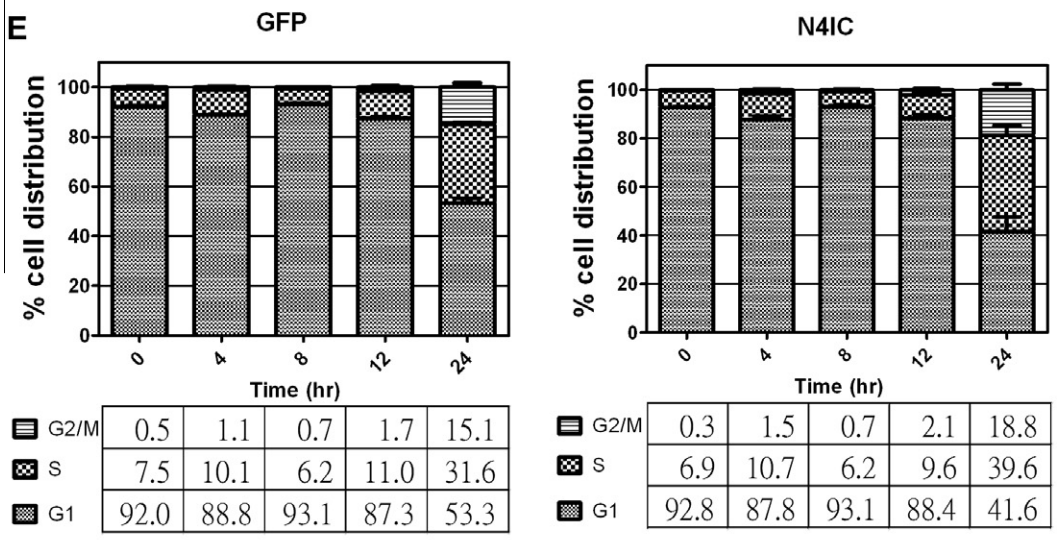


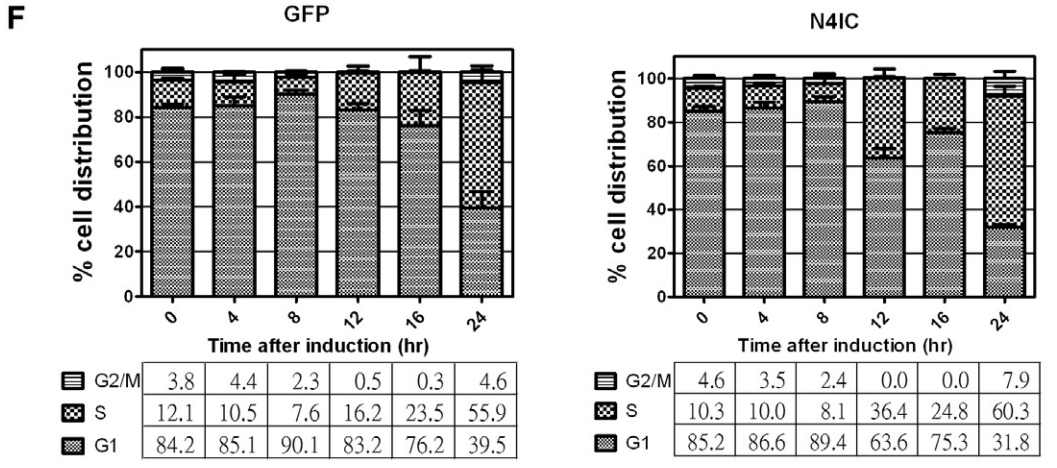
Fig. 1. Expression of Notch receptors during 3T3-L1 adipogenesis and the transient overexpression of human N4IC modulated Notch downstream and adipogenic genes in 3T3-L1 cells. (A) Q-RT-PCR for Notch1, Notch2, Notch3 and Notch4 gene transcripts on the indicated differentiation days of 3T3-L1 cells. The data are presented as the means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Day 2. (B) Immunofluorescence staining for c-myc-N4IC proteins (red) of 3T3-L1 cells transfected with pcDNA3-N4IC for 48 h. Nuclei were counterstained with DAPI (blue). (C) Q-RT-PCR for transcripts of HES1, Hey1, Pref-1, C/EBPδ, C/EBPβ, C/EBPα and PPARγ genes in pcDNA3-N4IC transfected 3T3-L1 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. pcDNA3-GFP transfected cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PPARγ expression [18,19]. The activation of STAT3 during 3T3-L1 differentiation was analyzed using immunoblotting. The results showed that the activation of STAT3 (pSTAT3) was much higher in N4IC cells than in control cells on differentiation Day 2 (Fig. 4C). The effect of STAT3 activation influenced the increased expression of PPARγ in N4IC cells on Day 2 (Fig. 4A).





