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# MicroRNA-139-5p Suppresses 3T3-L1 Preadipocyte Differentiation Through Notch and IRS1/PI3K/Akt Insulin Signaling Pathways

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## ABSTRACT

MicroRNAs (miRNAs) participate in the regulation of adipogenesis. Identification of the full repertoire of miRNAs expressed in adipose tissue is likely to significantly improve our understanding of adipose tissue growth and development. Here, miR-139–5p was identified as an inhibitor of 3T3-L1 adipocyte differentiation with significantly down-regulating the expression levels of adipogenic marker genes *PPAR* γ ($P < 0.01$), *aP2* ($P < 0.01$) and *FAS* ($P < 0.01$). Importantly, flow cytometry and EdU incorporation assay indicated that this inhibition was partly due to the dysfunction of clonal expansion. Furthermore, we firstly demonstrated that miR-139–5p blocked adipogenesis via directly targeted the 3′ untranslated regions (UTRs) of *Notch1* and *IRS1* mRNAs, a key member of Notch signaling and IRS1/PI3K/Akt insulin signaling, respectively. In addition, the overexpression of Notch1 or IRS1 partially restored the suppressive effects miR-139–5p on differentiation of 3T3-L1 cells. To our knowledge, this was the first report that miR-139–5p functioned negatively by targeting Notch1 and IRS1 during 3T3-L1 adipogenesis, regulating the transition from clonal expansion to terminal differentiation. J. Cell. Biochem. 116: 1195–1204, 2015. 

**KEY WORDS:** ADIPOCYTE DIFFERENTIATION; IRS1/PI3K/Akt INSULIN SIGNALING; miR-139-5p; 3T3-L1; NOTCH SIGNALING

MicroRNAs (miRNAs) are endogenously expressed noncoding RNAs of 19 to 22 nucleotides that negatively regulate gene expression by binding to the 3′ untranslated region (3′UTR) of target mRNAs (Lagos-Quintana et al., 2001; Bagga et al., 2005). MiRNAs possess high conservation, tissue specificity and temporality (Bartel, 2004). It is reported that miRNAs are involved in numerous physiological and pathological processes, including development timing (Reinhart et al., 2000), cell proliferation (Kim et al., 2012; Qin et al., 2012), apoptosis (Cimmino et al., 2005), tumorigenesis (Kumar et al., 2007; Hatley et al., 2010), and neuronal development (Fiore et al., 2008).

Adipocyte differentiation is a dynamic and complex process, including clonal expansion, cell cycle exit and terminal differentiation into mature adipocytes (Rosen and Spiegelman., 2000; Feve, 2005). Recent researches have indicated that miRNAs play some roles in regulatingadipocyte differentiation (Li et al., 2013). The

Abbreviation: PPARγ, Peroxisome proliferator activated receptor γ; aP2, Adipocyte fatty acid-binding protein 4; FAS, Fatty acid sythetase; Notch1, Notch homolog 1; IRS1, Insulin receptor substrate 1; Hes-1, Hairy and Enhancer of split 1; Hey-1, Hairy/enhancer-of-split related with YRPW motif 1; Pref-1, Preadipocyte factor 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT1, AKR mouse T-cell lymphoma oncoprtein; mmu, Mus musculus; ssc, Sus scrofa; hsa, Homo sapiens; ppy, Pongo pygmaeus; mml, Macaca mulatta; rno, Rattus norvegicus; cgr, Cricetulus griseus; eca, Equus caballus.

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first described adipogenesis-related miRNA was miR-143 which regulated adipogenesis by extracellular signal-regulated kinase (ERK5) (Esau et al., 2004). Shortly after, an increasing number of miRNAs were found to be involved in adipogenesis. For example, miR-103 (Xie and Lim Lodish., 2009), miR-30c (Karbiener et al., 2011) and miR-375 (Ling et al., 2011) promoted adipocyte differentiation, while miR-155 (Liu et al., 2011) and miR-224 (Peng et al., 2013) impaired adipocyte differentiation. Additional study showed that let-7 suppressed clonal expansion to block 3T3-L1 preadipocyte differentiation (Sun et al., 2009).

MiR-139–5p was first to be studied in the process of neurodegeneration and was upregulated in neurodegenerative disease- (Saba et al., 2008). Functional studies have shown that miR-139–5p participates in several physiological processes. MiR-139–5p could inhibit breast cancer progression via targeting TGFβ, Wnt and RTK-induced MAPK and PI3K signaling pathway (Krishnan et al., 2013). And also, it inhibited growth transformation dependent protein (HGTD-P) and regulated neuronal apoptosis induced by hypoxia-ischemia (Qu et al., 2014). Recent research have showed that miR-139–5p inhibited cell proliferation, metastasis, and promoting apoptosis and cell cycle arrest by targeting oncogenic Notch1 in colorectal cancer (Zhang et al., 2014). Interestingly, although most studies have focused on the role of miR-139–5p as a tumorigenic inhibitor, little is known about its role in adipocyte differentiation. Our previous work using high throughput sequencing identified that the expression level of miR-139–5p in adipose tissue from 240-day-old Rongchang pigs was 2.2041 folds higher than that of 7-day-old piglets (Li et al., 2011). The expression pattern of miR-139–5p suggested that it might be involved in adipogenesis.

To explore the role of miR-139–5p in 3T3-L1 adipogenic differentiation, we overexpressed miR-139–5p in 3T3-L1 preadipocyte. The results showed that miR-139–5p impaired adipocyte clonal expansion to inhibit the differentiation. Further evidence demonstrated that miR-139–5p suppressed 3T3-L1 preadipocyte differentiation through Notch signaling and IRS1/PI3K/Akt insulin signaling.

## MATERIALS AND METHODS

### ANIMAL STUDIES

Adult Kunming mice obtained from Fourth Military Medical University (Xi′an, China) were maintained in the animal facility of the Laboratory of Animal Fat Deposition and Muscle Development. All procedures of animal handling were approved by the Northwest A&F University Committee on Animal Care. Adipose tissue, skeletal muscle, heart, liver, spleen, and kidney from 12-week-old mice were harvested for miR-139–5p expression analysis by real-time qPCR. Tissues from four mice were pooled for each RNA sample preparation.

### CELL CULTURE

3T3-L1 cells were maintained in growth medium (GM, Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% fetal bovine serum (FBS)), and incubated in 37°C with 10% $CO_2$. To initiate differentiation, after 2 days′ confluence (designated as day 0), medium was changed to differentiation medium DM (GM with 0.5 mM 3-isobutyl-1-methylxanthine, 1μM dexamethasone, and 5 μg/ml of insulin). After 2 days, medium was changed to GM with 5 μg/ml insulin and refreshed with GM every 2 days till harvest.. The whole adipogenic process extended to day 6 of differentiation. HEK293T cells were cultured in GM, and the medium was changed every 2 days.

