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Original Paper

Inhibition of Notch Signaling Promotes the Adipogenic Differentiation of Mesenchymal Stem Cells Through Autophagy Activation and PTEN-PI3K/AKT/mTOR Pathway

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Key Words
Notch signaling • Mesenchymal stem cells • Adipogenesis • Autophagy • Phosphoinositide 3-kinase • Akt • mTOR

Abstract
Background: The Notch signaling pathway is implicated in a broad range of developmental processes, including cell fate decisions. This study was designed to determine the role of Notch signaling in adipogenic differentiation of human bone marrow derived MSCs (BM-MSCs). ***Methods:*** The Notch signaling was inhibited by the γ-secretase inhibitor N-[N-(3,5-difluor- ophenacetyl-L-alanyl)]-S-phenylglycine t-butylester (DAPT). The markers involving adipogenic differentiation of MSCs, the relative pathway PTEN-PI3K/Akt/mTOR and autophagy activation were then analyzed. Furthermore, the autophagy inhibitor chloroquine (CQ) and 3-methyladenine (3-MA) were used to study the role of autophagy in the DAPT-induced the adipogenic differentiation of MSCs. ***Results:*** We first confirmed the down-regulation of Notch gene expression during MSCs adipocyte differentiation, and showed that the inhibition of Notch signaling significantly enhanced adipogenic differentiation of MSCs. Furthermore, Notch inhibitor DAPT induced early autophagy by acting on PTEN-PI3K/Akt/mTOR pathway. The autophagy inhibitor CQ and 3-MA dramatically abolished the effects of DAPT-induced autophagy and adipogenic differentiation of MSCs. ***Conclusion:*** Our results indicate that inhibition of Notch signaling could promote MSCs adipogenesis mediated by autophagy involving PTEN-PI3K/Akt/mTOR pathway. Notch signaling could be a novel target for regulating the adipogenic differentiation of MSCs.

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Introduction

Human adult mesenchymal stem cells (MSCs) are a population of stromal cells present in bone marrow and many other tissues, which can differentiate into various cell types including osteoblasts, chondrocytes and adipocytes [1-3]. During the process of aging or in some pathological conditions, including aplastic anemia, multiple myeloma, and anorexia nervosa, the cell lineage commitment of MSCs shift to adipocytes, especially in bone marrow [4-7]. Over adipogenesis will usually lead to obesity, a major risk factor for certain chronic diseases, such as cardiovascular disease and type 2 diabetes [8, 9]. As adipocytes can be traced back to a common precursor MSCs, the precise molecular mechanism governing the adipogenic differentiation of MSCs becomes an important issue in this field.

Previous studies have shown that the adipogenic differentiation of MSCs is a complicated physiological process that involves a large number of molecular events. Various reports have demonstrated that under defined conditions *in vitro*, MSCs can differentiate into adipocytes depending on the presence of some particular cytokines (e.g, IFN-γ, TNF-α, TGF-β, and BMPs) and the extracellular matrix [10, 11]. It's also documented that the microRNAs (miRNAs) exert essential regulatory functions in adipocyte development [12]. However, the cell-intrinsic mechanism of this cell lineage commitment switch is poorly understood. Autophagy is a multifaceted process to degrade intracellular components by forming autophagosomes, which then fuse with lysosomes to form autolysosomes [13, 14]. Growing evidence suggests that autophagy is implicated in adipogenesis [15] and adipogenic differentiation [16].

Notch signaling is a developmental pathway that regulates several fundamental cellular processes including cell fate and differentiation [17, 18]. Four transmembrane Notch receptors (Notch-1, -2, -3, -4), the five canonical ligands (DLL1, DLL3, DLL4, JAGGED1,and JAGGED2), and the two non-canonical ligands (DLK1 and DLK2) constitute the Notch system [19]. Notch activation occurs when the ligand engagement renders the Notch intracellular domain to be cleaved from the cell membrane and translocate into the nucleus, where it forms a transcriptional activator complex with the CSL family of transcription factors (C-promoter binding factor 1/recombination signal sequence binding protein Jκ, suppressor of hairless, and Lag-1) and modulates the expressions of target genes such as Hes family of transcription factors members and p21 [20, 21]. The final cleavage that untethered Notch receptors from the cell membrane is mediated by the enzymatic action of γ-secretase and can be blocked with pharmacological inhibitors. Follow-up studies demonstrated that γ-Secretase inhibitors (DAPT) successfully prevent the final enzymatic step required for Notch cleavage and activation and can block Notch signaling *in vitro* and *in vivo* [18, 22]. Moreover, recent evidence has found that the components of the Notch pathway are expressed and activated in T-cell development, megakaryocyte development, marginal zone B cells development and HSC differentiation [23-26]. However, little is known about the effect of the Notch signaling pathway on human mesenchymal stem cell differentiation.

The present study was designed to determine the role of Notch signaling involved in human bone marrow derived MSCs (BM-MSCs) in terms of adipogenic differentiation and further disclose the potential molecular mechanism. The data reveal that inhibition of Notch signaling promotes BM-MSCs adipogenesis mediated by autophagy activation involving PTEN-PI3K/Akt/mTOR pathway.

Materials and Methods

Reagents and antibodies

3-methyladenine (3-MA), chloroquine (CQ) and DAPT were purchased from Sigma (St. Louis, MO, USA). The following antibodies were from Cell Signaling Technology (Beverly, MA, USA): anti-β-actin, anti-LC3-II/I, anti-mTOR, anti-phosphorylation of mTOR (p-mTOR), anti-Akt, anti-phosphorylation of Akt (p-Akt). The anti-bodies of anti-PI3K and anti-phosphorylation of anti-PI3K were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-beclin1 was obtained from Pepro Tech (Proteintech, USA).