	GFP 0	GFP 4	GFP 8	GFP 12	GFP 24	N4IC 0	N4IC 4	N4IC 8	N4IC 12	N4IC 24
G2/M	0.5	1.1	0.7	1.7	15.1	0.3	1.5	0.7	2.1	18.8
S	7.5	10.1	6.2	11.0	31.6	6.9	10.7	6.2	9.6	39.6
G1	92.0	88.8	93.1	87.3	53.3	92.8	87.8	93.1	88.4	41.6



	GFP 0	GFP 4	GFP 8	GFP 12	GFP 16	GFP 24	N4IC 0	N4IC 4	N4IC 8	N4IC 12	N4IC 16	N4IC 24
G2/M	3.8	4.4	2.3	0.5	0.3	4.6	4.6	3.5	2.4	0.0	0.0	7.9
S	12.1	10.5	7.6	16.2	23.5	55.9	10.3	10.0	8.1	36.4	24.8	60.3
G1	84.2	85.1	90.1	83.2	76.2	39.5	85.2	86.6	89.4	63.6	75.3	31.8

Fig. 2. N4IC promoted cell proliferation through modulating the ERK activation and the cell cycle in 3T3-L1 cells. (A) RT-PCR for human Notch4 gene transcripts in 3T3-L1 N4IC stable cells. Lane 1, without reverse transcriptase; lane 2, 3T3-L1 GFP cells; and lane 3, 3T3-L1 N4IC cells. (B) The cells were seeded in 96-well plates, and cell proliferation was analyzed on the indicated days using MTT assay. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. 3T3-L1 GFP cells on the same day. (C) Immunoblot detection of N4IC (c-myc), phosphorylated-ERK-1/2 (pERK-1/2), ERK-2 and α-tubulin in pcDNA3-GFP and N4IC transfected 3T3-L1 cells. Lane 1, pcDNA3-GFP transfected cells; and lane 2, pcDNA3-N4IC transfected cells. (D) 3T3-L1 GFP and N4IC cells were treated with the MEK inhibitor, U0126, for 3 days and cell proliferation was analyzed using MTT assay. $^{**}P < 0.01$ vs. 3T3-L1 GFP cells at the same U0126 concentration. DMSO treatment was designated as 100%. The cell cycle analyses for (E) serum-starved 3T3-L1 GFP and N4IC cells re-cultured in serum-restored media and (F) confluent 3T3-L1 GFP and N4IC cells treated with DMI for the indicated times. The cells were stained with propidium iodine and the distribution of the cell populations at G1, S and G2/M stages was determined using flow cytometry.