### TRANSFECTION OF MIRNA MIMIC

3T3-L1 cells cultured in 6-well plates (∼90% confluence) were with miR-139–5p mimic or miR-NTC (50 nM) (Genepharm, Shanghai, China) using 5.0 μl X-tremeGENE HP DNA Transfection Reagent (Roche) following manufacturer′s instructions at the density of ∼90%. The cells were harvested at 48 h after transfection and on day 6 and 8 of differentiation for quantitative realtime PCR (qRT-PCR), Oil Red O staining and Western blotting.

### BIOINFORMATICS ANALYSIS

MiRNA targets were predicted by TargetScan (http://www.targetscan.org/vert50//), miRanda (http://www.microrna.org/microrna/), and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/ rnahybrid/submission.html).

### 3′UTR LUCIFERASE REPORTER ASSAYS

The 3′UTR containing the binding site of miR-139–5p of *Notch1* or *IRS1* mRNA were amplified by RT-PCR from total RNA extracted from 3T3-L1 cells, using primers tagged with Xho I and Not I as cutting sites (primer sequences in Table S1). Subsequently, reporter vectors with point mutations at positions 3–5 of the *Notch1* and *IRS1* seed region were generated using overlap-extension PCR (primer sequences in Table S1). All constructs were confirmed by sequencing. The fragments were cloned into psiCHECK$^{TM}$-2 Vectors (Promega, Madison, WI) at the 3′-end of the Renilla gene. HEK293T cells were seeded in 96-well plates with the density of $2 \times 10^4$/well, and 24 h later, 100 ng of vector constructs along with either 50 nM of miR-139–5p mimic or miR-NTC were transfectioned using 1.0 μl X-tremeGENE HP DNA Transfection Reagent (Roche). Cells were harvested at 48 h after transfection. Luciferase activity was measured using the DualGlo Luciferase Assay System (Promega). Renilla luciferase activity was measured and normalized to corresponding firefly luciferase activity.

### PLASMID CONSTRUCTION AND TRANEFECTION

The full length IRS1 gene coding domain sequence (CDS) and Notch1 Intracellular Domain (NICD) were amplified by PCR reaction and cloned into pcDNA3.1 construct to generate the pCDNA3.1_IRS1 or pcDNA3.1_NICD constructs. 3T3-L1 cells in six-well plates were first transfected with miR-139–5p mimics or miR-NTC (50 nM) in six-well plates when cells were at the density of 90%. On day 2 of differentiation, 2.0 μg of pcDNA3.1_IRS1 or pcDNA3.1_NICD constructs were added. Cells were harvested on day 6 of differentiation for tests. Sequences of primers used for PCR amplification are summarized in Table S2.

### OIL RED O STAINING AND EXTRACTION

3T3-L1 cells were washed with PBS and fixed in 4% paraformaldehyde at 37°C for 40 min. After washing three times, the cells were stained with Oil Red O (0.5 g Oil Red O (Sigma) in 100 ml isopropanol

diluted with water (60:40) and filtrated at 37°C for 30 min, washed twice in water and then photographed. To extract and quantify intracellular Oil Red O, the stained lipid droplets were dissolved with pure isopropanol for 10 min at room temperature. The optical density (OD value) was measured at 510 nm on a spectrophotometer, which was an indicator intracellular lipid accumulation by quantification.

### REAL-TIME QPCR

Total RNA or mouse tissue RNA was extracted from cells or tissues with Trizol reagent (TaKaRa, Otsu, Japan). After quantification, cDNA was synthesized using reverse transcription kits (TaKaRa). Real-time qPCR reaction was performed in triplicate using SYBR green kits on a Bio-Rad iQTM5 system (Bio-Rad, Hercules, CA). The mRNA level was normalized to β-actin as a house keeping gene(primer sequences in Table S3). For miRNA quantification, stem-loop reverse transcription-polymerase chain reaction (RT-PCR) was executed and total RNA was reversely transcribed using reverse transcription kits (TaKaRa). Primers specific to mouse miR-139–5p were used and values were normalized to mouse U6 as housekeeping gene. All miRNA primers were bought from RiboBio.

### WESTERN BLOTTING ANALYSIS

Cell cultures of 3T3-L1 were homogenized in ice-cold protein lysis buffer (RIPA, Beyotime, Shanghai, China) containing protease inhibitor (Pierce, Rockford, IL, USA). The lysates were centrifuged to remove cell debris. Proteins were boiling in 5× protein loading buffer sample buffer for 15 min. A total of 20 μg of protein was electrophoresed on a 12% SDS-polyacrylamide gel and transferred to PVDF membranes (polyvinyldene difluoride membrane) (CST, Boston, MA). The membrane was blocked in 5% skim milk, and then incubated at 4°C overnight with primary antibodies. Antibodies against PPARγ, NICD, Hes-1, Hey-1, Pref-1 (Abcam, Cambridge, UK), aP2, FAS, β-actin (Santa Cruz, Dallas, TX), Notch1 and IRS1 (Boster, Wuhan, China) were used. The results were visualized with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and enhanced chemiluminescence. The protein bands were quantified using the Image J program (National Institutes of Health, Bethesda, MD).

### FLOW CYTOMETRY

3T3-L1 cells were seeded in 60 mm dishes ($1.6 \times 10^5$ cells per dish). 24 h later after seeding, cells reached confluence and were transfected by miR-139–5p mimic or miR-NTC using X-tremeGENE HP DNA Transfection Reagent (Roche). 3T3-L1 cells were harvested by trypsin digestion on day 2 of differentiation, and washed three times with PBS to remove cell debris. The cells were then fixed in cold 70% ethanol overnight, treated with 1 mg/mL RNase A for 40 min at 37°C, and then stained with 20 mg/mL propidium iodide (PI). The cells were analyzed using a FACScan argon laser cytometer (Becton Dickinson, Franklin Lakes, NJ).

### EDU INCORPORATION ASSAY

EdU labeling was used to detect DNA synthesis. 3T3-L1 cells from each group were cultured in 96-well plates and treated with EdU (30 mg/mL) for 3 h before fixation. Then medium was removed and EdU incorporation was measured using the Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen). Finally, the cells were examined using a Nikon TE2000 microscope (Nikon, Tokyo, Japan).

