miR-135a-5p inhibits 3T3-L1 adipogenesis through activation of canonical Wnt/β-catenin signaling

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Abstract
MicroRNAs are endogenous, conserved, and non-coding small RNAs that function as post-transcriptional regulators of fat development and adipogenesis. Adipogenic marker genes, such as CCAAT/enhancer binding protein α (Cebpα), peroxisome proliferator-activated receptor γ (Pparg), adipocyte fatty acid binding protein (Ap2), and fatty acid synthase (Fas), are regarded as the essential transcriptional regulators of preadipocyte differentiation and lipid storage in mature adipocytes. Canonical Wnt/β-catenin signaling is recognized as a negative molecular switch during adipogenesis. In the present work we found that miR-135a-5p is markedly downregulated during the process of 3T3-L1 preadipocyte differentiation. Overexpression of miR-135a-5p impairs the expressions of adipogenic marker genes as well as lipid droplet accumulation and triglyceride content, indicating the importance of miR-135a-5p for adipogenic differentiation and adipogenesis. Further studies show that miR-135a-5p directly targets adenomatous polyposis coli (Apc), contributes to the translocation of β-catenin from cytoplasm to nucleus, and then activates the expressions of cyclin D1 (Ccnd1) and Cmyc, indicating the induction of canonical Wnt/β-catenin signaling. In addition, inhibition of APC with siRNA exhibits the same effects as overexpression of miR-135a-5p. Our findings demonstrate that miR-135a-5p suppresses 3T3-L1 preadipocyte differentiation and adipogenesis through the activation of canonical Wnt/β-catenin signaling by directly targeting Apc. Taken together, these results offer profound insights into the adipogenesis mechanism and the development of adipose tissue.

Key Words
- miR-135a-5p
- 3T3-L1 preadipocyte differentiation
- adipogenesis
- APC
- Wnt/β-catenin

Introduction
Excessive accumulation of body fat probably results in the development of obesity. The adipose tissue is mainly composed of adipocytes deriving from mesenchymal stem cells, which undergo two phases, namely, commitment to preadipocyte lineage and terminal differentiation, and finally differentiate into mature adipocytes (Lefterova & Lazar 2009). Adipose tissue development is a complex process involving a cascade of multiple adipogenic

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transcription factors C/EBPβ, C/EBPδ, ADD1/SREBP1c, PPARY, and C/EBPz. Among these transcription factors, C/EBPz and PPARY are two master regulators of preadipocyte differentiation (Lefterova & Lazar 2009). The imbalance between energy intake and expenditure and the dysregulation of adipokines in adipose tissue are associated with disorders of the immune response, glucose metabolism, and lipid metabolism (Hotamisligil et al. 1993, Kahn & Flier 2000). Accordingly, clarifying the definite mechanisms of adipogenesis is most important.

miRNAs (microRNAs) are endogenous, conserved, and non-coding small RNAs that function as post-transcriptional regulators in diverse biological processes (Bartel 2004). Increasing evidence demonstrated that miRNAs exert a significant effect on adipose tissue development and adipocyte differentiation. For example, miR-143, the miR-17-92 cluster, and miR-375 accelerate 3T3-L1 adipogenic differentiation (Esau et al. 2004, Wang et al. 2008, Ling et al. 2011, Yi et al. 2011), while let-7 and the miR-27 family impair preadipocyte differentiation (Karbiener et al. 2009, Sun et al. 2009, Kim et al. 2010). In addition, miR-210 has been reported to promote adipogenesis by inhibiting the canonical Wnt/β-catenin signaling pathway (Qin et al. 2010).