Cell culture and differentiation

This study was approved by the Institutional Review Board of Chinese Academy of Medical Sciences and Peking Union Medical College. Bone marrow samples were obtained from donors with written informed consent. The isolation and expansion of BM-MSCs were performed as described previously described [4]. BM-MSCs were maintained in DMEM/F-12 medium (Gibco) containing 10% fetal bovine serum (FBS, HyClone), 2mM glutamine, 100U/ml penicillin-streptomycin, and 10ng/ml epidermal growth factor (EGF; Pepro Tech), incubated at 37°C and a 5% CO_2 humid atmosphere, cells from passage 3-5 were used for the following experiments. DAPT was dissolved in DMSO, and was freshly diluted to the desired concentration with culture medium. The final concentration of DMSO was at 0.05% (v/v). The DMSO group cells received the vehicle only.

For osteogenic induction, basal medium was replaced with differentiation medium consisting of IMDM supplemented with 0.1μM dexamethasone, 200μM ascorbic acid-2-phosphate, 10mM β-glycerophosphate, and 10% FBS. For adipogenic induction, medium consisted of IMDM supplemented with 1μM dexamethasone, 10μM insulin, 0.5mM isobutylmethylxanthine (IBMX), 60μM indomethacin and 10% FBS. The mediums were replaced three times a week.

Oil Red O staining

After adipogenic induction, BM-MSCs were stained with Oil red O. Cells were washed twice with PBS and fixed in 4% paraformaldehyde at room temperature for 30 min. Then, the cells were stained with an Oil red O solution (60% Oil Red O stock solution and 40% H_2O) for 15 min and then washed three times with PBS. The stained lipid drops were observed using an inverted microscope.

Quantitative real-time PCR

Total RNA was extracted from cells using TRIZOL® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech China Co.,Ltd. Beijing). Real-time PCR was carried out with an ABI 7500 real-time PCR system using SYBR Green mix (ABI, USA). The fold-change in gene expression relative to the control was calculated by $2^{-(\Delta\Delta CT)}$. The experiments were repeated 3 times and every sample was performed in triplicate. Primers used in the experiment were listed in Table 1.

Table 1. Sequence of primers used in real-time PCR

Genes	Forward primer	Reverse primer
NOTCH 1	5'-TCAGCGGGATCCACTGTGAG-3'	5'-ACACAGGCAGGTGAACGACTTG-3'
NOTCH 2	5'-ACAGTTGTGTCTGCTCACCAGGAT-3'	5'-GCGGAAACCATTCACACCGTTGAT-3'
NOTCH 3	5'-GATGGCATGGATGTCAATGTTCGT-3'	5'-TGCCTCATCCTCTTCAGTTGGCAT-3'
NOTCH 4	5'-TCAAACAGAGGTGGATGAGTGCCT-3'	5'-AGTTGGCCTTGTCTTTCTGGTCCT-3'
JAGGED1	5'-ACTGCTCACACCTGAAAGACCACT-3	5'-AGGACCACAGACGTTGGAGGAAAT-3'
JAGGED2	5'-TGCTGTGGAGGTGGCTATGTCT-3'	5'-TGTTTCCACCTTGACCTCGGT-3'
DLL1	5'-TGTGACGAGTGTATCCGCTATCCA-3'	5'-AGGGCTTATGGTGTGTGCAGTAGT-3'
DLL3	5'-TCCCGGATGCACTCAACAACCTAA-3'	5'- TTCAGGGCGATTCCAATCTACGGA-3'
DLL4	5'-ACTGCGAGAAGAAAGTGGACAGGT-3'	5'-ACATGAGCCCATTCTCCAGGTCAT-3'
DLK1	5'-TGGACGATGGCCTCTATGAATGCT-3'	5'-TCTCGCAGAAATTGCCTGAGAAGC-3'
DLK2	5'-CCATTGTGTGTGCTTACCAGGCTT-3'	5'-CGCATCAGGCAGTCATCCACATTT-3'
HES1	5'-GTCAACACGACACCGGATAAACCA-3'	5'-TTTCCAGAATGTCCGCCTTCTCCA-3'
HEY1	5'-AGAGTGCGGACGAGAATGGAAACT- 3'	5'-ACCAGCCTTCTCAGCTCAGACAAA-3'
HEY2	5'-AGATGCTTCAGGCAACAGGG-3'	5'-CAAGAGCGTGTGCGTCAAAG-3'
PPARγ	5'-AGCCTCATGAAGAGCCTTCCA-3'	5'-ACCCTTGCATCCTTCACAAGC-3'
C/EBPα	5'-GAAGTTGGTGGAGCTGTCGG-3'	5'-TGAGGTATGGGTCGTTGCTGA-3'
GAPDH	5'- CGGATTTGGTCGTATTGGGC -3'	5'- CTTCCCGTTCTCAGCCTTG -3'

Western blot analysis

BM-MSCs were collected, washed, and lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology, China) supplemented with PMSF (Invitrogen). Total protein was extracted and the protein concentration of the lysates was quantified by the BCA protein assay kit (Pierce). Protein (30μg) was denatured, separated by SDS-PAGE electrophoresis and transferred to a PVDF membrane. The transferred membranes were blocked using 5% BSA in TBST and incubated with appropriate primary antibodies overnight and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at 1:2,000 dilution for 2 h. Bands were visualized using enhanced chemiluminescence (ECL, Thermo Scientific, CA) detection reagents and scanned images were quantified using Image J software. The ratio of target gene to β-actin was used for semi-quantification and comparison among different groups.