### STATISTICAL ANALYSIS

All quantitative data are expressed as means mean ± S.D. Differences between groups were analyzed by applying Student′s two-tailed *t*-test for independent samples. A value of $P < 0.05$ (significant) or $P < 0.01$ (extremely significant) was considered to be statistically significant.

## RESULTS

### MIR-139–5p IS WIDELY EXPRESSED IN VARIOUS MOUSE TISSUES AND INCREASES DURING ADIPOGENESIS OF 3T3-L1 CELLS

Mature miR-139–5p sequence is highly conserved in many mammals including mouse, human and pig (Fig. 1A). Real-time qPCR results demonstrated that miR-139–5p was relatively highly expressed in muscle and subcutaneous adipose tissue (Fig. 1B). Then the expression level of miR-139–5p was investigated during adipocyte differentiation. 3T3-L1 cells were induced to differentiate with DM after 2 days of confluence. RNA was extracted from adipogenic cells at -2, 0, 2, 4, 6, 8 d of differentiation. Our data revealed that miR-139–5p decreased first and then increased in the later phase during 3T3-L1 cell adipogenesis (Fig. 1C). We speculated that this transient decrease in miR-139–5p expression might be related with clonal expansion (see below). Taken together, miR-139–5p was likely to be associated with adipogenesis.

### MIR-139–5p SUPPRESSES 3T3-L1 PREADIPOCYTE DIFFERENTIATION

To investigate the potential effect of miR-139–5p on adipogenesis, miR-139–5p mimic or negative control (miR-NTC) were transfected to 3T3-L1 cells. Then we detected the overexpression efficiency of miR-139–5p in 3T3-L1 cells by real-time qPCR. Results showed that the mimic transfection resulted in a 7000-fold overexpression of miR-139–5p at 48 h after transfection and the enhanced miR-139–5p could be kept to at least day 8 of differentiation (Fig. 2A). Ectopic miR-139–5p expression in 3T3-L1 cells with DM inhibited their differentiation as detected by Oil Red O staining and extraction on day 6 of differentiation ($P < 0.01$) (Fig. 2B). The mRNA levels of adipogenic markers, *PPARγ* ($P < 0.01$) and *aP2* ($P < 0.01$), were significantly lower in cells transfected with miR-139–5p mimic compared with control on the days shown (Fig. 2C). Then we detected the protein levels of PPARγ, aP2 and FAS by Western Blotting. Likewise, all of them were declined by elevation of miR-139–5p on day 6 of differentiation (Fig. 2D). These data indicated that miR-139–5p inhibited 3T3-L1 preadipocyte differentiation.

### MIR-139–5p IMPAIRS THE CLONAL EXPANSION OF 3T3-L1 CELLS

3T3-L1 cells with adipogenic inducer treatment are triggered a series of complex events, including clonal expansion, growth arrest and terminal differentiation (Rosen and Spiegelman., 2000). Because of its decrease on day 2 of adipogenesis and then ascend during differentiation, miR-139–5p might play a critical role in transmission from clonal expansion to terminal differentiation. To test this

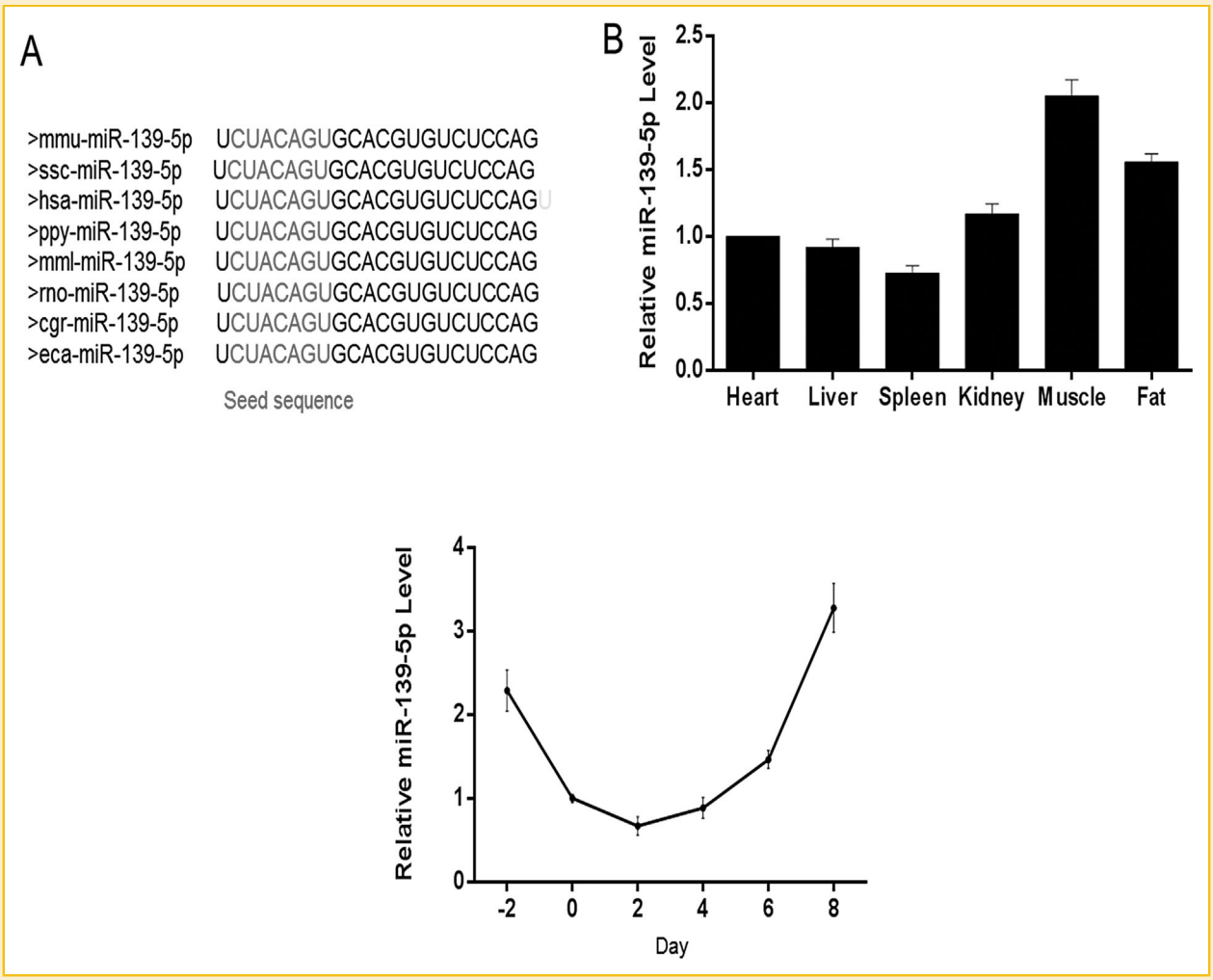


**Fig. 1.** Expression of miR-139-5p in mouse tissues and during 3T3-L1 preadipocyte differentiation. A: Mature miR-139-5p sequence is conserved among species. Expression of miR-139-5p was surveyed by real-time qPCR at the time points indicated in six different mouse tissues (B) and during 3T3-L1 cell differentiation (C). U6 small nuclear RNA was used as a reference gene. All measurements shown are the means ± S.D. of three independent experiments.