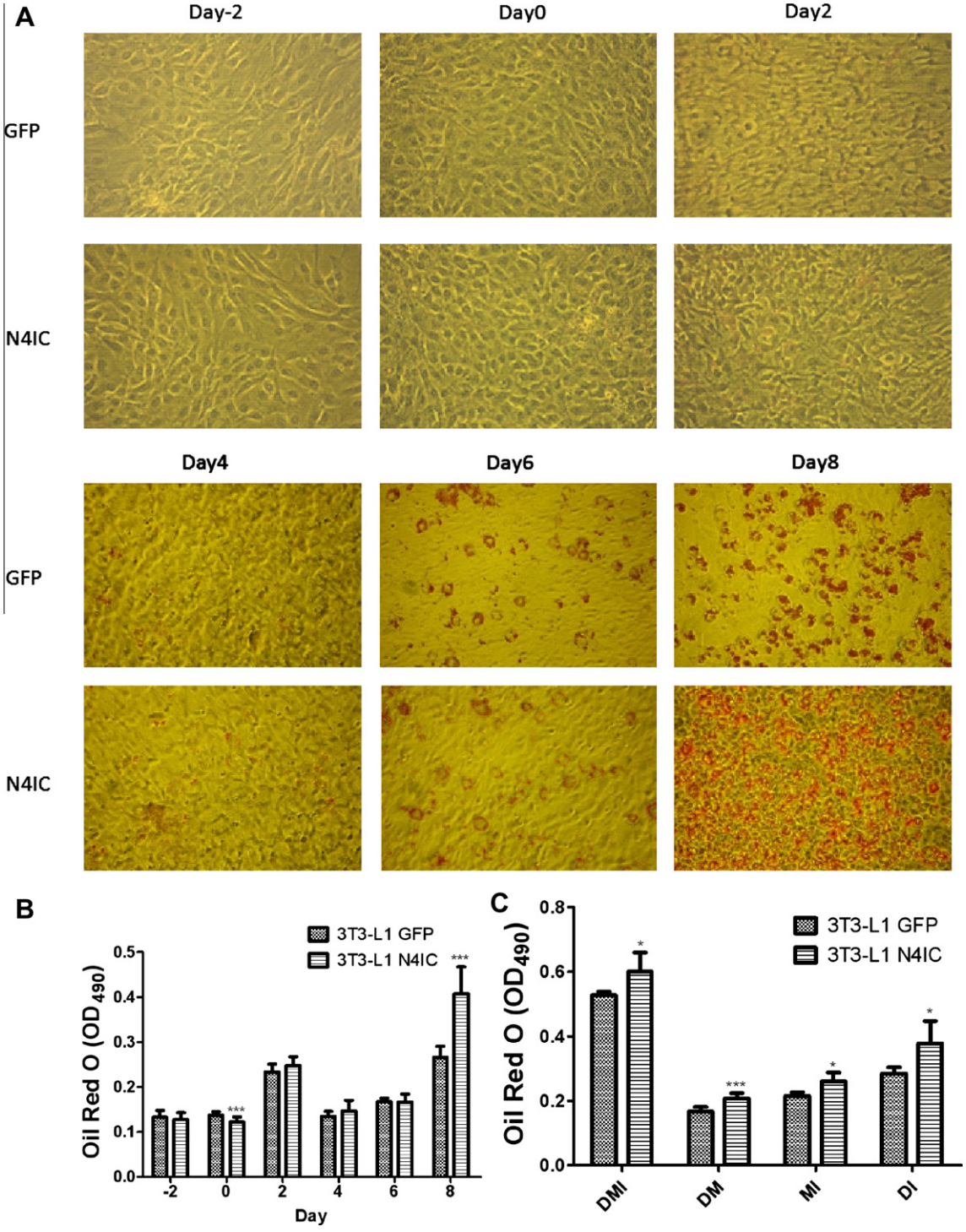


Fig. 3. N4IC facilitated 3T3-L1 adipogenesis. (A) 3T3-L1 GFP and N4IC cells were induced to differentiation and stained lipids with Oil Red O on the indicated differentiation days. (B) Oil Red O stained-cells were solubilized using isopropanol and the absorbance was measured at 490 nm. $^{***}P < 0.001$ vs. 3T3-L1 GFP cells on the same day. (C) The adipogenesis of 3T3-L1 GFP and N4IC cells was induced through DMI, DM (insulin dropout), MI (dexamethasone dropout), or DI (IBMX dropout) for 12 days. The lipids were stained with Oil Red O and the absorbance of the isopropanol extracts was measured at 490 nm. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. 3T3-L1 GFP cells.