Immunofluorescence

After treated according to protocol, cells were fixed in 4% paraformaldehyde, permeabilized in 0.25% Triton, and blocked with Blocking Buffer (1×PBS + 5% normal serum + 0.3% Triton™X-100). Then incubated overnight with rabbit polyclonal anti-LC3B antibody (CST, Beverly, MA, USA), after this incubation, cells were stained for 1 h with goat anti-rabbit IgG peroxidase antibody (Sigma, St Louis, MO) counterstained with DAPI (Sigma, St Louis, MO), the cells were subsequently viewed by fluorescence microscopy.

Statistical analysis

The data were presented as mean ± SD, comparisons of quantitative data were analyzed using Student's t-test between two groups or by one-way ANOVA followed by the Newman–Keuls test for multiple groups. Statistical significance was set at $P < 0.05$. All experiments were performed at least three times.

Results

Basal characterization of BM-MSCs

Elongated spindle-shaped or rhomboid BM-MSCs grew colonially and displayed a rather homogeneous confluent population (Fig. 1A). The cells possessed multipotent differentiation potential, as they could be induced into osteoblasts and adipocytes under standard *in vitro* differential conditions (Fig. 1B and C). Cells showed positive expression of CD44, CD73, CD90, CD105, CD166, but negative for CD14, CD19, CD34, CD45, HLA-DR (Fig. 1D).

Notch Signaling is downregulated during adipocyte differentiation

To understand the role of Notch signaling during adipocyte differentiation, we investigated the modulation of Notch signaling during adipocyte differentiation through the gene expression of Notch receptors, ligands and the transcription factors by real-time PCR. As show in Fig. 2A, adipogenic differentiation of BM-MSCs was associated with a lower Notch receptors, Notch1, Notch2 and Notch3 than in undifferentiated group. Notch4 expression appeared to be also decrease, although their decrease was not significant. The expression of Notch canonical ligands DLL1, DLL3, DLL4, and the two non-canonical ligands (DLK1and DLK2) were significantly decreased compared to undifferentiated group, whereas there were no significant changes in JAGGED1 and JAGGED2 gene expression (Fig. 2B and C). The gene expressions of the downstream effector genes activated by Notch signaling, HES1, HEY1, and HEY2 were also analyzed. Only HEY1 gene expression was significantly decreased compared to the undifferentiated group, the expression levels of HES1 and HEY2 were also decreased, but they did not reach statistical significance (Fig. 2 D). These findings suggested that Notch signaling could be potential key targets in the adipogenic differentiation of BM-MSCs.

Adipogenic differentiation of BM-MSCs induces early autophagy

To explore the role of autophagy in adipogenesis, we quantified the levels of autophagy during adipogenic differentiation of BM-MSCs, We found that during initial stages of differentiation, the conversion of LC3-I to autophagosome associated LC3-II, as a marker of autophagy, reaching its maximum at day 3 and then rapidly declined at later stages of

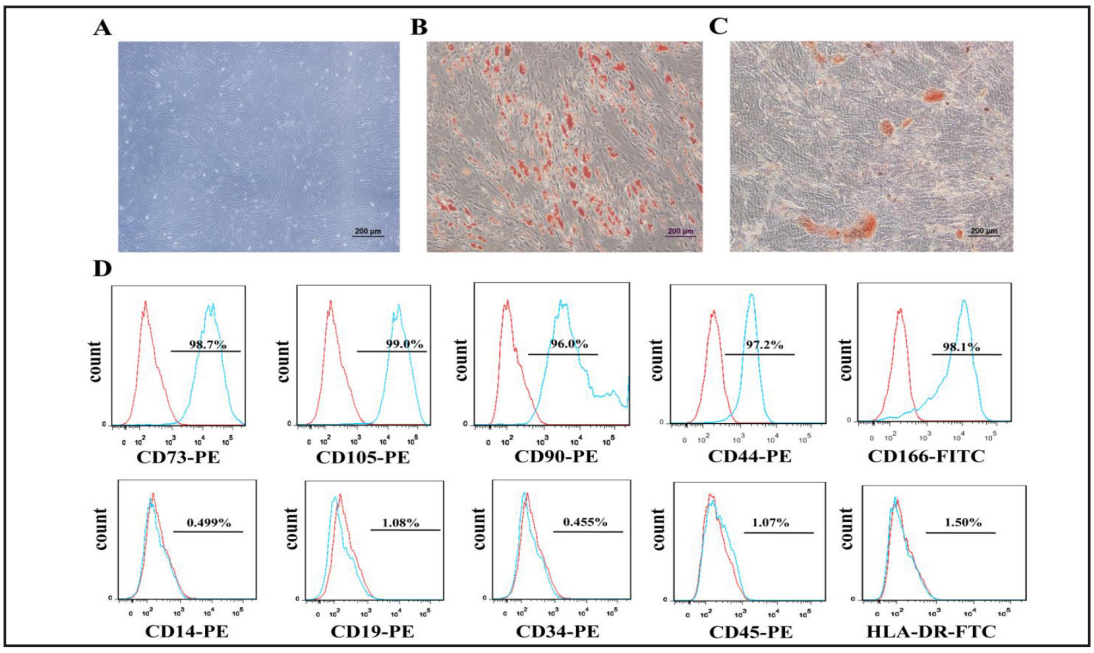

Fig. 1. Characterization of bone marrow derived mesenchymal stem cells (BM-MSC). (A) Morphology of BM-MSCs after culturing the cells for 6 days. (B) Adipocyte induction for 14 days, stained by lipid accumulation with Oil Red O, (C) osteoblast induction for 21 days, mineralized nodules were detected after Alizarin Red S staining. Scale bars = 200μm. (D) Immunophenotyping of BM-MSCs by flow cytometry.