speculation, 3T3-L1 cells were transfected with either miR-139-5p mimic or miR-NTC and were induced to initiate differentiation to day 2. On day 2 of differentiation, flow cytometric analysis demonstrated that when miR-139-5p was overexpressed, the fraction of S-phase cells descended ($P < 0.01$) while $G_0/G_1$ phase proportion increased ($P < 0.01$), indicating that miR-139-5p led to a cell-cycle delay (Fig. 3A) (the differentiation status in Figure S1). Consistently, EdU incorporation showed relative positive cells were reduced ($P < 0.01$) after miR-139-5p mimic transfection (Fig. 3B). Moreover, several cell cycle genes including *Cyclin D1* and *p21* were declined ($P < 0.01$) and increased ($P < 0.01$) by miR-139-5p at the mRNA level, respectively (Fig. 3C). Collectively, these data supported that miR-139-5p impaired clonal expansion to suppress adipocyte differentiation.

### MIR-139-5p TARGETS THE 3′UTR OF NOTCH1 AND IRS1

To explicit how miR-139-5p exert its effect on adipocyte differentiation, three computational prediction programs were applied to select miR-139-5p targets: TargetScan, miRanda and RNAhybrid (Fig. 4A). Among candidate target genes, Notch1 and IRS1 were identified as miR-139-5p assumed direct targets on adipogenesis. Interestingly, mRNA levels of *Notch1* and *IRS1* were rapidly upregulated, with levels peaking on day 2 and then downregulated during adipocyte differentiation (Fig. 4B), which was generally inverse to the endogenous expression of miR-139-5p.

In order to further validate the predicted interaction of miR-139-5p with Notch1 and IRS1, we constructed luciferase reporter genes with Notch1 and IRS1 3′UTRs with or without mutation at the miR-139-5p binding regions and then co-transfected the vectors and miR-139-5p mimic or miR-NTC into HEK293T cells. Indeed, luciferase reporter assays manifested a ~25% or 30% decrease in luciferase activity ($P < 0.05$), respectively, when the Notch1 or IRS1 3′UTR was co-transfected with miR-139-5p mimic (Fig. 4C), while no decrease was found in cells transfected with mutant Notch1 or IRS1 3′UTR. These results demonstrated that Notch1 and IRS1 were targeted by miR-139-5p.

