γ -secretase inhibitor induces adipogenesis of adipose-derived stem cells by regulation of Notch and PPAR- γ

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Abstract

Objective: To determine the inhibitory effect and mechanism of Notch signalling on adipogenesis of mouse adipose-derived stem cells (mASCs).

Materials and methods: Varied concentrations of *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine t-butylester (DAPT) were added to mASCs 3 days before adipogenic induction with insulin-containing differentiation medium. The process of adipogenesis and ability of lipid droplet accumulation were analysed using oil red-O staining. The Notch signalling pathway (Notch-1, -2, -3, -4, Hes-1 and Hey-1) and adipogenesis-related factors (PPAR- γ , DLK-1/Pref-1 and Acrp) were tested using real-time PCR, Western blot analysis and immunofluorescence staining assays.

Results: We demonstrated that Notch-2-Hes-1 signalling pathway was inhibited dose-dependently by DAPT in mASCs. In addition, transcription of PPAR- γ was promoted by DAPT before adipogenic induction, while inhibitor of adipogenesis DLK-1/Pref-1 was further depressed. At early stages of differentiation (2–4 days), adipogenesis in mASCs was advanced and significantly enhanced in 5 and 10 μ M DAPT pre-treated cases. On day 4, in differentiated mASCs cases with DAPT pre-treatment, we also found promotion of activation of de-PPAR- γ and depression of HES-1, DLK-1/Pref-1 mRNA and protein expression.

Conclusions: We conclude that blocking Notch signalling with DAPT enhances adipogenesis of differentiated mASCs at an early stage. It may be due to depression of DLK-1/Pref-1 and promotion of de-PPAR- γ activation, which work through inhibition of Notch-2-Hes-1 pathway by DAPT.

Introduction

Mesenchymal stem cells (MSCs) are a group of multipotent adult-derived stem cells that can be isolated from organs and tissues including bone marrow, ligaments, muscular and adipose tissue (1,2). MSCs may undergo self-renewal for several generations while maintaining their capacity to differentiate into multi-lineage tissues such as bone, cartilage, muscles and fat (3). In our previous studies, we have investigated MSCs derived from adipose tissue of human and other species origin, reporting the multi-lineage differentiation potential of MSCs, especially in the adipogenic differentiation process (4).

MSCs derived from fat are termed adipose-derived stem cells (ASCs). They can be easily isolated, cultured and expanded and further differentiated into adipocytes with a traditional cocktail differentiation medium (DM) containing dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin and indomethacin (5). Therefore, ASCs provide an excellent model to uncover modes of regulation of adipogenic differentiation and insulin sensitivity of mature adipocytes, which are vital for treatment of obesity, insulin resistance and fat tissue regeneration. Recently, an increasing number of functional studies on adipogenic differentiation have been performed on pre-adipogenic progenitor cell lines such as 3T3-L1 (6). However, research based on the ASCs model would be more beneficial if there was translation of these data into clinical applications.

Notch signalling plays a critical role in development and regeneration of stem/progenitor cells as well as in

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controlling their fate (7–10). The Notch system is known to be an evolutionarily conserved mechanism that balances differentiation and proliferation of stem/progenitor cells (11). Notch signalling is mediated by intracellular interaction of type I transmembrane ligands, such as Delta and Serrate with Notch receptors (Notch-1, -2, -3 and -4) in vertebrates. Once bound to its ligand, Notch receptor is cleaved away by metalloprotease TNF- α converting enzyme and the γ -secretase complex respectively, at two sites, to generate the Notch intracellular domain (NICD). This translocates to the nucleus and binds a CCAAT-binding protein (CBF-1), also called CSL (12). In the absence of NICD, CBF-1 acts as a transcriptional repressor, which recruits a co-repressor complex and inhibits transcription of target genes that containing CCAAT binding sites (13,14). As a sequence of binding, NICD displaces the repressor complex of CBF-1 and recruits nuclear co-activators, such as mastermind-like 1 (MAML1) (15) and histone acetyltransferases, converting CBF-1 into a transcriptional activator. Notch activation through CBF-1-NICD interactions can in turn activate transcription of various target genes, including *Hes* (*Hairy/Enhancer of Split*) (15,16), Hes-related repressor protein (HERP) (17,18), nuclear factor- κ B (NF- κ B) (19) and PPAR (peroxisome-proliferator-activated receptor) (20).