4. Discussion

Previous studies have suggested that C/EBPδ, C/EBPβ, C/EBPα and PPARγ are major transcription factors involved in the regulation of adipogenesis. In addition, Notch pathway has been implicated in adipogenesis. In this study, we observed that the increase level of Notch4 mRNA was comparable to that of Notch1 during 3T3-L1 differentiation (Fig. 1A). Fig. 1C showed that transiently overexpressing N4IC up-regulated expression of Notch downstream genes, HES1 and Hey1, and adipogenic genes, C/EBPδ and PPARγ, in 3T3-L1 cells. In contrast, the expression of Pref-1, an adipogenic inhibitor, was down-regulated. C/EBPα was also down-regulated in N4IC-transfected cells to potentially promote cell proliferation, as a previous study showed that C/EBPα possessed antimitotic activity [20].

To delineate the role of N4IC signaling in adipogenesis, the 3T3-L1 cells stably expressing human N4IC were established. Human N4IC transcripts were detectable in N4IC stable cells, and the

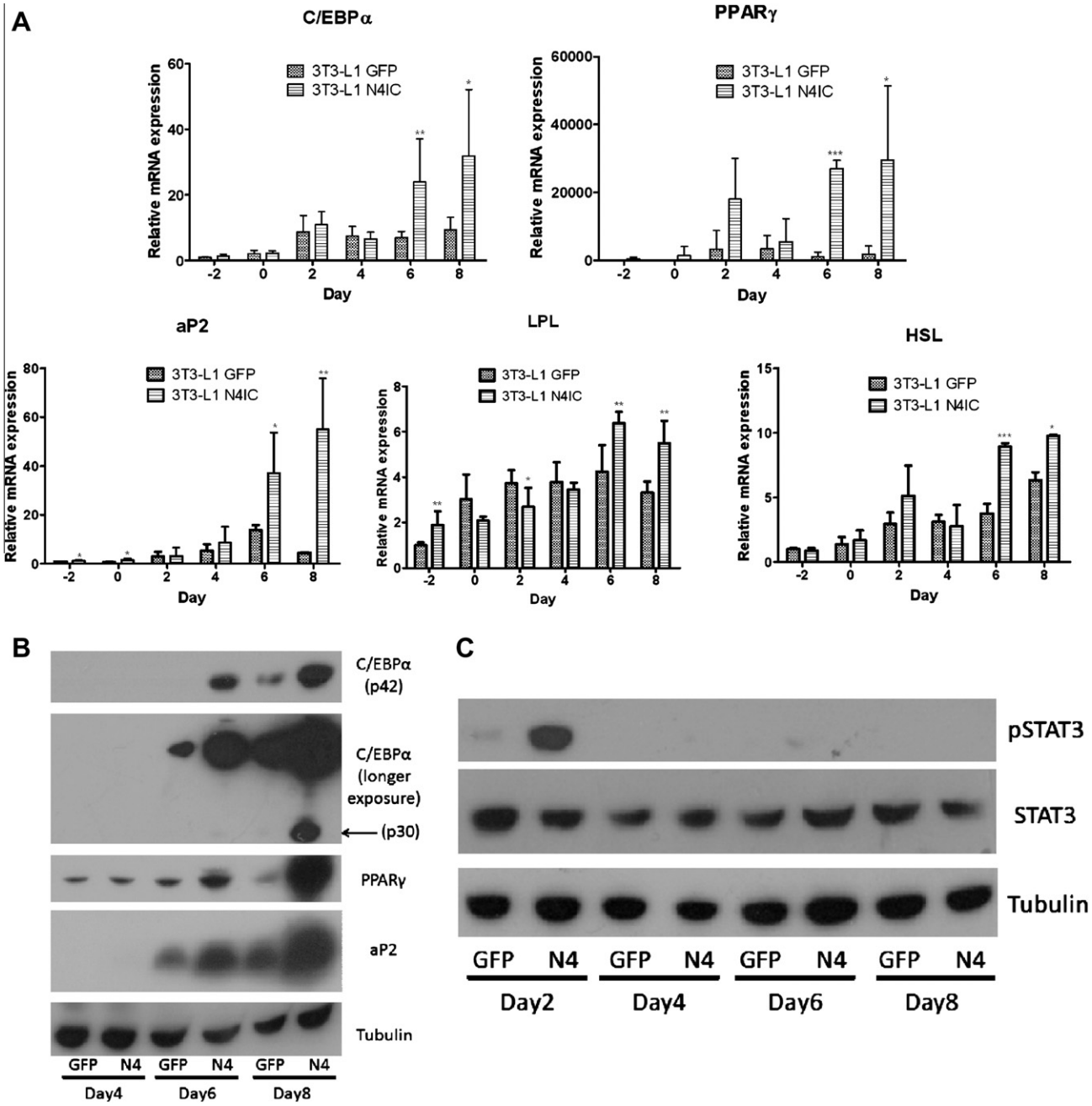


Fig. 4. N4IC facilitated 3T3-L1 adipogenesis through up-regulating adipogenic signals. (A) Q-RT-PCR for C/EBPα, PPARγ, aP2, LPL and HSL adipogenic gene transcripts in 3T3-L1 GFP and N4IC cells on the indicated differentiation days. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. 3T3-L1 GFP cells on the same day. (B) Immunoblot detection of C/EBPα, PPARγ, aP2 and α-tubulin in 3T3-L1 GFP and N4IC cells on the indicated differentiation days. In addition to the p42 isoform of C/EBPα, the p30 isoform is also showed in the second panel. (C) Immunoblot detection of phosphorylated-STAT3 (pSTAT3), total STAT3 and α-tubulin in 3T3-L1 GFP and N4IC cells on the indicated differentiation days.

expression levels remained stable for the duration of this study. However, we failed to detect N4IC proteins using immunoblot analysis in stable cells, although these proteins were readily detectable in pcDNA3 N4IC-transient transfected cells (Fig. 2C). These phenomena might indicate a shortened half-life of N4IC proteins in stable cells [14,21], as FBW7 E3 ubiquitin ligase affected Notch4 stability [14,22,23]. FBW7 was highly expressed in preadipocytes to promote the phosphorylation-dependent