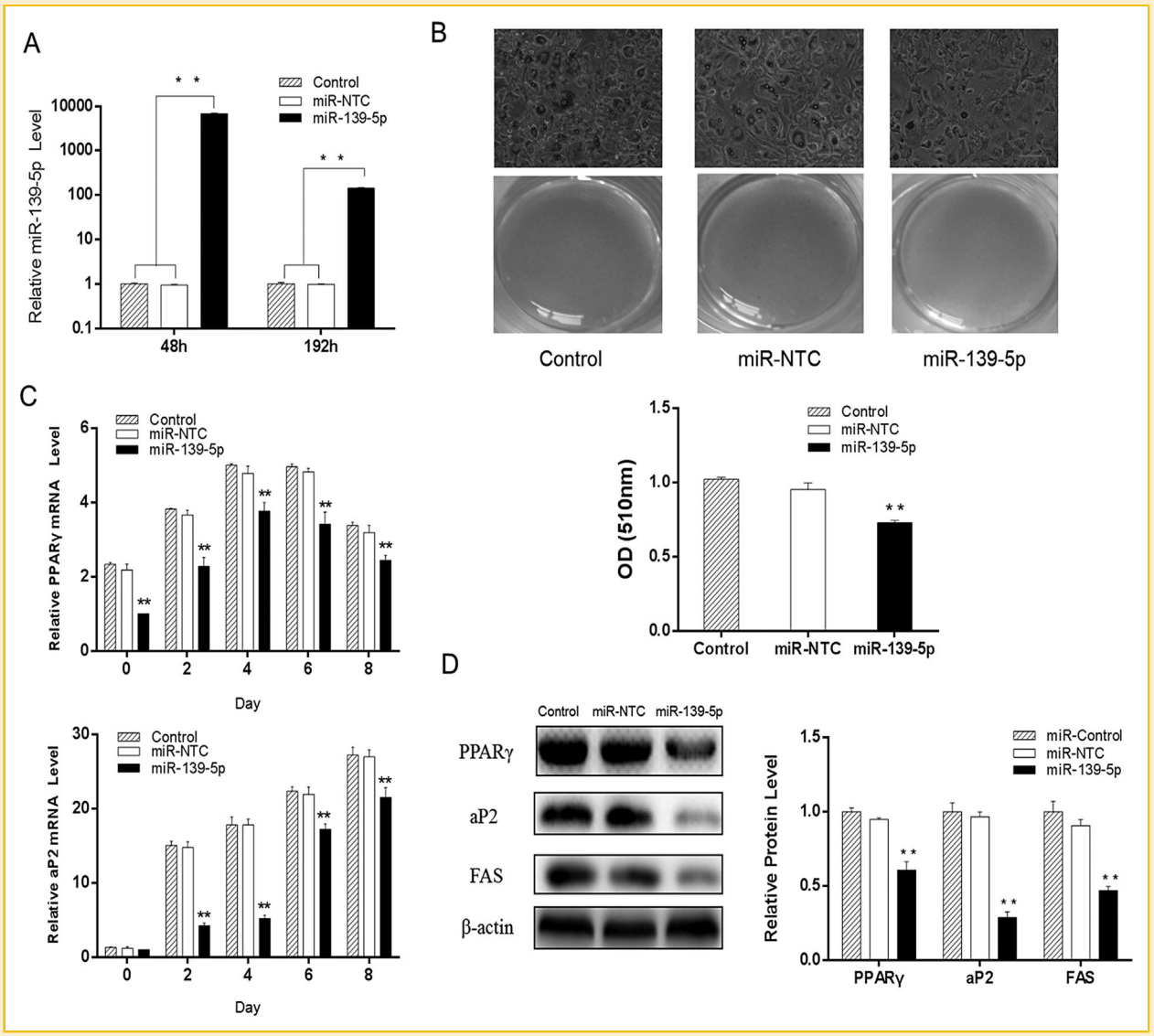


**Fig. 2.** MiR-139-5p impairs 3T3-L1 cells differentiation. MiR-139-5p mimic or negative control (miR-NTC) were transfected into 3T3-L1 cells when reaching 90% confluence, and 48 h later, cells were subject to adipogenic induction. A: The overexpression of miR-139-5p was detected at 48 h after transfection and day 8 of differentiation, respectively. B: Lipid accumulation during 3T3-L1 cell differentiation into adipocytes was monitored by Oil Red O staining on day 6 of differentiation. C: mRNA expression of various adipogenic genes was assayed on the days shown by real-time qPCR in differentiated 3T3-L1 preadipocytes that were treated with miR-139-5p mimic or miR-NTC. D: The protein levels of various adipogenic genes were detected on day 6 of differentiation by Western Blotting. β-actin as a reference gene. All measurements shown are the means ± S.D. of three independent experiments. ** indicates $P < 0.01$.

### MIR-139-5p REGULATES 3T3-L1 PREADIPOCYTE DIFFERENTIATION THROUGH NOTCH AND IRS1/PI3K/AKT SIGNALING PATHWAYS

Subsequently, we detected the mRNA of *Notch1* and *IRS1* on the days shown during adipogenesis after the transfection of miR-139-5p mimic or miR-NTC into 3T3-L1 cells. We found that both of them presented significant declines on the days shown when miR-139-5p overexpression ($P < 0.01$) (Fig. 5A). The protein levels of Notch1 and IRS1 were also decreased in miR-139-5p transfected groups on day 6 of differentiation (Fig. 5B).

To further clarify the targets of miR-139-5p in 3T3-L1 adipogenesis, we adopted a "rescue" methodology to examine the functional relevance of miR-139-5p/Notch1 or IRS1 interaction in 3T3-L1 cells. Obviously, lipid accumulation was rescued on day 6 of differentiation when pcDNA3.1_NICD or pcDNA3.1_IRS1 was transfected into 3T3-L1 cells that had been treated with miR-139-5p mimics (Fig 5C). In agreement with the restored lipid accumulation, the increased protein levels of PPARγ and aP2 (cells transfected with pcDNA3.1_NICD or pcDNA3.1_IRS1 and miR-139-5p mimic vs cells transfected with miR-139-5p mimic) were also observed in 3T3-L1 cells transfected with pcDNA3.1_NICD or pcDNA3.1_IRS1 construct following the treatment of miR-139-5p mimics on day 6 of differentiation (Fig 5D), suggesting that Notch1 and IRS1 were involved in the miR-139-5p-mediated suppression of 3T3-L1 cells metastasis. These data provided further evidence that