Conflicting findings have been reported concerning the roles of Notch signalling in adipocyte differentiation of pre-adipocytes. It has been argued by Nichols *et al.* that Notch is dispensable in adipocyte specification (21). However, more evidence has been presented demonstrating that inhibition of Notch signalling pathway or its target genes can inhibit or promote differentiation of pre-adipocytes (22–24). γ -secretase inhibitors (GSIs) including *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butylester (DAPT) are a family of chemical compounds that can efficiently block the γ -secretase complex (25). Considering that γ -secretase can cleave four Notch receptors, thereby blocking all Notch response, we sought to investigate the interplay between adipogenesis and Notch signalling.

As there are large numbers of Notch-regulated genes and proteins with potential cross-talk between Notch and other signalling cascades, it is difficult to predict the outcome of Notch signalling in differentiation of pre-adipocytes in diverse contexts. There is much work to be carried out in understanding the crosstalk between Notch and adipogenesis signalling pathways. Here, we have concentrated on the relationship between Notch and PPAR- γ . There are three homologues in the PPAR family (PPAR- α , PPAR- δ and PPAR- γ) – all members of the nuclear hormone receptor superfamily (26). Recent studies have shown that PPAR- γ is one of the most critical transactivators in initiation of adipogenesis (27–30). Some have

observed the potential crosstalk between Notch and delta-like 1 homologue (DLK-1)/pre-adipocyte factor-1 (Pref-1). DLK-1/Pref-1 has been long considered to be a marker of undifferentiated pre-adipocytes; expression of DLK-1/Pref-1 sharply decreases during early adipogenesis (31,32).

In this study, we have attempted to compare insulin-dependent adipogenesis between DAPT pre-treated mASC groups and a control group of cells in diverse stages of differentiation. We first investigated transcription levels of Notch-2, Hes-1, Hey-1 and DLK-1/Pref-1 in DAPT-treated mASCs in gradient concentrations. Then, we induced DAPT pre-treated mASCs into adipocytes in cocktail DM and investigated the adipogenic progress daily. Having observed the effect of DAPT on adipogenetic progress, we further examined transcription and expression of Notch signalling factors, PPAR- γ , DLK-1/Pref-1 and adipocyte complement-related protein (Acrp), a marker of mature adipocytes. Dephosphorylated and phosphorylated forms of PPAR- γ proteins were detected using Western blotting and immunofluorescence staining techniques, respectively.

Materials and methods

Isolation and culture of mASCs

Six-week-old Kunming mice from the Sichuan University Animal Center (33,34) were used in this study, in accordance with the International Guiding Principles for Animal Research (1985). All surgical procedures were performed under approved anaesthetic methods using Nembutal at 35 mg/kg. Inguinal fat pads were dissected from the mice and were washed extensively with sterile PBS, to remove contaminating debris. Then they were incubated with 0.075% type 1 collagenase (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 60 min at 37 °C, with agitation. After neutralization of the collagenase, cells released from adipose specimens were filtered and collected by centrifugation at 1200 *g* for 10 min. The resulting pellet was resuspended, washed three times in medium and cells were seeded in plastic flasks in control medium (α -MEM, 10% FBS) (35). Cultures were maintained in a humidified atmosphere of 5% CO₂ at 37 °C and mASCs were passaged three times prior to differentiation or measurement.

DAPT treatment, adipogenic induction and oil red-O staining

The fourth passage of mASCs was seeded into flasks, and on glass coverslips into six-well plates at an appropriate initial density. Once 60% confluent, each was divided into

