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Journal of Cellular Biochemistry 116:2658–2666 (2015)

Journal of Cellular Biochemistry

MiR-25 Suppresses 3T3-L1 Adipogenesis by Directly Targeting KLF4 and C/EBPα

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ABSTRACT

In the past decade, miRNA emerges as a vital player in orchestrating gene regulation and maintaining cellular homeostasis. It is well documented that miRNA influences a variety of biological events, including embryogenesis, cell fate decision, and cellular differentiation. Adipogenesis is an organized process of cellular differentiation by which pre-adipocytes differentiate towards mature adipocytes. It has been shown that adipogenesis is tightly modulated by a number of transcription factors such as PPARγ, KLF4, and C/EBPα. However, the molecular mechanisms underlying the missing link between miRNA and adipogenesis-related transcription factors remain elusive. In this study, we unveiled that miR-25, a member of miR-106b-25 cluster, was remarkably downregulated during 3T3-L1 adipogenesis. Restored expression of miR-25 significantly impaired 3T3-L1 adipogenesis and downregulated the expression of serial adipogenesis-related genes. Further experiments presented that ectopic expression of miR-25 did not affect cell proliferation and cell cycle progression. Finally, KLF4 and C/EBPα, two key regulators of adipocyte differentiation, were experimentally identified as *bona fide* targets for miR-25. These data indicate that miR-25 is a novel negative regulator of adipocyte differentiation and it suppressed 3T3-L1 adipogenesis by targeting KLF4 and C/EBPα, which provides novel insights into the molecular mechanism of miRNA-mediated cellular differentiation. J. Cell. Biochem. 116: 2658–2666, 2015.

KEY WORDS: MicroRNA; miR-25; 3T3-L1; ADIPOGENESIS

INTRODUCTION

As an emerging global epidemic, obesity is a well-established risk factor for a variety of life-threatening diseases such as Type 2 diabetes, cardiovascular disease, osteoarthritis, and multiple types of cancer [Hotamisligil, 2006]. Characterized by deposition of excess adipose tissue, obesity arises from disruption of energy homeostasis and metabolic network, which eventually results in uncontrollable growth and expansion of adipocytes. As a well characterized in vitro model for the investigation of adipogenesis, 3T3-L1 has been extensively

All authors declare that they have no potential conflict of interest.

Grant sponsor: Croucher Foundation; Grant sponsor: National Basic Research Program; Grant number: 2012CB911201; Grant sponsor: National Natural Science Foundation; Grant numbers: 31171397, 31271533.

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Manuscript Received: 2 September 2014; Manuscript Accepted: 22 April 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 29 April 2015

DOI 10.1002/jcb.25214 • © 2015 Wiley Periodicals, Inc.

utilized to study the biological process of adipogenesis [Green and Kehinde, 1975]. The entire progression of 3T3-L1 adipogenesis comprises of serial cellular events including growth arrest induced by contact inhibition, clonal expansion triggered by hormonal induction, and terminal adipogenic differentiation.

During clonal expansion, a large number of adipocyte genes such as C/EBPα, PPARγ, aP2, and KLF4 would be transactivated and eventually initiate terminal adipocyte differentiation [Rosen and MacDougald, 2006]. Among the aforementioned transcription factors, C/EBPα has been shown to directly transactivate various adipogenic genes such as aP2 and SCD1 [Christy et al., 1989]. Moreover, the C/EBPα−/− mice displays shorter life span and profound hypoglycaemia and almost complete absence of white-adipose tissue in many depots except mammary gland, suggesting a vital role for C/EBPα in regulating the biogenesis of white adipose tissue [Linhart et al., 2001]. Aside from C/EBPα, several members of C/EBP family such as C/EBPβ and C/EBPγ, are preferentially expressed in mature adipocytes. The activation of these transcription factors during adipogenesis has been shown to be essential for terminal differentiation from pre-adipocytes towards adipocytes. On the other hand, as a novel regulator of adipogenesis, KLF4 was found to be a vital early modulator of adipogenesis. It was reported that KLF4 presented dramatic upregulation in pre-adipocytes within 30 min and reached its peak at 2 h in response to hormonal induction [Birsoy et al., 2008]. Further experiments demonstrated that KLF4 directly regulated the expression of C/EBPβ by cooperating with Krox2 and subsequently activated adipogenesis [Birsoy et al., 2008].