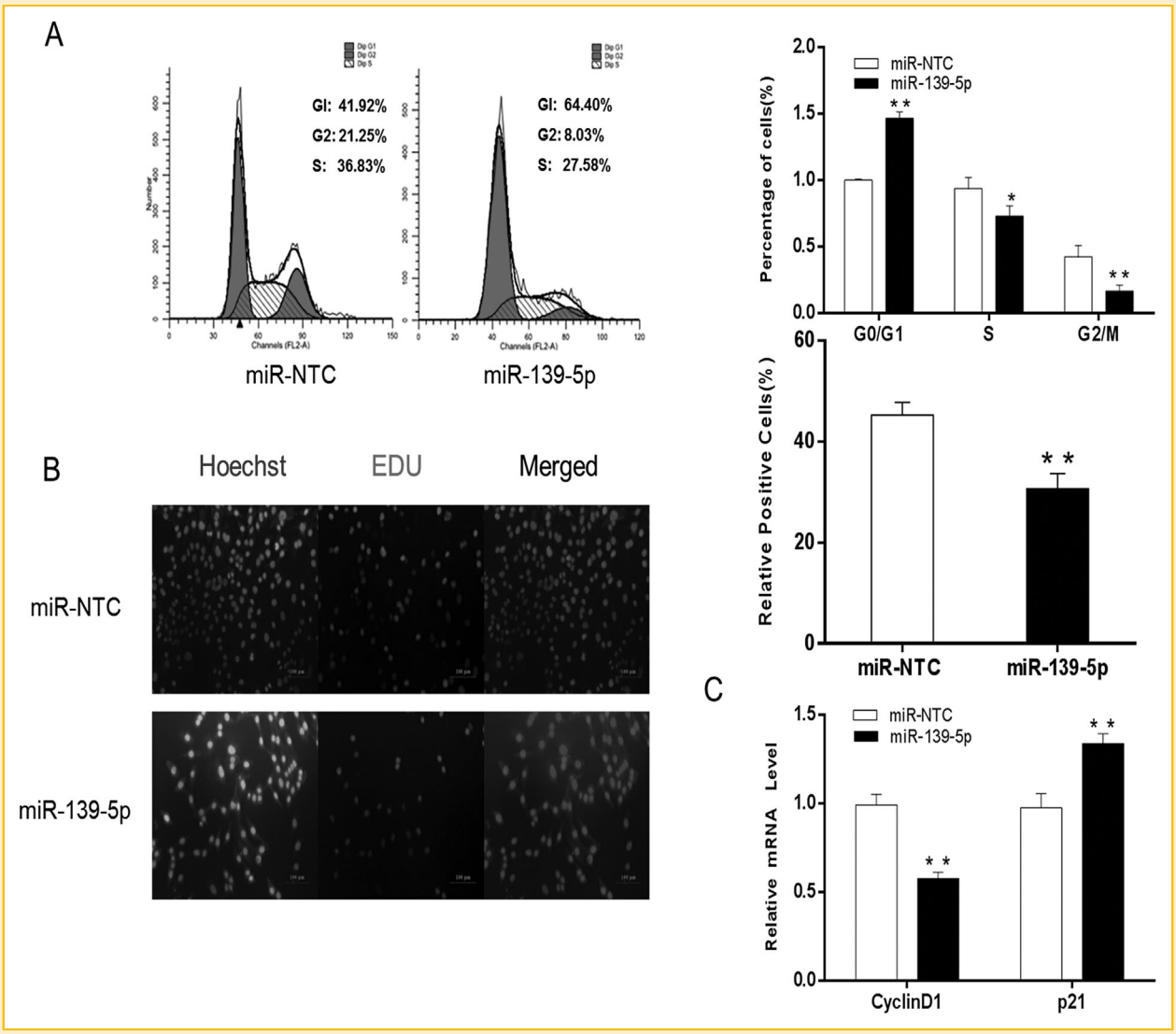


**Fig. 3.** MiR-139-5p impairs the clonal expansion of 3T3-L1 cells. A: Cell-cycle analysis of 3T3-L1 cells using flow cytometry on day 2 of differentiation. B: Fluorescence attributable to EdU incorporation assay of 3T3-L1 cells day 2 of differentiation. C: Expression levels of cell cycle genes from 3T3-L1 cells transfected with miR-139-5p or miR-NTC was measured by real-time qPCR day 2 of differentiation. All measurements shown are the means ± S.D. of three independent experiments. * indicates $P < 0.05$, ** indicates $P < 0.01$.

Notch1 and IRS1 were direct and functional targets of miR-139-5p in 3T3-L1 cells.

Notch intracellular domain (NICD), the active form of Notch1, was downregulated dramatically ($P < 0.05$) (Fig. 6A). In addition, we detected the protein levels of the downstream target genes, Hes-1 and Hey-1. Compared with the negative control, both of them presented a noteworthy dip ($P < 0.05$) (Fig. 6A). On the contrary, the expression level of Pref-1 was upregulated ($P < 0.01$) in the miR-139-5p mimic transfected cells (Fig. 6A). These data showed that miR-139-5p inhibited adipogenesis through Notch Signaling. The decline of IRS1/PI3K/Akt insulin signaling pathway activity was also determined with the decreased phosphorylation levels of IRS1, PI3K and Akt by Western Blotting when miR-139-5p was over-expressed (Fig. 6B).

## DISCUSSION

Mature miR-139-5p is highly conserved among mammals, like human, pig, and mouse, among which miR-139-5p shares the same "seed" sequence. This might result in a similar function in these species. In this report, we found that miR-139-5p was decreased rapidly whereas increased again as the differentiation progressed and miR-139-5p was an inhibitor of adipocyte differentiation. Adipogenic stimulus initiates differentiation, which features 3T3-L1 cell clonal expansion, growth arrest and terminal differentiation in sequence (Rosen and Spiegelman., 2000). Flow cytometry and EdU incorporation showed that when miR-139-5p was overexpressed, clonal expansion of 3T3-L1 cells was impaired.

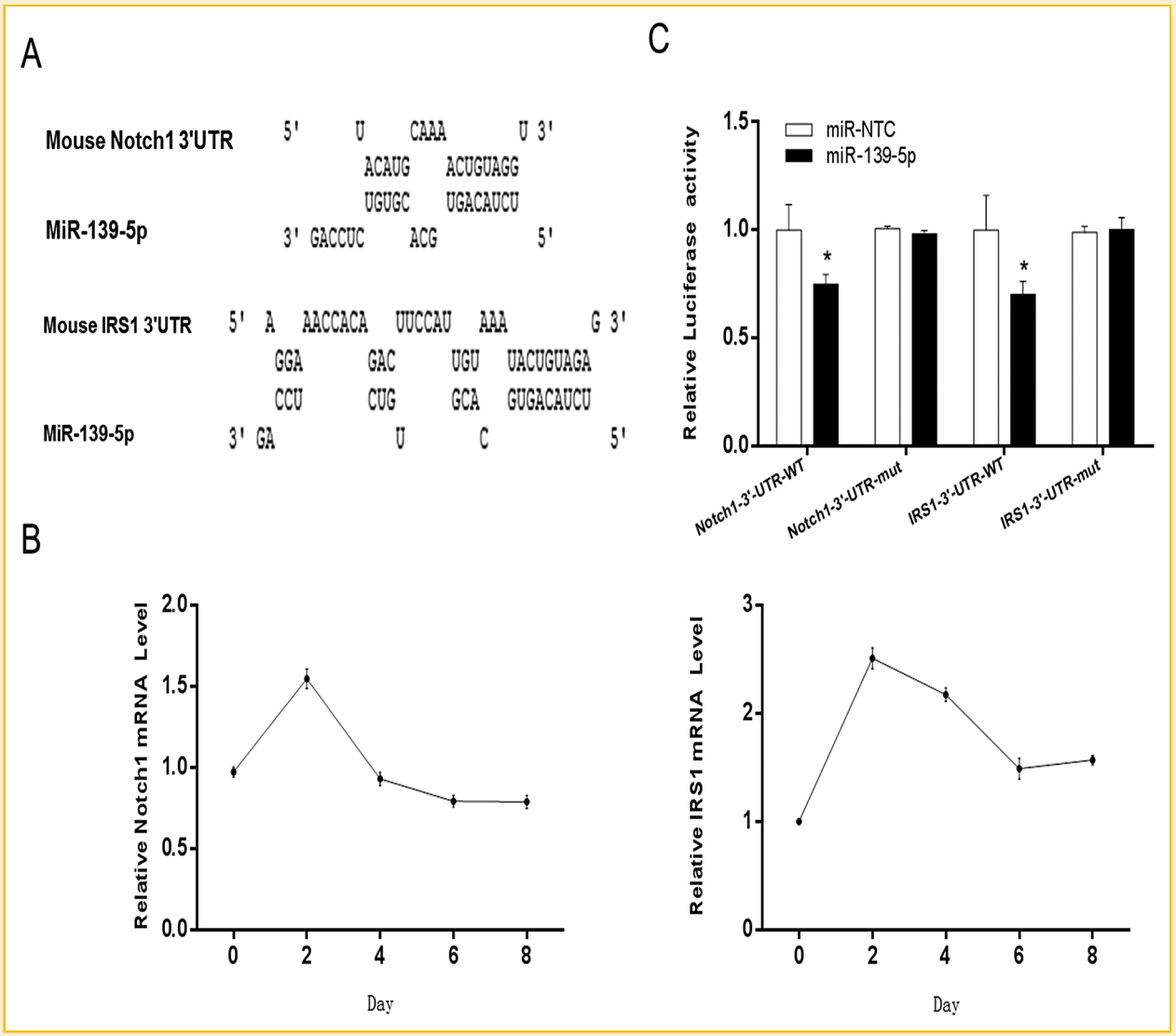


**Fig. 4.** MiR-139-5p targets Notch1 and IRS1 3′UTR. A: Target site of miR-139-5p within mouse Notch1 and IRS1 mRNA 3′UTR. B: Notch1 and IRS1 mRNA levels were evaluated by real-time qPCR at the indicated time points after treatment of 3T3-L1 cells with DM. C: The wild type 3′UTR of Notch1 (Notch1-3′UTR-WT) or IRS1 (IRS1-3′UTR-WT), or 3′UTRs with mutated miR-139-5p seed matches (Notch1-3′UTR-mut and IRS1-3′UTR-mut, respectively), were co-transfected with 50 nM miR-139-5p mimic or miR-NTC into HEK293 cells. Cells were harvested and luciferase reporter assays were performed after 48 h. For each sample, Renilla luciferase activity was normalized to firefly luciferase. All measurements shown are the means ± S.D. of three independent experiments. * indicates $P < 0.05$.