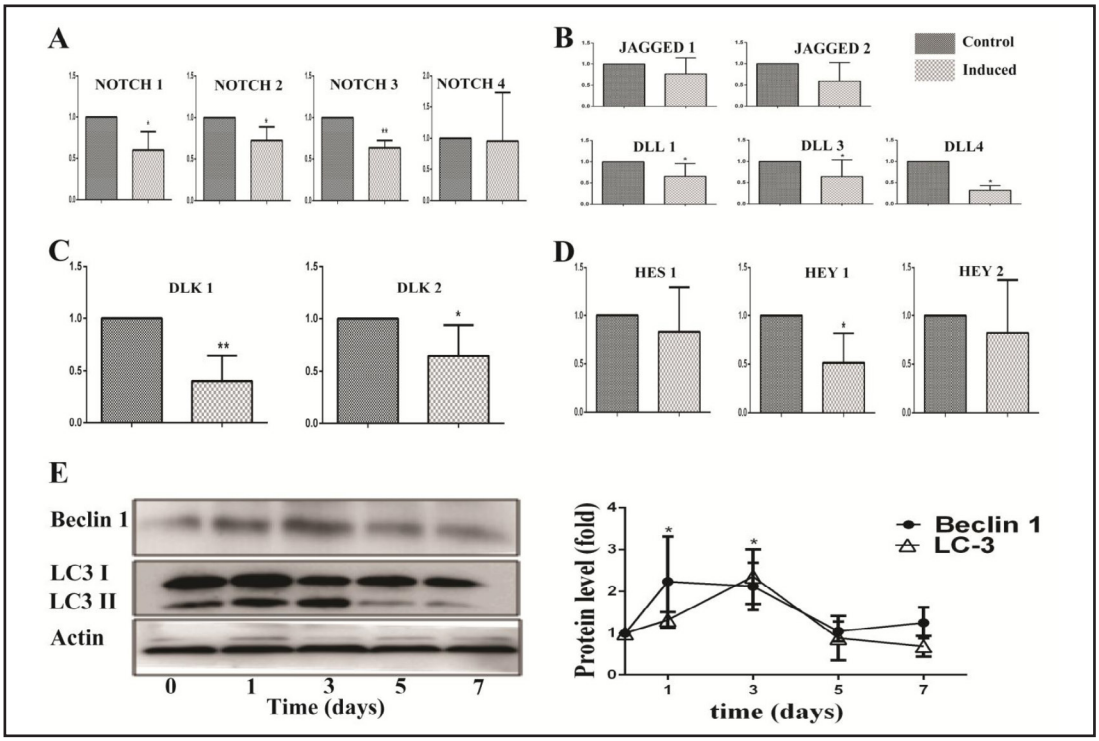

Fig. 2. The changes of Notch signaling and autophagy during adipocyte differentiation. (A) Quantitative real-time PCR was used to determine the four Notch receptor NOTCH1, NOTCH2, NOTCH3 and NOTCH4, (B) Notch canonical ligand genes, Jagged1, Jagged2, DLL1, DLL3, DLL4, (C) Notch non-canonical ligandDLK1 and DLK2. (D). Notch-dependent transcription factors, HES1, HEY2, and HEY2 relative mRNA expression levels, BM-MSCs incubated in differentiation medium for 14 days. (E) Autophagy activation was assessed by western blot at 0, 1, 3, 5, and 7 d after adipogenic induction.$^{*}p < 0.05$, $^{**}p < 0.01$.