Our previous research revealed that miR-135a-5p is possibly a negative regulator during adipose tissue development (Chen et al. 2012). Moreover, an earlier study showed that human miR-135 family induces downstream canonical Wnt/β-catenin signaling pathway activity (Nagel et al. 2008). The fact that the canonical Wnt/β-catenin signaling pathway is a molecular switch during adipogenesis has been established in recent years. Adipogenesis is inhibited when Wnt/β-catenin signaling is activated, and vice versa (Ross et al. 2000, Christodoulides et al. 2009). These results indicate that miR-135a-5p may work in adipogenesis. miR-135a-5p has been believed to be involved in colorectal cancer, osteoblast differentiation, and blood pressure regulation (Li et al. 2008, Sober et al. 2010). However, so far, the role of miR-135a-5p in adipogenesis has not been reported. The mouse 3T3-L1 preadipocyte line as an adipogenesis model is often used to clarify the molecular process of adipocyte differentiation. In this study, we investigated the function of miR-135a-5p in 3T3-L1 preadipocyte differentiation and adipogenesis. Our results indicated that miR-135a-5p is an important negative regulator of adipogenesis by targeting adenomatous polyposis coli (Apc) and activating the canonical Wnt/β-catenin signaling pathway. Taken all together, this research provides more insights into the mechanism of adipocyte differentiation.

**Materials and methods**

**Cell culture and differentiation**

3T3-L1 preadipocytes and BHK cells were cultured in DMEM (Genom, Hangzhou, Zhengjiang, China) supplemented with 10% fetal bovine serum (FBS, Gibco) in a 5% CO₂ humidified atmosphere at 37 °C. For 3T3-L1 adipocyte differentiation, confluent preadipocytes were incubated for 2 days (with the first designed as day 0) in the medium comprising DMEM supplemented with 10% FBS, 8.61 × 10⁻⁷ mol/l insulin (INS), 1 × 10⁻⁶ mol/l dexamethasone (DEX), and 5 × 10⁻⁵ mol/l 3-isobutyl-1-methylxanthine (IBMX). On day 2, cells were cultured in DMEM containing 10% FBS and 1.72 × 10⁻⁷ mol/l INS, and on day 4 and every 2 days thereafter, cells were fed with 10% FBS.

**Transfection assays**

3T3-L1 preadipocytes were plated in six-well plates, 2 days after the cells reached confluence they were transfected with 2 × 10⁻⁵ mol/l of miR-135a-5p mimics, negative control (NC), inhibitor, NC inhibitor, siRNA oligonucleotides targeting Apc mRNA (siApc-1 and siApc-2), or a non-targeting control (Apc NCi) (GenePharma, Shanghai, China) by using FuGENE HD reagent (Roche) according to the manufacturer’s instructions. At 2 days after transfection (designed as day 0), 3T3-L1 preadipocytes were induced to undergo adipogenic differentiation. The oligonucleotide sequences are listed in Table 1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession no.</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-135a-5p mimics</td>
<td>MIMAT000147</td>
<td>UAUGGCUUUUUAUUC-CUAUGUGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UCACAUAUG-GAAUAAGGCAAUA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UUCUCGAACGUGUCAGC-GAATT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACGUGACAGGUGCCGA-GAATT</td>
</tr>
<tr>
<td>miR-135a-5p NC</td>
<td></td>
<td>UCACAUAUG-GAAUAAGGCAAUA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UCCUCGAACGUGUCAGC-GAATT</td>
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<tr>
<td>miR-135a-5p inhibitor</td>
<td></td>
<td>UGAAACCAUGCAUGGUGUAG-GAAT</td>
</tr>
<tr>
<td>miR-135a-5p NC inhibitor</td>
<td>NM_007462.3</td>
<td>UGAAAGCAAGGCAUGCAUGCAUG</td>
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<tr>
<td>SiApc-1</td>
<td></td>
<td>UCCUCGAACGUGUCAGC-GAATT</td>
</tr>
<tr>
<td>SiApc-2</td>
<td></td>
<td>UCCUCGAACGUGUCAGC-GAATT</td>
</tr>
<tr>
<td>Apc NCi</td>
<td></td>
<td>UCCUCGAACGUGUCAGC-GAATT</td>
</tr>
</tbody>
</table>