Both of *Notch1* and *IRS1* mRNAs were upregulated significantly and then declined, which was inversely correlated with miR-139-5p expression. Previous studies have demonstrated that overexpression of Notch1 decreased the expression levels of PPARγ, aP2 and adiponectin and inhibited adipogenesis in human bone marrow-derived stromal cells (Ugarte et al., 2009). However, in 3T3-L1 cells transfected with antisense oligo of Notch1, PPARγ and PPARδ were downregulated and further prevent adipogenesis (Garces et al., 1997). Consistently, our data also showed that the inhibition of Notch1 blocks 3T3-L1 cells adipogenesis. In addition, Notch1 is a key regulator of Notch signaling, which is involved in adipogenesis (Ross and Rao Kadesch., 2004; Osathanon et al., 2012). Hes-1, a target gene of Notch, inhibited adipocyte differentiation (Ross and Rao kadesch., 2004). However, the interference of Hes-1 mRNA impaired adipogenesis in 3T3-L1 cells (Ross and Rao Kadesch., 2004). Further investigation revealed that Hes-1 promoted adipogenesis through down-regulating Pref-1, an adipogenic inhibitor (Ross and Rao kadesch., 2004; Wang and K.-A. Kim, 2006). In line with the research above, in this study, we found that protein levels of Hes-1 and Hey-1 showed similar descends, while that of Pref-1 increased in 3T3-L1 cells transfected with miR-139-5p mimic.

Our study indicated that miR-139-5p inhibited 3T3-L1 cells adipogenesis via IRS1/PI3K/Akt insulin signaling pathway. Activated form of PI3K, a critical target of IRS1 downstream, led to phosphorylation of phosphatidyl inositides and then activated the downstream main target Akt, which is pivotal in regulating 3T3-L1 preadipocyte differentiation (Hirsch et al., 2000; Xu and Liao., 2004). Obviously, in this research, miR-139-5p was

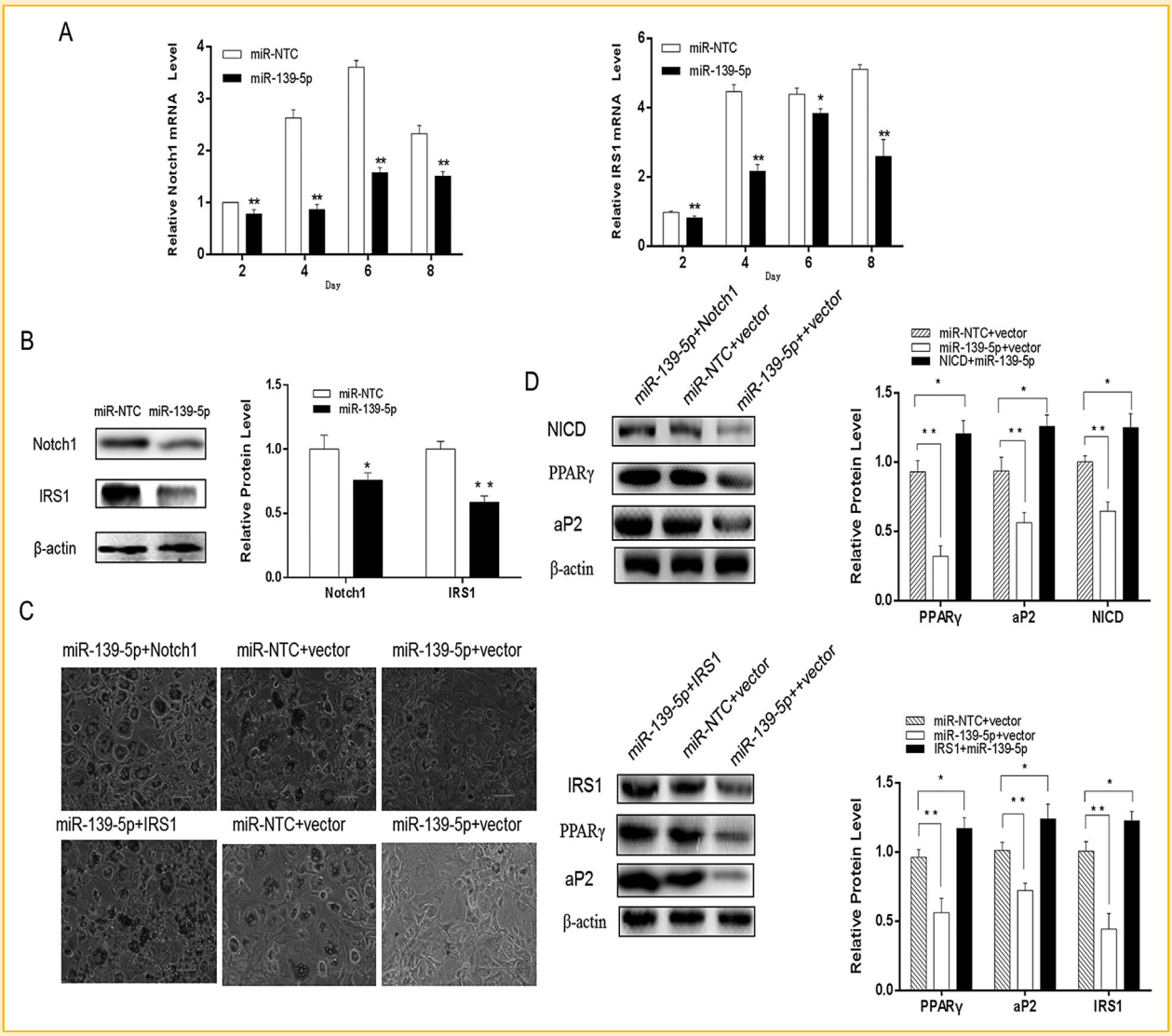


**Fig. 5.** Notch1 and IRS1 are involved in miR-139-5p-mediated suppression of 3T3-L1 cell differentiation A: The mRNA levels of Notch1 and IRS1 were analyzed after transfection on the days shown during 3T3-L1 cells adipogenesis. B: The protein levels of Notch1 and IRS1 were detected on day 6 of differentiation by Western Blotting. C: Formation of lipid droplets in the cells that co-transfection with pcDNA3.1_NICD or pcDNA3.1_IRS1 were observed as miR-139-5p mimics treatment on day 6 of differentiation by Oil Red O. D: Upon co-transfection with pcDNA3.1_NICD or pcDNA3.1_IRS1 construct, the expression levels of NICD, IRS1, *PPAR γ* and *aP2* were detected on day 6 of differentiation by Western Blotting. All measurements shown are the means ± S.D. of three independent experiments. * indicates $P < 0.05$, ** indicates $P < 0.01$.