one control group and four DAPT pre-treated groups (DAPT groups), with at least three parallel wells in each group. DAPT (Sigma-Aldrich) was dissolved in 100% dimethyl sulphoxide (DMSO) to obtain a stock solution of 1 mM, which was then diluted with α -MEM (10% FBS) to the desired concentrations. α -MEM with 0.5% DMSO was set as the control group. In DAPT groups, mASCs were incubated with DAPT (Sigma) in gradient concentrations (1, 2, 5 and 10 μ M) for 4 days. DAPT pre-treated cells were then cultured in adipogenic differentiation medium including 10% FBS, dexamethasone (1 μ M), insulin (10 μ M), indomethacin (200 μ M) and isobutyl-methylxanthine (0.5 mM) (Sigma-Aldrich). Formation of lipid droplets in differentiated mASCs was analysed using oil red-O staining as follows. Cells on coverslips were fixed in 10% formaldehyde solution for 20 min, washed with PBS, and stained with oil red-O (Amresco, Solon, OH, USA) solution (in 60% isopropanol) for 5 min. To quantify adipogenic progress of mASCs, 10 random microscopic fields (amplification time: 10 $\times$ 10) were observed using the DMi 6000-B microscope (Leica, Bensheim, Germany) on each coverslip. Images were captured for each field and image analysis was carried out using *Image-Pro® Plus 6.0.0.260* (Media Cybernetics, Silver Spring, MD, USA). Average areas of red-stained droplets (pixels per one cell) were measured, representing approximate lipid content of the cells.

Extraction of total RNA, RT-PCR and real-time PCR

We assessed transcriptional levels of Notch-1, -2, -3 and -4, Hes-1, Hey-1, PPAR- γ 2, DLK-1/Pref-1 and Acrp in mASCs by real-time PCR assay. Initially, total RNA of mASCs was extracted using the Total Tissue/cell RNA Extraction Kit (Watson, China) according to the manufacturer's protocol. Total RNA (11 μ l) was reverse

transcribed into cDNA in a 20 μ l reverse transcription system (Fermentas, Vilnius, Lithuania) according to the First Strand cDNA Synthesis Kit handbook. Total RNA and cDNA of each sample were examined using agarose electrophoresis according to the protocol outlined in *Molecular Cloning: A Laboratory Manual* (2001, 3rd edition). To establish the standard curve of a certain gene, cDNA samples were amplified using an RT-PCR kit (Tiangen, China) with primers as displayed in Table 1. Expression of certain genes in mASCs was then quantified with real-time PCR, utilizing SYBR® Premix Ex Taq™ (Perfect Real Time) kit (Takara, Japan). Reactions were carried out on an ABI 7300 system (ABI, Foster City, CA, USA). For each reaction, a melting curve was generated to test primer dimer formation and false priming. Then relative quantification of mRNA levels was carried out by means of a double standard curve method. To compare transcription level of target genes in different amounts of samples, expression of β -actin was used for normalization of real-time PCR results.

Determination of PPAR- γ and DLK-1/Pref-1 protein expression

After 4 days adipogenic induction, relative level of PPAR- γ and DLK-1/Pref-1 protein expression was analysed using Western blot assay as follows: before cell dissolution cell layers were washed three times in PBS buffer, then total proteins were extracted using Protein Extract Reagents (Pierce, Rockford, IL, USA) and centrifuged (4 °C, 13 000 *g* for 10 min) to remove cell debris. Protein concentration was assessed using BCA kit (Pierce) according to the manufacturer's instructions. Forty micrograms of total protein were analysed by Western blotting using anti-PPAR- γ antibody (Abcam, Cambridge, U.K.), anti-PPAR- γ (phosphor 112) antibody (Abcam, Cambridge,

Table.1. Primer sequences of target genes and β -actin for real-time PCR assay

Genes	NM	Primer sequence (5'-3')	Tm (°C)	Product (bp)
PPAR- γ	011146.3	F: TGCACTGCCTATGAGCACTT	59.62	131
		R: TGATGTCAAAGGAATGCGAG	59.80	
Notch-2	010928.2	F: CTGCTATCCCACCACCACATC	62.66	111
		R: GTTCATCTCCACACGGTTCATC	61.72	
Hes-1	008235.2	F: GCCAATTTGCCTTTCTCATC	59.65	120
		R: AGCCACTGGAAGGTGACACT	59.76	
Hey-1	010423.2	F: TTTTCCTTCAGCTCCTTCCA	59.93	92
		R: ATCTCTGTCCCCAAGGTCT	59.93	
Pref-1	010052.4	F: TGTCATGGAGTCTGCAAGG	59.83	87
		R: CAAGCCGAACGTCTATTTT	59.71	
Acrp	028840.2	F: CCAGCTACGGGATTAGCAG	59.86	128
		R: CAAGCAAAGCTGAGTGAACG	59.78	
β -actin	007393.2	F: TGTTACCAACTGGGACGACA	60.00	139
		R: CTGGGTCATCTTTTCACGGT	59.97	

U.K.) or DLK-1 antibody (AbCam). Immunocomplexes were visualized using enhanced chemiluminescence reagent (Pierce) according to the manufacturer's instructions. Gel electrophoresis images were acquired using Gel Doc RX documentation system (Bio-Rad Lab., Hercules, CA, USA) and analysed using the Quantity One 4.6.2 according to the manufacturer's instructions.