**Table 1 Oligonucleotide sequences of miR-135a-5p and Apc siRNA**
expression levels of genes were calculated using the Lightcycler 480 machine (Roche), and the relative level was measured by the stem-loop QPCR method (Chen et al. 2005), and U6 small nuclear RNA (snRNA) was used as an internal control. QPCR analysis was carried out using a Supermix (Takara, Dalian, Liaoning, China) with Bactin as a housekeeping gene for normalization. miR-135a-5p was synthesized using the RevertAid first-strand cDNA synthesis kit (Fermentas (Waltham, Massachusetts, USA), according to the manufacturer’s protocol. In QPCR analysis, the mRNA levels of Pparg, Cebpa, Ap2, Fas, Apc, Ccnd1, Cmyc, and Bcina were quantified using SYBR Green Supermix (Takara, Dalian, Liaoning, China) with Bactin as a housekeeping gene for normalization. miR-135a-5p level was measured by the stem-loop QPCR method (Chen et al. 2005), and U6 small nuclear RNA (snRNA) was used as an internal control. QPCR analysis was carried out using a Lightcycler 480 machine (Roche), and the relative expression levels of genes were calculated using the 2^{\Delta\Delta CT} method. Three independent experiments were conducted and the primer sequences are listed in Table 2.

Quantitative PCR (QPCR) assay

Total cellular RNA from 3T3-L1 cells was harvested using Trizol reagent (Invitrogen). One microgram of total RNA was incubated with DNase I (Fermenta) to rule out the possibility of DNA contamination. For the RT-PCR, cDNA was synthesized using the RevertAid first-strand cDNA synthesis kit (Fermentas (Waltham, Massachusetts, USA), K1622) according to the manufacturer’s protocol. In QPCR analysis, the mRNA levels of Pparg, Cebpa, Ap2, Fas, Apc, Ccnd1, and Cmyc were quantified using SYBR Green Supermix (Takara, Dalian, Liaoning, China) with Bactin as a housekeeping gene for normalization. miR-135a-5p level was measured by the stem-loop QPCR method (Chen et al. 2005), and U6 small nuclear RNA (snRNA) was used as an internal control. QPCR analysis was carried out using a Lightcycler 480 machine (Roche), and the relative expression levels of genes were calculated using the 2^{\Delta\Delta CT} method. Three independent experiments were conducted and the primer sequences are listed in Table 2.

Table 2 Primers sequences for QPCR analysis

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession no.</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
<th>Stem-loop primer (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pparg</td>
<td>NM_011146.3</td>
<td>AACAGCTGACCCAAATGGTGA</td>
<td>ACCCTTGACTTCATCAACAG</td>
<td>CTAACCTGTGGCGATGCTGTGG</td>
</tr>
<tr>
<td>Cebpa</td>
<td>NM_007678.3</td>
<td>CGCAAGAGCGAGGAGAATAG</td>
<td>CGGTTTCATGGAAGACGCAG</td>
<td>CTAACCTGAACTGCTTCATTCA</td>
</tr>
<tr>
<td>Ap2</td>
<td>NM_024406.2</td>
<td>TCCACTGGGAAGACGACCTCT</td>
<td>GGGAGTTGGATGAGCGGAT</td>
<td>CTAACCTGAACTGCTTCATTCA</td>
</tr>
<tr>
<td>Fas</td>
<td>NM_007988.3</td>
<td>GGAGGTGGTGATAGCCGGTAT</td>
<td>TGGTTACCTTCCAGCCAG</td>
<td>CTAACCTGAACTGCTTCATTCA</td>
</tr>
<tr>
<td>Apc</td>
<td>NM_007462.3</td>
<td>GAGAGCTGCTGCAAATGGAA</td>
<td>GACTTCACACATCTGGCAG</td>
<td>CTAACCTGAACTGCTTCATTCA</td>
</tr>
<tr>
<td>Ccnd1</td>
<td>NM_007631.2</td>
<td>TATAAGGACCCAGAGGAA</td>
<td>GGGCTGGTGCTGTCTTTGC</td>
<td>CTAACCTGAACTGCTTCATTCA</td>
</tr>
<tr>
<td>Cmyc</td>
<td>NM_007631.2</td>
<td>TCAAATCTTCCTTCTGGTAT</td>
<td>TTGATGACCGAGTTACTTGGAG</td>
<td>CTAACCTGAACTGCTTCATTCA</td>
</tr>
<tr>
<td>Bcina</td>
<td>NM_001177352.1</td>
<td>CAGCCTTCCTTCTTCTGGAT</td>
<td>GGGCTGGTGCTGTCTTTGC</td>
<td>CTAACCTGAACTGCTTCATTCA</td>
</tr>
<tr>
<td>U6</td>
<td>NM_007988.3</td>
<td>TCTGCTTGCGACACATATAC</td>
<td>TGCTGTTGGTGGGTGATTGAT</td>
<td>CTAACCTGAACTGCTTCATTCA</td>
</tr>
<tr>
<td>miR-135a-5p</td>
<td>MIMAT0000147</td>
<td>CGTGGATGTATGGCTGTGATTTTATTC</td>
<td>CGTGGATGTATGGCTGTGATTTTATTC</td>
<td>CTAACCTGAACTGCTTCATTCA</td>
</tr>
</tbody>
</table>