demonstrated to decrease the tyrosine phosphorylation of IRS1, phosphorylation of p85 subunit of PI3K and also serine phosphorylation of Akt.

The interaction of Notch1 and PI3K/Akt signaling was observed in T-cell acute lymphoblastic leukemia (T-ALL) (Palomero et al., 2007). Specifically, NICD reduces PTEN levels by Hes1 and then actives PI3K/Akt signaling. However, there are still no compelling evidence for the similar interplaying between Notch1 and PI3K/Akt signaling in 3T3-L1 adipogenesis. Further evidences, therefore, are still required to clarify the mechanisms by which miR-139-5p affects 3T3-L1 adipogenesis.

In conclusion, our studies provided new insights into the fundamental role of miR-139-5p in 3T3-L1 preadipocyte differentiation. We showed for the first time that miR-139-5p functioned by targeting Notch1 and IRS1 during 3T3-L1 preadipogenesis, affecting the transition of preadipocytes from clonal expansion to terminal differentiation. Our study also provided mechanistic insights into the molecular processes of miR-139-5p as a potential therapeutic target of obesity and related metabolic diseases.

## ACKNOWLEGEMENT

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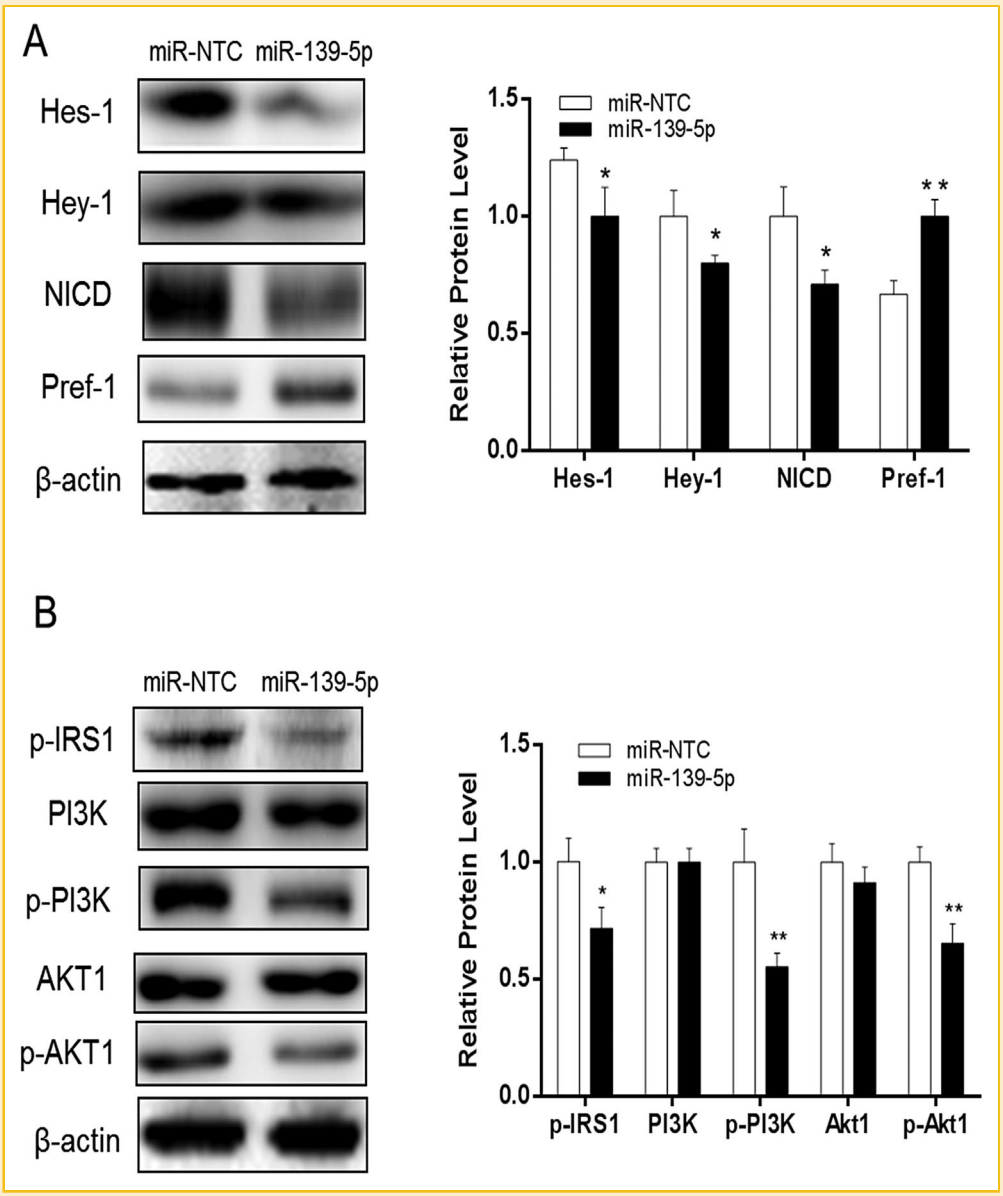


**Fig. 6.** MiR-139-5p suppresses 3T3-L1 cells adipogenesis through Notch and IRS1/PI3K/Akt signaling pathways. A: The protein levels of several signal molecules of Notch signaling pathway were detected on day 6 of differentiation by Western Blotting. B: The protein levels of several signal molecules of IRS1/PI3K/Akt signaling pathway were detected on day 6 of differentiation by Western Blotting. All measurements shown are the means ± S.D. of three independent experiments. * indicates $P < 0.05$, ** indicates $P < 0.01$.

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