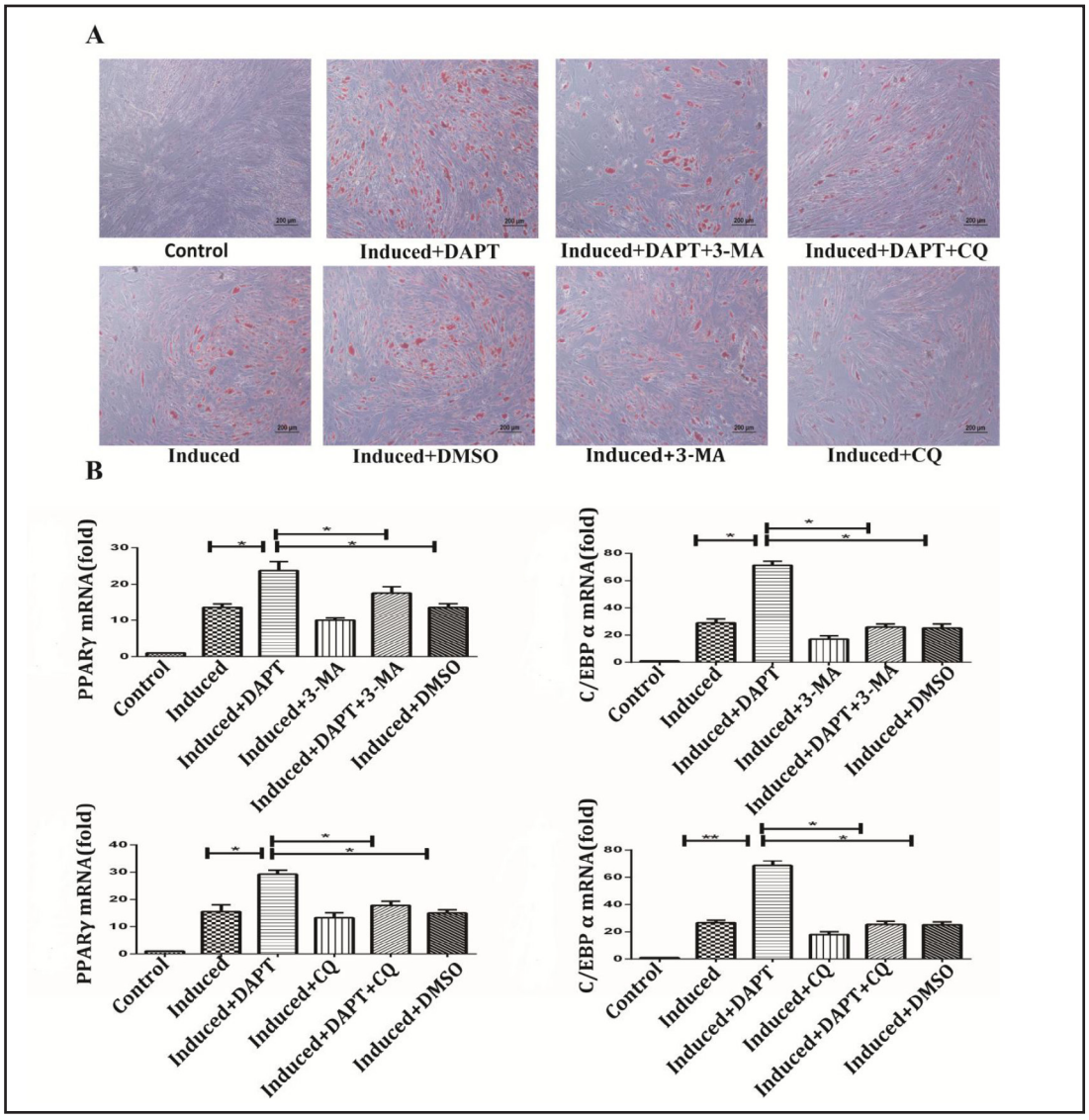

Fig. 3. DAPT promoted adipogenesis by activating autophagy. (A)BM-MSCs were cultured in control mediumor adipogenic medium, in the presence of 5μM DAPT alone, or combine with 3-MA (5mM) or CQ (20μM) for 72 h, after that the cells were subjected to Oil Red O staining (Original magnification, 200×); B. mRNA expression of PPARγ and C/EBPα were assessed by real-time PCR. *p <0.05.

differentiation. Changes in the LC3-II/LC3-I ratio were correlated with the extent of pro-autophagic protein beclin-1, which reached its maximum at day 1 and day 3 after initiation of differentiation (Fig. 2E). The results suggest that autophagy seems a key target in efficient MSC *adipogenic* differentiation and function.

DAPT promotes the adipogenic differentiation of BM-MSCs through the activation of autophagy

To evaluate the effects of Notch signaling on the adipogenic differentiation of BM-MSCs, BM-MSCs were allowed to differentiate into adipocytes in the absence or presence of Notch inhibitor DAPT (5μM) during the entire differentiation period 14 days. Following adipocyte induction, significantly increased lipid droplets (Fig. 3A) and mRNA levels of PPAR-γ and C/EBP-α (Fig. 3B) were observed in DAPT treated group in comparison with the induced group or DMSO-alone group. These results collectively demonstrate that Notch signaling is

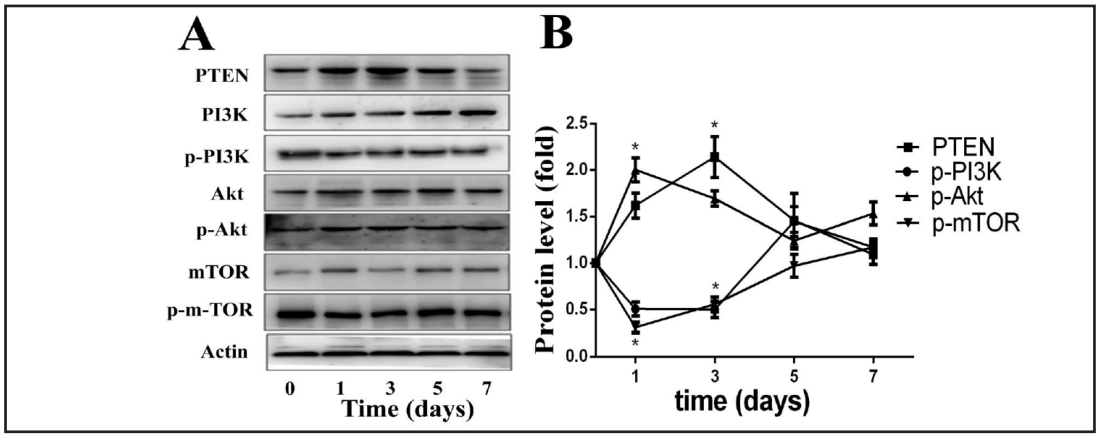

Fig. 4. Modulation of PI3K/Akt/mTOR signaling during adipogenic differentiation of BM-MSCs, the expression of PI3K/Akt/mTOR signaling pathway members was assessed by western blot at the indicated time points. The densitometry data are significantly different in comparison with day 0. $^{*}p < 0.05$.

a critical regulator of adipogenesis and inhibition of Notch signaling promotes adipogenic differentiation of BM-MSCs.