Luciferase vector construction and reporter assays

The 3’-UTR of mouse Apc, containing two potential target sites of miR-135a-5p predicted by Targetscan (http://www.targetscan.org/) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), was amplified from mouse cDNA with the primers listed in Table 3. The PCR product was double digested with Xhol and NotI, and was subcloned into the XhoI and NotI sites of the psi-CHECK2 vector (Promega). This wild plasmid was named Apc-w. During the construction of mutated plasmids, PCR site-directed mutagenesis (Ho et al. 1989) was adopted to introduce point mutations respectively into the two predicted binding sites in the 3’-UTR of Apc. The plasmids with the first- and second-binding site mutations were designated as Apc-m1 and Apc-m2 respectively. The vector with both binding site mutations was named Apc-m3. The primers used are also listed in Table 3. In luciferase reporter assay, using lipofectamine 2000 (Invitrogen), 0.4 μg of miR-135a-5p mimics or NC was co-transfected with 0.4 μg Apc-w, Apc-m1, Apc-m2, or Apc-m3 into BHK cells seeded in a 24-well plate. Cell lysates were obtained 24 h after transfection and the luciferase activity was analyzed by using the Dual Luciferase Reporter Assay System (Promega).

Table 3 Primers sequences for construction of Apc 3’ UTR and its mutants

<table>
<thead>
<tr>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
<th>Stem-loop primer (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apc-w</td>
<td>CCGCTCAGAAAA-GAGCTGCGAAGATGTAATGTTG</td>
<td>AATGGAATTCTGAGAGCGAGAGCATCATTTCAATTCATATTTTTCG</td>
</tr>
<tr>
<td>Apc-m1</td>
<td>CGTGGTAACTTACAGCGGCCGAGGATCTGCTTCATTCAATTCATTTTTCG</td>
<td></td>
</tr>
<tr>
<td>Apc-m2</td>
<td>CGTGGTAACTTACAGCGGCCGAGGATCTGCTTCATTCAATTCATTTTTCG</td>
<td></td>
</tr>
</tbody>
</table>
Western blot analysis

3T3-L1 cells were lysed on ice with RIPA lysis buffer (Beyotime, Nantong, Jiangsu, China) after being washed with PBS. The cellular protein samples were then obtained by centrifugation. Nuclear protein samples were also collected with a nucleoprotein extraction kit (Beyotime). Protein samples denatured by boiling in 5× SDS buffer were subjected to 5% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Boston, MA, USA). The membranes were incubated with primary antibodies against APC (Abcam, Cambridge, UK), β-catenin (EPI), β-actin (Santa-Cruz), and histone H3 (Bioworld, St Louis Park, MN, USA). The immunoblotted membranes were finally probed with corresponding HRP-conjugated secondary antibodies (KPL). The chemiluminescence was detected using an ECL assay (Beyotime).

Statistical analysis

All the experiments were carried out in triplicate and the results were presented as mean ± S.E.M. The data were analyzed using SPSS 16.0. Statistical significance between samples was calculated by one-way ANOVA. A P value of <0.05 was deemed statistically significant (*P<0.05 and **P<0.01). In QPCR analysis, the relative expression levels of genes were calculated using the 2−ΔΔCt method.

Results

Downregulation of miR-135a-5p during 3T3-L1 preadipocyte differentiation

We assessed the expression level of miR-135a-5p during 3T3-L1 preadipocyte differentiation using stem-loop QPCR assay. The result indicated that the level of miR-135a-5p was significantly downregulated during adipogenesis (Fig. 1).