Immunofluorescence staining of de-PPAR- γ and ph-PPAR- γ

To demonstrate the distribution of PPAR- γ proteins, the mASCs were seeded on glass coverslips for immunofluorescence (IF) staining. Before the process, mASCs were treated with 5 and 10 μ M DAPT in expansion medium for 3 days, while controls were treated with 0.5% DMSO instead. mASCs were next induced into adipogenesis in DM for 4 days. Cells were then washed briefly in PBS, fixed in cold acetone for 30 min at room temperature, then blocked in 0.5% bovine serum albumin for 15 min. Coverslips were subsequently incubated overnight at 4 °C with rabbit polyclonal antibody against PPAR- γ (1:100) (AbCam, Cambridge, U.K.) or rabbit monoclonal antibody against PPAR- γ with phosphoserine at residue 82 (1:100) (Upstate, Lake Placid, NY, USA). Sequentially, slides were incubated with secondary antibodies conjugated to rhodamine (Pierce) for 1 h at room temperature and nuclei were stained with DAPI (Molecular Probes, Eugene, OR, USA) for 1 min. After rinsing in PBS, cells were observed and photomicrographs were taken using a DMi 6000-B fluorescence microscope (Leica). Images were analysed using Image-Pro® Plus 6.0.0.260 and integral optical density (IOD) was measured to evaluate PPAR- γ concentration.

Data analysis

All experiments were repeated a minimum of three times and representative data are presented as mean $\pm$ SD. ANOVA was used to analyse difference within groups in all assays. To specify significant difference between experimental groups and the control, Dunnett *t*-test was conducted. To determine effectiveness of different DAPT concentrations, data were also analysed using the LSD *t*-test. $P < 0.05$ shows significant differences in all *t*-tests.

Results

DAPT inhibited Notch-2, Hes-1 and DLK-1/Pref-1 transcription dose dependently

To evaluate the inhibitory effect of DAPT on Notch signalling, transcription level of Hes-1 and Hey-1 was

detected in mASCs, before differentiation. After 3 days treatment in gradient concentrations of DAPT (1, 2, 5 and 10 μ M), mRNA level of Hes-1 reduced to $76.8 \pm 8.2\%$ of its previous value (1 μ M DAPT), $60.3 \pm 11.4\%$ (2 μ M DAPT), $31.2 \pm 9.1\%$ (5 μ M DAPT) and $27.0 \pm 4.3\%$ (10 μ M DAPT), with significant differences from control values ($P < 0.05$, Dunnett *t*-test, $n = 9$). Hey-1 mRNA was moderately depressed by DAPT in DAPT groups at the same time, although without significant difference in 1 and 2 μ M DAPT groups from controls ($P > 0.05$, Dunnett *t*-test). Furthermore, DLK-1/Pref-1 transcription was significantly depressed in all DAPT groups, $64.9 \pm 10.5\%$ (1 μ M DAPT), $53.6 \pm 5.7\%$ (2 μ M DAPT), $37.6 \pm 9.3\%$ (5 μ M DAPT) and $40.4 \pm 5.3\%$ (10 μ M DAPT) of controls respectively (all $P < 0.05$, Dunnett *t*-test) (Fig. 1a). Interestingly, Notch-2 transcription was also inhibited by DAPT after 3 days treatment (Fig. 1a), reducing to $83.1 \pm 8.0\%$ (1 μ M DAPT), $50.6 \pm 12.0\%$ (2 μ M DAPT), $36.5 \pm 4.3\%$ (5 μ M DAPT) and $28.3 \pm 6.9\%$ (10 μ M DAPT) respectively ($P < 0.05$, Dunnett *t*-test); Notch-1, -3 and -4 were not significantly inhibited by DAPT (Fig. 1b). As a whole, inhibition effectiveness for Hes-1, Notch-2 and DLK-1/Pref-1 was DAPT dose-dependent ($P < 0.05$, LSD *t*-test).