As the activation of autophagy happened after adipogenic induction, particularly during the first three days, we postulated that autophagy might play an important role during adipogenesis stage. To investigate whether the DAPT-induced adipogenesis is dependent on autophagy activation, cells were incubated with autophagy inhibitor 3-MA (5 mM) and CQ (20 μM) either alone or in combination with DAPT during adipogenic differentiation conditions for early 3 days. Adipogenesis was assessed by oil O staining and PPARγ and C/EBPα mRNA expression (Fig. 3A, B). We confirmed the addition of 3-MA or CQ to DAPT reversed the pro-adipogenic effect. Taken together, these results show that the effect of DAPT on the adipogenic differentiation of BM-MSCs is partly due to the activation of early autophagy.

Adipogenic differentiation of BM-MSCs is associated with time-dependent modulation of PI3K/Akt/mTOR signaling

To clarify the further molecular mechanism of Notch signaling and autophagy during adipogenic differentiation of BM-MSCs, we examined the expression profiles of PI3K, PTEN, Akt and mTOR at 0, 1, 3, 5, and 7 days after inducing adipogenesis. As shown in Fig. 4A, B, an inverse activation pattern was observed with p-PI3K, demonstrating an early decrease at day 1 followed by activation from day 3 onwards. This process was associated with PTEN increase and rapid phosphorylation of Akt, which reached its peak at day 3 and day 1 respectively, and then gradually declined. The decrease in p-mTOR has roughly paralleled that of p-PI3K, reaching its maximum at day 1 and remaining high during the rest of the differentiation period. These data demonstrated a complex, time-dependent modulation of PI3K/Akt/mTOR signaling during adipogenic differentiation of BM-MSCs.

DAPT induces autophagy in BM-MSCs via inhibition of PI3K/Akt/mTOR pathway

In order to check whether DAPT could induce autophagy of BM-MSCs and elaborate the underlying mechanism, BM-MSCs were treated with DAPT at 0, 2.5, 5 and 10μM for 72 h, we found that there was a low to moderate basal level of autophagy in BM-MSCs. DAPT treatment of BM-MSCs induced remarkable autophagy in a concentration-dependent manner. Incubation of the cells with DAPT at 2.5, 5.0 and 10μM significantly increased the autophagy related proteins LC3II/I and Beclin-1 respectively, compared to the control cells. Meanwhile, we also investigated the effect of DAPT treatment on PI3K/Akt/mTOR pathway expression in BM-MSCs. With increasing concentrations of DAPT, The p-PI3K/PI3K ratio was significantly decreased, indicating a clear dose dependence of PI3K decrease by DAPT. As a downstream

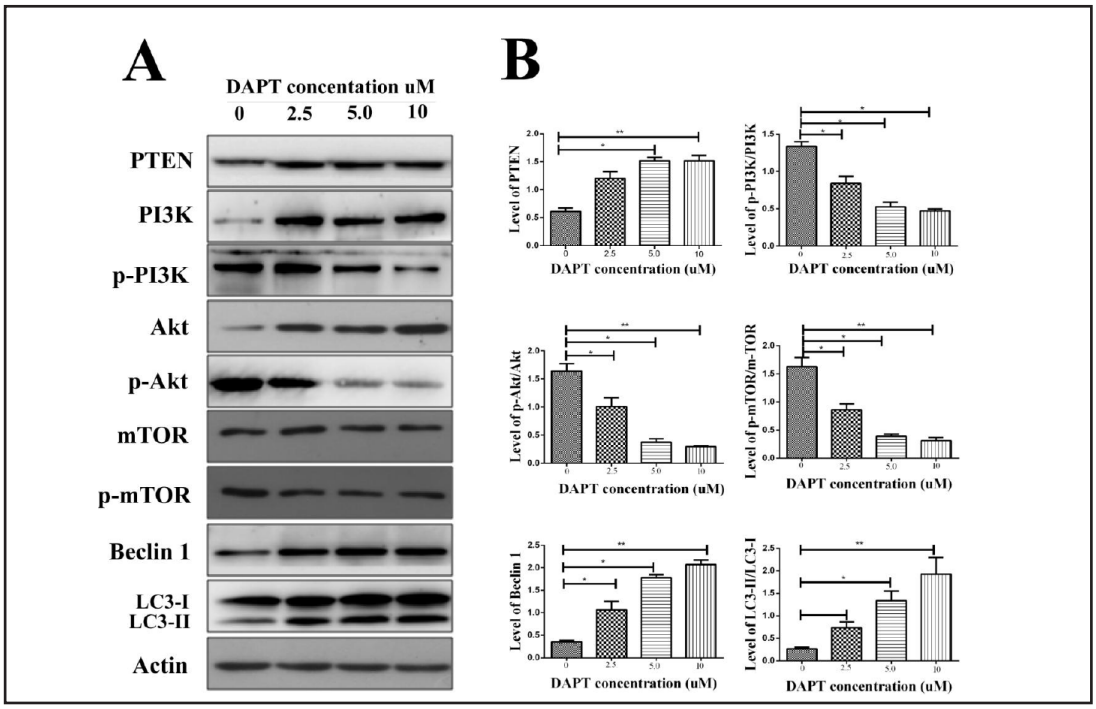

Fig. 5. Effect of DAPT treatment on the expression levels of PTEN, phosphorylated PI3K, PI3K, phosphorylated Akt, Akt, phosphorylated mTOR , mTOR, beclin-1 and LC3-I, LC3-II in BM-MSCs. (A) Representative blots of the proteins measured. (B) Levels of the proteins PTEN, Beclin 1 and ratios of phosphorylated PI3K, phosphorylated Akt, and phosphorylated mTOR over the corresponding protein without phosphorylation and LC3-II over LC3-I, β-actin was used as the internal control. $^{*}p < 0.05$, $^{**}p < 0.01$.