Inhibition of 3T3-L1 adipogenic differentiation and adipogenesis by miR-135a-5p

To examine the role of miR-135a-5p in 3T3-L1 preadipocyte differentiation and adipogenesis, miR-135a-5p mimics or NC respectively were used to transfect 3T3-L1 preadipocytes, which were then subjected to adipogenic differentiation. The abundance of miR-135a-5p was detected, and QPCR analysis verified that there was a marked increase in miR-135a-5p in the mimics group when compared with that in the controls (Supplementary Figure 1, see section on supplementary data given at the end of this article). As shown by Oil Red O staining and triglyceride content assays, the amount of lipid droplets in 3T3-L1 cells was significantly decreased in the miR-135a-5p mimics group (Fig. 2A), which were further quantified by measuring OD values at 510 nm in Oil Red O extractions. The overexpression of miR-135a-5p reduced the absorbance by approximately 20% (Fig. 2B, left), and resulted in a 72.2 and 56.2% decrease in triglyceride content in comparison with that in the positive and NC groups respectively (Fig. 2B, right).

In order to elucidate the mechanism by which miR-135a-5p inhibited 3T3-L1 adipogenesis, we explored the expression changes of peroxisome proliferator-activated receptor γ (Pparg) and CCAAT/enhancer binding protein α (Cebpα), two master regulators that are important for adipocyte differentiation, at various stages of adipogenic differentiation, including day 2, 4, 7, and 9 after differentiation. We found that miR-135a-5p was able to repress the transcriptional induction of Pparg and Cebpα during the process of adipogenic stimulation (Fig. 2C). Furthermore, the expressions of adipocyte fatty acid binding protein (Ap2) and fatty acid synthase (Fas), two other adipogenic marker genes, were also blunted in the miR-135a-5p mimics group (Fig. 2D). These results demonstrated that miR-135a-5p inhibited preadipocyte differentiation and adipogenesis by impeding the transcriptional induction of adipogenic marker genes such as Pparg, Cebpα, Ap2, and Fas.

Activation of the canonical Wnt/β-catenin signaling pathway by targeting Apc during adipogenesis by miR-135a-5p

We used bioinformatics target prediction software including Targetscan and RNAhybird to predict the potential
Apc

miR-135a-5p

and D). Moreover, the reduction in miR-135a-5p caused by suppressed the mRNA and protein expression levels of respectively. It was found that miR-135a-5p drastically were detected by QPCR and western blot analysis 3T3-L1 cells. The mRNA and protein expression levels of when compared with the positive and NC groups (Fig. 3C

miR-135a-5p. These findings indicated that miR-135a-5p drastically

Apc

completely abolished the inhibitory effects of miR-135a-5p on mutated

reporter, (see Materials and methods). Unlike the Apc luciferase reporters, named Apc-m1, Apc-m2, and Apc-m3 respectively (Fig. 3A, right) (see Materials and methods). Unlike the Apc-w luciferase reporter, Apc-m1, and Apc-m2 significantly, and Apc-m3 completely abolished the inhibitory effects of miR-135a-5p on mutated Apc 3'-UTR (Fig. 3B). Then, we investigated the repressive effect of miR-135a-5p on endogenous Apc in 3T3-L1 cells. The mRNA and protein expression levels of Apc were detected by QPCR and western blot analysis respectively. It was found that miR-135a-5p drastically suppressed the mRNA and protein expression levels of Apc when compared with the positive and NC groups (Fig. 3C and D). Moreover, the reduction in miR-135a-5p caused by miRNA inhibitor, as expected, led to an increase in the protein expression level of Apc (Fig. 3D, bottom). These findings indicated that Apc was a direct target of miR-135a-5p.

In the canonical Wnt/β-catenin signaling pathway, APC is an important factor and it tends to be combined with AXIN, GSK3β, and β-catenin to form a complex which evidently affects adipogenesis. To test whether miR-135a-5p regulated 3T3-L1 preadipocyte differentiation and adipogenesis by influencing the canonical Wnt/β-catenin signaling pathway, we carried out immunoblotting analysis to investigate the effect of miR-135a-5p on the nuclear β-catenin. As shown in Fig. 4A, the expression of nuclear protein of β-catenin was increased in 3T3-L1 cells treated with miR-135a-5p mimics when compared with that in the positive and NC groups. Furthermore, the overexpression of miR-135a-5p led to a significant increase in mRNA levels of Ccnd1 and Cmyc which are targets of the APC pathway (Fig. 4B). These results proved that miR-135a-5p suppressed 3T3-L1 adipogenic differentiation and lipid droplets accumulation by activating the canonical Wnt/β-catenin signaling pathway by targeting Apc.