DAPT promoted adipocyte differentiation and accumulation of lipid droplets

After 3 days treatment with DAPT (5, 10 μ M), mASCs were cultured in 24-well plates in insulin-containing cocktail DM. At 48, 72 and 96 h differentiation, oil red-O staining was performed (Fig. 2a) to analyse adipogenesis. At early time points (48 and 72 h), morphological transformation of mASCs was observed. Cells were found changed to having more spherical shapes initially, with any processes contracted. Next after this, large numbers of small lipid droplets appeared in cells, throughout the cytoplasm. At 48 h, oil red-O positively stained adipocytes were evident in 16.67% cases of control cells, 33.33% in the 5 μ M DAPT group and 66.67% in the 10 μ M DAPT group. At 72 h, level of adipogenesis rose to 33.33% in the control group, 50% in the 5 μ M DAPT-treated group and 83.33% in the 10 μ M DAPT-treated group, ultimately reaching 66.67%, 100% and 100% respectively on day 4. This strongly suggests that DAPT could significantly advance differentiation and the process of adipogenesis. Furthermore, image analysis of lipid droplet formation was evaluated for the groups. Average droplet densities were obtained for each group on day 2, 3 and 4 (Fig. 2b). The lipid droplets in the 10 and 5 μ M DAPT groups were significantly more concentrated than in the control at each time point ($P < 0.05$, by Dunnett *t*-test, $n = 10$).

Figure 1. DAPT (1, 2, 5 and 10 μM) treatment (3 days) inhibits transcription of Notch-2, Hes-1, Hey-1 and DLK-1/Pref-1 in mASCs; 0.5% DMSO was used as control. (a) Transcription of Hes-1 and DLK-1/Pref-1 was simultaneously reduced by DAPT in a dose-dependent manner within 1–5 and 10 μM respectively ($^{*}P < 0.05$). (b) Notch-2 mRNA was dose-dependently depressed by DAPT of 1–5 μM ($^{*}P < 0.05$), while Notch-1, -3 and -4 were not significantly inhibited ($^{*}P > 0.05$). * Significantly different from control level and specified DAPT group respectively ($P < 0.05$ by * Dunnett t -test and * LSD t -test, $n = 9$). Transcription level of genes was analysed using real-time PCR as described in the Materials and methods section.

DAPT promoted PPAR- γ , and inhibited DLK-1/Pref-1, Notch-2, Hes-1 and Hey-1 transcription after adipogenic induction

Three days after induction of differentiation, PPAR- γ 2, DLK-1/Pref-1, Notch-2, Hes-1 and Hey-1 mRNAs were detected using real-time PCR. In the DAPT groups, there were 2.34 ± 0.12 (5 μM DAPT) and 1.75 ± 0.31 (10 μM DAPT)-fold increases in PPAR- γ 2 transcription ($P < 0.05$, Dunnett t -test, $n = 9$) (Fig. 3). However, transcription of the gene coding for Acrp was similar to that of the control at 1.03 ± 0.153 (5 μM DAPT) and 0.9 ± 0.206 (10 μM DAPT) ($P > 0.05$, Dunnett t -test). In line with the advanced appearance of lipid droplets in DAPT pre-treated cases, DLK-1/Pref-1 transcription was found continuously depressed to $30.5 \pm 9.1\%$ (5 μM DAPT) and $81.0 \pm 17.2\%$ (10 μM DAPT) in DAPT groups respectively ($P < 0.05$, Dunnett t -test). Notch-2 and Hes-1 transcription was maintained at lower levels, with $43.0 \pm 8.5\%$ (5 μM DAPT) and $49.2 \pm 12.7\%$ (10 μM DAPT) for Notch-2 ($P < 0.05$), and $30.4 \pm 5.0\%$ (5 μM DAPT) and $41.2 \pm 7.9\%$ (10 μM DAPT) for Hes-1 ($P < 0.05$, Dunnett t -test) respectively. However, the further target gene, Hey-1, did not decline as sharply as Hes-1, $85.1 \pm 9.1\%$ (5 μM DAPT) ($P > 0.05$, Dunnett t -test) and $68.5 \pm 7.2\%$ (10 μM DAPT) ($P < 0.05$, Dunnett t -test) of that in the control. Western blot analysis also demonstrated that DLK-1/Pref-1 protein expression was depressed 0.76 and 0.32 fold ($P < 0.05$, Dunnett t -test) in DAPT groups (Fig. 4).