effector of PI3K, Akt can activate mTOR, DAPT at 2.5-10μM did not significantly affect the expression of Akt. However a reducing phosphorylation of Akt was observed, indicating a clear dose dependence of Akt phosphorylation inhibition by DAPT. The expression of PTEN, a negative regulator of the PI3K/Akt pathway, in BM-MSCs was significantly increased by DAPT at 0.5, 2.5, and 10μM. mTOR plays a key role in cell growth, autophagic cell death, and homeostasis, incubation of BM-MSCs with DAPT at 2.5-10μM, the ratio of phosphorylation of mTOR level was significantly decreased in comparison with the control cells (Fig. 5A , B).

Chloroquine and 3-methyladenine represse DAPT-induced autophagy

To identify that increased autophagosome formation by DAPT treatment was due to increased autophagic flux, BM-MSCs were treated with autophagy inhibitor CQ and 3-MA. CQ inhibits the last step in autophage induced additional accumulation of LC3-II, meanwhile 3-MA blocks the formation of autophagosomes by inhibiting the class III phosphatidylinositol 3-kinase. The addition of CQ accumulated LC3-II expression and 3-MA significantly reduced the expression of LC3-II.

As expected, compared with the control group or DMSO-alone group, autophage activity was increased in the DAPT group with a high LC3-II/LC3-I ration and beclin-1 expression. The addition of CQ accumulated LC3-II expression in BM-MSCs pretreated with DAPT and 3-MA significantly reduced the expression of LC3-II (Fig. 6A, B). The DAPT-induced abnormal expression of Beclin1 was significantly reduced by CQ and 3-MA (Fig. 6A, B). We also measured the autophagic vacuole formation by immunofluorescent staining. The results showed that addition of DAPT drastically increased the accumulation of LC3-II expression and the formation of LC3 puncta, compared to the control group. Treatment of the cells with CQ and 3-MA significantly reduced the DAPT-induced autophagy (Fig. 6C), suggesting that DAPT induced autophagy can be repressed significantly by CQ and 3-MA.

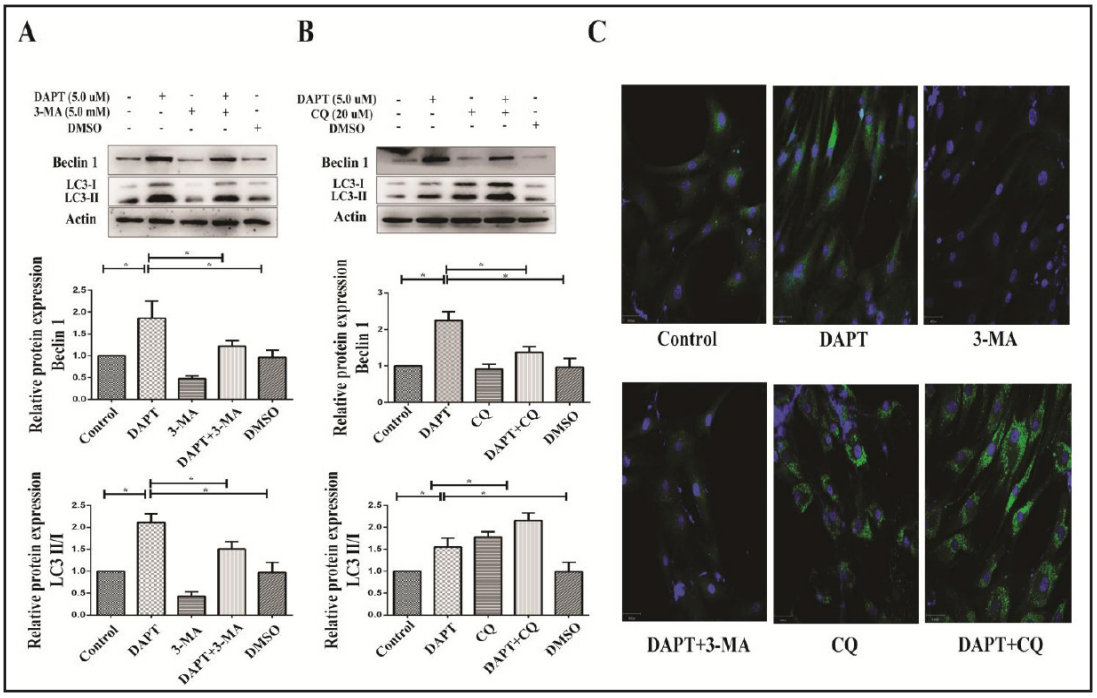

Fig. 6. Cells were treated with DAPT (5μM) alone or combination with 3-MA (5mM) and CQ (20μM) for 72 h. A. Western blot showing the effect of CQ and 3-MA on Beclin-1 and LC3II/I protein levels in DAPT treated BM-MSCs; B. LC3 protein levels were analyzed by confocal microscopy, Original magnification, 600×. $^{*}p<0.05$, $^{**}p<0.01$.