MiRNA, a class of small but functional RNA molecules, modulates gene expression at the post-transcriptional level and hence tightly controls a number of physiological or pathological progression including developmental timing, organ morphogenesis, cell differentiation and tumorigenesis [Alexander et al., 2011; Hilton et al., 2013; Qi et al., 2013; Ceppi and Peter, 2014]. Overexpression of miR-143, miR-210, and miR-17-92 promoted 3T3-L1 adipogenic differentiation [Wang et al., 2008; Yi et al., 2011; Liang et al., 2013], while enhanced expression of miR-27a/b and let-7 in 3T3-L1 cells impaired adipogenesis [Karbiener et al., 2009; Sun et al., 2009; Kim et al., 2010]. Previous studies showed that the miR-17-92 cluster was significantly increased during 3T3-L1 adipogenesis and elevated expression of miR-17-92 cluster accelerated 3T3-L1 adipocyte differentiation by targeting Rb2/p130 [Wang et al., 2008]. However, the function of the miR-106b-25, a paralog of polycistron miR-17-92 cluster, has not been previously demonstrated. In an effort to unveil the biological significance of miR-106b-25 cluster, we used 3T3-L1 preadipocytes to monitor their expression profiles during hormonal induction and verified that miR-25 displayed dramatic decrease during 3T3-L1 adipogenesis. Subsequent serial experiment showed that ectopic introduction of miR-25 reduced adipogenic ability through targeting KLF4 and C/EBPα, two important adipogenic factors.

MATERIALS AND METHODS

CELL CULTURE AND ADIPOCYTE DIFFERENTIATION

The mouse 3T3-L1 preadipocytes and human HEK293 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, USA). Two days post-confluence, the 3T3-L1 pre-adipocytes were subsequently subjected to adipogenesis for three days in the medium containing 10 μg/mL insulin (Sigma-Aldrich, USA), 0.5 μM dexamethasone (Sigma-Aldrich, USA) and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, USA). Then, the 3T3-L1 cells were maintained in the DMEM with 10%FBS and 10 μg/mL insulin until they were fully differentiated into mature adipocytes.

OIL RED O STAINING

At day 7, mature 3T3-L1 adipocytes were fixed with 10% formalin for 30 min. After fixation, the lipid droplets were visualized by 0.5% Oil Red O staining for 20 min. To quantify the intracellular lipid droplets accumulation, the stained cells were eluted with isopropanol and the optical density was measured at a wavelength of 510 nm by a microplate reader (Biorad, USA).

TRANSFECTION ASSAYS

MiRNA mimics were all synthesized by GneParma Co. (Shanghai, China). A small RNA with minimal sequence identity in mouse was used as negative control. The mouse KLF4 expression vector was a kind gift from Prof. Faye Tsang (The Chinese University of Hong Kong, Addgene No. 24193). The WZLneo-C/EBPα vectors were purchased from Addgene (No. 34567). All the oligonucleotide and plasmid transfections were performed by using Lipofectamine 2000 (Invitrogen, USA). For adipocyte differentiation, the 3T3-L1 cells were cultured in the six-well plates and transfected with 20 μL of 5 μM miRNA mimics or 500 ng plasmid. To enhance the transfection efficacy, the transfection was repeated at day −2, 0, and 2. For the luciferase reporter assay, HEK293 cells were culture in 24-well plates and transfected with miRNA mimics and the luciferase vectors containing 3′UTR fragments from KLF4 and C/EBPα.

RNA EXTRACTION AND REAL-TIME PCR

Total RNA was extracted by Trizol Reagent (Invitrogen, USA). The RNA reverse transcription were conducted by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) or One Step Primer Script miRNA cDNA Synthesis Kit (Takara, Japan). The relative mRNA expression level was quantitated using the Fast start Universal SYBR Green Master (Roche) in Viia 7 Real Time PCR System (Applied Biosystems, USA) according to the manufacturers' recommendations. The software RQ manager (Applied Biosystems) was utilized in the RT-PCR data analysis and the relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. The relative fold changes of candidate adipogenic marker genes were normalized to the house keeping gene GAPDH. Primer sequences for RT-PCR are listed in Table I.

XTT ASSAYS

Cell proliferation was measured using the XTT colorimetric assays (Promega, USA) following the manufacturer's recommendation. Briefly, 3T3-L1 cells transfected with oligonucleotides were seed into 96-well plate and incubated with colorimetric reagents XTT. Cell growth was monitored by measurement at the wavelength of 490 nm using the microplate reader (Biorad, USA).