DAPT promoted PPAR- γ protein expression after 4 days adipogenic induction

PPAR- γ is commonly activated by dephosphorylation in the cytoplasm. Dephosphorylated PPAR- γ moves into nuclei and functions as an intranuclear transcriptional factor (36,37). In this study, expression level, dephosphorylation and phosphorylation states, and intracellular location of PPAR- γ were investigated using Western blot and IF assays, in differentiated mASCs. We demonstrated that after 4 days, dephosphorylated PPAR- γ was promoted 1.74 (5 μM DAPT) and 2.48 (10 μM DAPT)-fold ($P < 0.05$, Dunnett t -test, $n = 6$) in two DAPT groups. Under the same conditions, phosphorylated PPAR- γ expression increased 1.49 (5 μM DAPT) and 1.86 (10 μM DAPT) fold (Fig. 4) ($P < 0.05$, Dunnett t -test). Furthermore, to localize PPAR- γ protein after 4 days differentiation, IF was conducted with anti-de-PPAR- γ (Fig. 5a) and anti-ph-PPAR- γ (Fig. 5b) antibodies respectively. We observed that de-PPAR- γ was condensed in nuclei, with significantly higher IOD in DAPT groups ($2.415 \times 10^{-2} \pm 4.31 \times 10^{-3}$ and $4.015 \times 10^{-2} \pm 5.89 \times 10^{-3}$ for 5 and 10 μM DAPT groups respectively) than in controls ($1.02 \times 10^{-2} \pm 2.91 \times 10^{-3}$) ($P < 0.05$, Dunnett t -test) (Fig. 5c). According to analysis of IF staining images, concentration of de-PPAR- γ in nuclei corresponded positively to dose of DAPT 4 days after induction ($P < 0.05$, LSD t -test, $n = 6$). IOD of ph-PPAR- γ protein was also found to be increased but without significant difference between two DAPT concentration groups ($P > 0.05$, Dunnett t -test) (Fig. 5c).

Figure 2. DAPT groups significantly promoted adipogenic ability. (a) At 48, 72 and 96 h in differentiated mASCs, oil red-O staining was carried out to investigate the adipogenic process. (b) Average area (pixels) of droplets per cell was measured at every checkpoint and adipogenic ability of each group was presented. This demonstrates that DAPT pre-treatment promoted adipogenesis dose-dependently between 5 and 10 μM ($\#$, $^*P < 0.05$). $\#$, * Significantly different from control and specified DAPT group respectively ($P < 0.05$ by $\#$ Dunnett *t*-test, and * LSD *t*-test, $n = 10$).

Figure 3. Three days after adipogenic induction, DAPT pre-treatment increased transcription of PPAR- γ 2 ($\#P < 0.05$) and retained depression of Notch-2, DLK-1/Pref-1 and Hes-1 on lower levels than controls ($\#P < 0.05$). Surprisingly, Acrp mRNA level was not up-regulated as had been predicted. Hey-1 declined in the 10 μM DAPT group ($\#P < 0.05$). Inhibition effectiveness for DLK-1/Pref-1 and Hes-1 was dose-dependent on DAPT of 5–10 μM . $\#$, * Significantly different from control and specified DAPT group respectively ($P < 0.05$ by $\#$ Dunnett *t*-test, and * LSD *t*-test, $n = 9$).

Discussion

In this study, we demonstrate that DAPT pre-treatment promotes adipogenesis and early differentiation of mASCs *in vitro* (on days 2–4). Simultaneously, 5 and 10 μM DAPT significantly inhibited mRNA expression of Notch-

2, Hes-1 and DLK-1/Pref-1. As a critical marker of adipocyte differentiation, PPAR- γ was significantly higher at both transcription and expression levels in two DAPT groups at the early differentiation stage (days 3 and 4). Inhibition of DLK-1/Pref-1 at transcription level was also observed in DAPT pre-treatment groups. Moreover, loss