Inhibition of 3T3-L1 adipogenic differentiation and adipogenesis by downregulating APC with siRNA

To investigate the influence of APC downregulation on 3T3-L1 preadipocyte differentiation, siApc-1 or siApc-2, siRNA against Apc respectively, were used to transfect 3T3-L1 preadipocytes which would experience adipogenic
differentiation. Western blot analysis verified that siApc-1 effectively suppressed the protein expression of APC (Fig. 5A). The Oil Red O staining and triglyceride content assays clearly showed that the downregulation of APC also resulted in reductions in the accumulation of lipid droplets and triglyceride content (Fig. 5B and C). Meanwhile, the reduction in APC significantly inhibited the expression levels of adipogenic marker such genes as Pparg, Cebpa, Ap2, and Fas, as indicated by QPCR analysis (Fig. 5D). To further detect whether the decrease in APC influenced the activity of the canonical Wnt/β-catenin signaling pathway, we examined the expressions of Ccnd1 and Cmyc through QPCR assay, and we found that the levels of mRNA both genes were markedly increased in 3T3-L1 cells incubated with siApc-1 (Fig. 5E). Our data demonstrated that the downregulation of APC caused by siRNA effectively inhibited 3T3-L1 adipogenic differentiation and adipogenesis by activating the canonical Wnt/β-catenin signaling pathway.

Discussion

Recently, extensive reports have indicated that as a major class of gene-regulatory molecules, miRNAs, are critical to various biological processes, such as tissue development, the onset of diabetes, cell differentiation, and proliferation (Ambros 2003, Martinelli et al. 2010, Guo et al. 2012, Bhushan et al. 2013). It is well documented that miRNAs...
miRNA let-7, miR-130, and miR-224 impair adipogenesis and lipid metabolism (Sun et al. 2009, Lee et al. 2011, Peng et al. 2013). In addition, miRNAs could regulate adipocyte differentiation and adipogenesis by targeting the canonical Wnt/β-catenin signaling pathway (Kennell et al. 2008, Qin et al. 2010).

Previous studies demonstrated that miR-135a-5p plays a role in the development of colorectal cancer, differentiation of osteoblasts, and regulation of blood pressure (Li et al. 2008, Nagel et al. 2008, Söber et al. 2010). miR-135a-5p may be a negative regulator during adipose tissue development according to a previous study (Chen et al. 2012). Moreover, a former study showed that human miR-135 family targets the Apc gene and induces the downstream canonical Wnt/β-catenin signaling pathway (Nagel et al. 2008), which is a molecular switch during adipogenesis. When the switch is on, adipogenesis is inhibited (Ross et al. 2000, Christodoulides et al. 2009). However, the role of miR-135a-5p in adipogenesis has not been reported, and it is necessary to clarify the mechanism of adipogenesis from the standpoint of miR-135a. Taking all these above into consideration, we proposed the
hypothesis that miR-135a-5p might function by affecting the canonical Wnt/β-catenin signaling pathway during adipogenesis. In our research, miR-135a-5p was identified as a novel miRNA that plays a negative role in 3T3-L1 preadipocyte differentiation and adipogenesis.

Firstly, we examined the expression level of miR-135a-5p during 3T3-L1 adipogenesis and found that miR-135a-5p was down-regulated during the whole process of adipogenic differentiation from day 0 to day 9 (Fig. 1). This result indicated that miR-135a-5p may act in 3T3-L1 preadipocyte differentiation. In order to determine whether miR-135a-5p influenced 3T3-L1 preadipocyte differentiation and adipogenesis, miR-135a-5p mimics were used to transfect 3T3-L1 preadipocytes and the cells were induced to undergo adipogenic differentiation. The findings demonstrated that the mRNA expression levels of adipogenic marker genes such as Pparg, Cebpa, Cmyc, and Fas were inhibited by overexpression of miR-135a-5p during adipogenesis (Fig. 2C and D). Meanwhile, the fat accumulation and triglyceride content were also reduced when compared with that in the positive and NC groups (Fig. 2A and B). All these findings supported the conclusion that miR-135a-5p served as a negative regulator during adipogenesis.