Discussion

Adipogenesis, the formation of adipose tissue, has an important impact on different biological aspects of aging, insulin sensitivity, lipid metabolism, stress response and inflammation [27]. The imbalance of adipogenesis is often associated with pathophysiological conditions, such as obesity, aging, aplastic anemia and hematological malignancies [28, 29]. The regulatory mechanisms of adipogenic differentiation of MSCs remain to be elucidated. The present study demonstrated that inhibition of Notch signaling promoted the adipogenic differentiation of BM-MSCs. In addition, the autophagy plays an important role in DAPT-induced adipogenic differentiation of BM-MSCs involving PTEN-PI3K/Akt/mTOR pathway.

The Notch signaling is highly conserved among multi-cellular organisms and has been demonstrated to participate in a broad range of developmental processes in part through the regulation of cell fate decisions [30]. To clarify the molecular mechanism of how Notch signaling mediates adipocytes differentiation, we focused on the well-known signaling pathways of the phosphatidylinositol 3-OH kinase (PI3K) and the serinehreonine kinase AKT, which are key intermediates of several cell-extrinsic signals that are involved in cell growth and survival [24]. Several lines of evidence now point to a link between the Notch and PI3K pathways via PTEN, a negative regulator of the PI3K pathway [31, 32]. Of interest several reports indicated that the Notch and PI3K signaling pathways interact at several levels and in various cellular contexts [33, 34]. One of Akt's key targets is the mTOR pathway. mTOR belongs to a family of PI3K-related kinases and is a key regulator of cell growth and metabolism. It resides in two distinct signaling complexes, TORC1 and TORC2 [35, 36]. The inhibitory function of mTOR complex 1 (mTORC1) in autophagy is well established. Therefore, mTORC1 regulates autophagy is of great importance because it may link others signals to regulation of autophagy [37]. Autophagy is essential under both physiological and pathological conditions. Basal autophagy exerts ''quality control'' function, that is, eliminating old organelles or turning over long-lived proteins, to maintain

cellular homeostasis. Autophagy has also been involved in adipogenic differentiation, since its impairment in Atg5 and Atg7 knockout mice compromises fat cell maturation [38, 39]. Furthermore, autophagy also favors adipogenic differentiation by affecting cellular shape [40]. The relationship between autophagy and adipogenesis has been characterized in various contexts, however, it has not been extensively studied in MSCs [15, 16, 41]. Therefore autophagy may involve the modulation of the Notch signaling pathway in the adipogenic differentiation of mesenchymal stem cells.

In the first part of this study, we investigated Notch signaling and autophagy alterations during adipocyte differentiation. The reduced gene expression of Notch receptors NOTCH1, NOTCH2, NOTCH3, Notch canonical ligands DLL1, DLL3, DLL4, the two non-canonical ligands DLK1, DLK2, and downstream effector HEY1 observed during adipocyte differentiation. Meanwhile, basal autophagy increased during the initial stages of adipogenic differentiation of BM-MSCs. Next, we found that when BM-MSCs were treated with Notch inhibitor DAPT for 14 days in the presence of adipogenic induction media, there was a significant increase in adipocytes as evidenced by increase in Oil Red O staining positive cells as well as a higher mRNA levels for PPARγ and C/EBPα. Meanwhile, DAPT treatment of BM-MSCs induced a remarkable autophagy in a concentration-dependent manner via inhibition of PI3K/Akt/mTOR pathway. Based on our data, we hypothesized that Notch inhibitor DAPT-mediated promotion of adipogenesis might be caused by the early autophagy activation. In order to determine whether the DAPT-induced autophagy could affect the differentiation potential of BM-MSCs, we treated BM-MSCs with autophagy inhibitor CQ and 3-MA for early 3 days and allowed the cells to differentiate into adipocytes in the addition of DAPT. The results showed that CQ and 3-MA could inhibit DAPT-induced autophagy, and significantly undermine the effects of DAPT-induced adipogenic differentiation of BM-MSCs. Therefore, our data showed that Notch signaling plays an important role in BM-MSCs adipogenic differentiation via early autophagy activation partly involves PTEN-PI3K-Akt-mTOR signaling pathway.

One limitation of this study is the lack of Notch receptor and ligand protein expression data, and didn't use specific inhibitors of such downstream proteins to block DAPT effect, so the actual regulatory effects controlling MSC adipogenesis of Notch signaling could not be determined either. Further studies are needed to explore the role of Notch signaling in regulating the adipogenic differentiation of MSCs.

Although previous studies have demonstrated that inhibition of Notch altered differentiation of MSCs and emphasized the important role for autophagy in the differentiation of MSCs. The current study not only confirmed the importance of Notch signaling in promoting MSCs adipogenesis, but also provides detailed evidence for autophagy involving the modulation of the Notch signaling pathway in the adipogenic differentiation of MSCs through the PTEN-PI3K/Akt/mTOR pathway. These results bring new insights into our understanding of the Notch signaling regulation of BM-MSCs adipogenesis appearing spontaneously during aging or pathological conditions, and suggest a clinical potential of MSCs in regenerative medicine.

Disclosure Statement

No potential conflicts of interest were disclosed.

Acknowledgments

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