Figure 4. DAPT promoted protein expression of de-PPAR- γ , ph-PPAR- γ , and inhibited DLK-1/Pref-1 in differentiated mASCs, after 4 days of induction. (a) Western blot assay was performed to analyse protein expression. (b) Adj. Volume (INT*mm², mean $\pm$ SD) of each protein measured using Quantity One 4.6.2, presented above. (c) Relative expression level of protein was obtained by normalization with β -actin. It demonstrates that de-PPAR- γ and ph-PPAR- γ proteins were up-regulated by DAPT, dose-dependently within 5–10 μ M ([#] P < 0.05), while DLK-1/Pref-1 expression remained at lower levels than controls ([#] P < 0.05). [#]Significantly different from control and specified DAPT group respectively (P < 0.05 by [#]Dunnett t -test, and *LSD t -test, n = 3).

of DLK-1/Pref-1 persisted to day 4 after adipogenic induction. The mASCs did not differentiate when continuously cultured in DAPT pre-treatment media (without

moving them to differentiation media). Taken together, these findings suggest that blocking Notch signalling enhanced adipocyte differentiation of mASCs via promotion of PPAR- γ and depression of DLK-1/Pref-1 at the early stages, likely to be through the Notch-2-Hes-1 pathway.

Notch signalling is a critical transcriptional factor in regulation of differentiation and proliferation of stem cells. γ -secretase targets a range of signalling pathways and plays an important role in regulating Notch signalling, which in turn, is involved in developmental decisions of a diverse range of progenitor cells (38). To date, neither their roles in MSC cell fate decisions have been explored completely nor has their effect on adipogenesis of mASCs. It has been reported that GSI has an inhibitory effect on chondrogenesis of bone marrow-derived hMSCs, by reducing expression of Hes-1 and Hey-1, in a DAPT concentration-dependent manner (39). Furthermore, the adipogenic effect can be achieved by addition of DAPT and 100 nM dexamethasone, which supports our observations. In our study, 1 μ M of dexamethasone was added to the adipogenic cocktail. In contrast, previous studies have not found further enhancement of adipogenesis when DAPT was added to hMSCs differentiating in adipogenic media. One explanation for this might be differences in MSCs of different species and derivations in sensitivity of DAPT. Another possible explanation is that differences in early adipogenesis that we demonstrate in this study have not been appreciated previously.

Our results are also supported by evidence that constitutive expression of Notch blocks adipogenesis of 3T3-L1 pre-adipocytes (23). The process of adipogenesis involves down-regulation of the gene encoding Hes-1, which is now considered to function both as a repressor and as an activator. However, promoter analyses of up-regulated and down-regulated genes in 3T3 pre-adipocytes has indicated that Notch most likely blocks adipogenesis through induction of Hes-1 homodimers, which repress transcription of key target genes (23). Furthermore, this effect could be mimicked by exposing the 3T3-L1 to Jagged-1, the canonical ligand of Notch receptors. This block was associated with complete loss of C/EBP- α and PPAR- γ (22). This is supported by the finding in our study that Hes-1 transcription in mASCs was depressed after 3 days of DAPT treatment and this depression could be sustained during subsequent adipocyte differentiation in absence of DAPT, for at least 4 days. It seems that Hey-1 was also inhibited by DAPT but not as dramatically as Hes-1, especially during early differentiation in the absence of DAPT. As neither Hey-1 nor Hes-1 is the only target of the Notch signalling pathway, inhibition of DAPT cannot be universal for all target genes of the Notch signalling pathway.

Figure 5. Immunofluorescence staining of de-PPAR-γ and ph-PPAR-γ in differentiated mASCs after 4 days adipogenesis (magnification 10×40). (a) de-PPAR-γ proteins were localizing and condensed in nuclei of differentiated mASCs in two DAPT groups. Also, de-PPAR-γ was detected at higher density and intensity in the 10 μM cases than the 5 μM cases. (b) Accumulation patterns of ph-PPAR-γ in two DAPT groups were quite different from controls. In the control cases, ph-PPAR-γ was concentrated in nuclei, while in the 5 μM DAPT cases, it had accumulated in the cytoplasmic compartment; in 10 μM DAPT cases, it was positive in both nuclei and cytoplasm. (c) IOD of de-PPAR-γ and ph-PPAR-γ was significantly higher than control ($^{#}P < 0.05$). Furthermore, IOD of de-PPAR-γ positively responded to dose of DAPT ($^{*}P < 0.05$). (c) * Significantly different from control and specified DAPT group respectively ($P < 0.05$, by $^{#}$ Dunnnett *t*-test, and * LSD *t*-test, $n = 6$).