To screen miR-135a-5p targets and identify the direct binding pattern between miR-135a-5p and its targets, we used Targetscan and RNAhybrid, two bioinformatics target prediction software packages, to predict the targets of miR-135a-5p. We found that Apc 3′-UTR has two potential binding sites for miR-135a-5p (Fig. 3A). Luciferase reporter assays revealed that luciferase activity of Apc-w was reduced by approximately 50% after miR-135a-5p was used compared with the NC group, and the reductive luciferase activity was abolished when co-transfected with Apc-m3 and miR-135a-5p (Fig. 3B). Meanwhile, the mRNA and protein expression levels of Apc were repressed when miR-135a-5p was overexpressed, and vice versa (Fig. 3C and D). These results revealed that Apc was the real target of miR-135a-5p. Moreover, of the two binding sites, the second one played the major role; however, both of them had a synergistic effect on the interaction between miR-135a-5p and Apc.

APC is a key member of the canonical Wnt/β-catenin signaling pathway and is indispensable to signal transduction. In the presence of WntS, the cytoplasmic complex containing APC, axin, GSK3β, and β-catenin is disassembled. β-catenin which translocates into nucleus, first combines with members of the LEF/TCF family, and then activates CCND1 and CMYC (DENND4A) genes which are considered to be the targets of the APC pathway (He et al. 1998, Mann et al. 1999, Shtutman et al. 1999, Tetsu & McCormick 1999). This contributes to the activation of the canonical Wnt/β-catenin signaling pathway (Christodoulides et al. 2009). Meanwhile, it is well known that the canonical Wnt/β-catenin signaling pathway is a negative molecular switch during adipogenesis (Ross et al. 2000, Longo et al. 2004). Therefore, the mechanism by which miR-135a-5p regulated 3T3-L1 preadipocyte differentiation was further examined. This study found that miR-135a-5p contributed to the translocation of β-catenin protein into the nucleus, and it increased the expression of Ccnd1 and Cmyc during 3T3-L1 adipogenesis (Fig. 4), resulting in the activation of the canonical Wnt/β-catenin signaling pathway.

Earlier studies showed that the reduction in APC gene expression induces the downstream canonical Wnt/β-catenin signaling pathway (Nagel et al. 2008, Miclea et al. 2011). To further ascertain whether the reduction in APC caused by miR-135a-5p induces the canonical Wnt/β-catenin signaling pathway, we utilized siRNA oligonucleotides against Apc to mimic the effect of miR-135a-5p. It was found that lipid droplet accumulation and triglyceride contents in 3T3-L1 cells treated with siApc were, as expected, decreased when compared with the controls (Fig. 5B and C). In addition, the expression of adipogenic marker genes, such as Pparg, Cebpa, Ap2, and Fas, was also significantly inhibited (Fig. 5D). In contrast, the expression of Ccnd1 and Cmyc was markedly boosted (Fig. 5E). These results further illustrated that the anti-adipogenic effect of miR-135a-5p was ascribable to the inhibition of Apc and the stimulation of the

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**Figure 6**

Model of how miR-135a-5p impairs 3T3-L1 preadipocyte differentiation and adipogenesis by activating the canonical Wnt/β-catenin signaling pathway. miR-135a-5p inhibited Apc, activated the canonical Wnt/β-catenin signaling pathway, and blocked the expression of adipogenic marker genes such as Pparg and Cebpa, leading to impaired adipogenesis.
canonical Wnt/β-catenin signaling pathway during 3T3-L1 adipogenesis.

Taken together, these lines of evidence indicated that miR-135a-5p triggered the canonical Wnt/β-catenin signaling pathway by targeting Apc, and that the activated Ccn1l and Cmyc suppressed the expression of Ppara and Cebpa (Freytag & Geddes 1992, Antson et al. 1995, Fu et al. 2005), resulting in the obstruction of 3T3-L1 preadipocyte differentiation and adipogenesis (Fig. 6).

Accordingly, the fine control of preadipocyte differentiation mediated by miR-135a-5p may be a novel negative regulatory mechanism that offers profound insights into adipogenesis and adipose tissue development.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-14-0013.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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