degradation of pro-adipogenic transcription factors, such as C/EBPα and SREBP1c, thereby attenuating adipogenesis [21]. The analysis of *Fbw7*$^{-/-}$ mice revealed that the levels of N4IC were elevated following *Fbw7* knockout [22,23]. Therefore, N4IC protein might easily to degrade in 3T3-L1 cells with high levels of FBW7 expression. After co-treatment of N4IC cells with MG132 and chloroquine, N4IC protein could be detected using immunocytochemistry, particularly in the nucleus (Supplementary Fig. 1). Although N4IC protein was not easily detectable in N4IC stable cells, N4IC still modulated 3T3-L1 cells in response to proliferation and differentiation signals.

Our data suggested that N4IC promoted the proliferation of 3T3-L1 cells through ERK activation and modulated the cell cycle during the early clonal expansion step of adipogenesis (Fig. 2F). The N4IC also activated adipogenic signals to facilitate 3T3-L1 differentiation (Fig. 4). The effect of N4IC might partially through activating STAT3 during the early stage of adipogenesis (Fig. 4C),

as previous studies showed that STAT3 is activated in the presence of active Notch through HES1 [24,25]. The activated STAT3 (pSTAT3) in turn up-regulated the expression of C/EBPβ and PPARγ [18,19], subsequently, these proteins up-regulated the terminal differentiation genes. Based on these results, we suggest that N4IC might first promote the proliferation and clonal expansion of 3T3-L1 cells to achieve cell confluence and initiate adipogenesis, respectively, followed by the up-regulation of adipogenic genes, such as C/EBPδ and PPARγ, and the down-regulation of Pref-1 to facilitate differentiation.

Although cell proliferation and differentiation should be mutually exclusive, our study showed that N4IC promotes both the proliferation and differentiation of 3T3-L1 cells. Previous studies concerning the involvement of Notch in adipogenesis have suggested that Notch signaling serves as a negative regulator; nevertheless, the results of the present study confirmed that Notch4 facilitates the differentiation of preadipocytes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.024.

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