In vitro adipocyte differentiation is coordinately activated by two transcription factors, PPAR-γ and CCAAT enhancer binding protein alpha (C/EBP-α), but it is inhibited by DLK-1/Pref-1 (40). We were interested in the relationship between GSI and down-regulation of DLK-1/Pref-1, which is the part of the Notch signalling pathway that controls various developmental processes (41). The inhibitory role for DLK-1/Pref-1 in adipogenesis has been suggested in a human model (42). Previous studies have reported down-regulation of DLK-1/Pref-1 by Notch signalling and Hes-1. Therefore, new downstream Notch-Hes-1 targeting DLK-1/Pref-1 in adipocyte development may exist. According to this theory, we would anticipate restoration of DLK-1/Pref-1 after inhibition of the Notch-Hes-1 signalling pathway. In contrast, we found down-regulation of DLK-1/Pref-1 with repression of the Notch-2-Hes-1 pathway by DAPT, before and after adipocyte differentiation. In addition, repressing level of these two genes *Notch-2* and *Hes-1* was similar. We hypothesize that: (i) the down-regulating effect of Hes-1 on DLK-1/Pref-1 is context-dependent, partly relating to the background of Notch signalling; (ii) DLK-1/Pref-1, as an EGF-like repeat containing protein, is likely to be inhibited by mechanisms triggered by GSI directly including, but not limited to, the Notch signalling pathway. Further investigation is necessary to reveal the regulation mechanism of DLK-1/Pref-1 by Notch or other transcriptional factors.

PPAR-γ is known to activate transcription of its target genes in adipogenic pathways (43–45). Several authors have reported that expression of PPAR-γ is turned on early in adipogenesis, following increase in transcription factors C/EBP-β and C/EBP-δ, and leads to an increase in C/EBP-α (46,47). In this study, we have demonstrated that transcription of PPAR-γ2, an isoform of murine PPAR-γ (48), was induced dose-dependently by DAPT. We have shown that during the process of MSC differentiation, PPAR-γ was activated by a process of dephosphory-

lation, and subsequently translocated to nuclei where it bound to target DNA sequences and have its effects (49). To analyse the role of DAPT in activation of PPAR- γ in adipogenesis, the active forms of PPAR- γ (de-PPAR- γ) and ph-PPAR- γ were assayed. After differentiation began, Western blot analysis was performed and it was demonstrated that the expression level of de-PPAR- γ was promoted significantly in DAPT pre-treated cases, dose-dependently. Furthermore, IF analysis demonstrated that in differentiated mASCs (on day 4) of 5 and 10 μ M DAPT groups, de-PPAR- γ pellets accumulated and condensed in nuclei with dramatically higher intensity than in controls. We also found that concentration of DAPT determined the expression level of de-PPAR- γ in nuclei. Our latest finding suggests that nuclear translocation and DNA-binding of de-PPAR- γ may be enhanced by DAPT. There are two reasonable interpretations for promotion of de-PPAR- γ : (i) activation of PPAR- γ dephosphorylation-related signaling pathway by DAPT; (ii) or level of de-PPAR- γ was simply elevated with increasing translation of PPAR- γ mRNA. Indeed, ph-PPAR- γ was also promoted with de-PPAR- γ , on a protein level, by DAPT in this study. However, the ratio of de-PPAR- γ versus ph-PPAR- γ was significantly promoted, with 0.392 in controls, 0.458 in the 5 μ M DAPT group and 0.525 in 10 μ M DAPT group. This suggests that a mechanism involving activation of PPAR- γ dephosphorylation may somehow be triggered by DAPT. More details of this potential mechanism remain to be elucidated. Taken together, increased de-PPAR- γ and its early condensation in nuclei provide a reasonable explanation for promoted adipogenesis process in DAPT cases.

In conclusion, our study suggests that adipogenesis can be enhanced by coordinated regulation of Notch and PPAR- γ . In brief, DAPT comprehensively inhibited the Notch signalling pathway and consequently influenced Hes-1 expression, which may directly or indirectly reduce DLK-1/Pref-1, an inhibitor of adipogenic transcription activator PPAR- γ . Continuous repression of DLK-1/Pref-1 and therefore activation of PPAR- γ dephosphorylation, together promote adipogenesis of mASCs.

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