TABLE I. Primers Used in This Work

Description	Primer sequence
RT-PCR primer	
KLF4_F	5′-GTGCCCCGACTAACCGTTG-3′
KLF4_R	5′-GTCGTTGAACTCCTCGGTCT-3′
C/EBPα_F	5′-GACATCAGCGCCTACATCGA-3′
C/EBPα_R	5′-TCGGCTGTGCTGGAAGAG-3′
C/EBPβ_F	5′-ATTTCTATGAGAAAAGAGGCGTATGT-3′
C/EBPβ_R	5′-AAATGTCTTCACTTTAATGCTCGAA-3′
PPARγ_F	5′-CAAGAATACCAAAGTGCGATCAA-3′
PPARγ_R	5′-GAGCTGGGTCTTTTCAGAATAATAAG-3′
GAPDH_F	5′-AGGTCGGTGTGAACGGATTTG-3′
GAPDH_R	5′-TGTAGACCATGTAGTTGAGGTCA-3′
Adiponectin_F	5′-TGTTCCTCTTAATCCTGCCCA-3′
Adiponectin_R	5′-CCAACCTGCACAAGTTCCCTT-3′
miR-106b	5′-TAAAGTGCTGACAGTGCAGAT-3′
miR-93	5′-CAAAGTGCTGTTCGTGCAGGTAG-3′
miR-25	5′-CATTGCACTTGTCTCGGTCTGA-3′
RNU19	5′-GCTCCAAGCACTGTTTTGGT-3′
Plasmid construction	
KLF4-3UTR_F	5′-ACACGAGCTCGGAAGACCAGGATTCCCTTG-3′
KLF4-3UTR_R	5′-GCTCTAGAGGCATACTTGGGAATAAACCA-3′
C/EBPα-3UTR_F	5′-ACACGAGCTCTAGGTTTCTGGGCTTTGTGG-3′
C/EBPα-3UTR_R	5′-GCTCTAGAAAGTCTTAGCCGGAGGAAGC-3′

FLOW CYTOMETRY ANALYSIS

3T3-L1 cells transfected with miR-25 mimics or negative control were cultured in six-well plates. Twenty-four hours post-transfection, 3T3-L1 cells were washed with cold PBS and fixed with 70% ethanol at 4°C overnight. The cells were subsequently stained with staining buffer containing 50 ug/ml Propidium Iodide, 50 ug/ml RNase, 0.37 mg/ml EDTA, and 1% Triton X-100 at 4°C for 30 min. The cell cycle progression was analyzed by using LSR Fortessa and FACSDiva software (BD Biosciences, USA).

WESTERN BLOT

Cellular protein was separated by 10% SDS-PAGE and then transferred to the PVDF membrane (Millipore, USA). The membranes were probed with the following antibodies: KLF4 (Santa Cruz, USA), C/EBPα (Santa Cruz, USA), C/EBPβ (Santa Cruz, USA), Egr1 (Santa Cruz, USA), and β-actin (Sigma, USA). The blots were detected on Super RX X-ray film (Fujifilm, Japan).

PLASMID CONSTRUCTION

The fragments of KLF4 and C/EBPα 3′UTR containing the putative binding sites for miR-25 were amplified from 3T3-L1 cDNA by PCR. These two fragments were inserted into pmir-GLO dual luciferase reporter vector (Promega, USA). The mutant constructs KLF4 or C/EBPα 3′UTR were generated by over-lapping PCR. Primer sequences were available in Table I.

LUCIFERASE REPORTER ASSAYS

HEK293 cells were seed onto 24-well plates and subsequently transfected with 10 μL of 5 μM miRNA mimics and 50 ng luciferase vectors harboring 3′UTR region from KLF4 or C/EBPα. 100 ng pRSV-β-Galactosidase vector was simultaneously transfected into HEK293 cells as an internal control for normalization. The firefly luciferase activity was monitored by using luciferase reporter gene assay kits (Roche, USA). The β-Galactosidase activity was quantitated by an o-nitrophenyl-β-galactoside (ONPG) colorimetric assays by using the microplate reader (Biorad, USA).

STATISTICAL ANALYSIS

All results were expressed as mean ± SD. Data were analyzed by Student′s independent t-test and the statistical differences were considered to be significant when $P < 0.05$.

RESULTS

MIR-25 SUPPRESSED 3T3-L1 ADIPOGENESIS

To shed light on the functional significance of miR-106b-25 cluster in adipogenesis, the 3T3-L1 preadipocytes after hormonal induction were used to monitor the temporal expression profiling of miR-106b-25 cluster. Quantitative RT-PCR (qRT-PCR) was conducted to evaluate the expression profile of miR-106b-25 cluster at specific time points (Day 0, 2, 4, 6, 8, and 10) during 3T3-L1 adipogenesis. Interestingly, we found only miR-25 showed dramatic downregulation in response to hormonal induction (Fig. 1A), while unexpectedly miR-106b and miR-93 did not present significant alteration during 3T3-L1 adipogenesis (Fig. 1B,C), suggesting distinctive regulatory mechanisms may exist within this cluster.

To characterize the biological function of miR-25 in adipocyte differentiation, 3T3-L1 cells were transfected with miR-25 mimics and subsequently subjected to hormonal induction to initiate 3T3-L1 adipogenesis. Afterwards, by periodically introducing miR-25 mimics into the 3T3-L1 cells, the lipid droplets were visualized by Oil Red O staining and quantified by the optical measurement. We found that less lipid droplets were formed in miR-25-treated group when compared with the control group, suggesting that miR-25 may impair lipogenesis (Fig. 1D). Thereafter, the respective mRNA levels of well-defined adipogenic markers were determined simultaneously and almost all adipogenic marker genes, including KLF4, C/EBPα, C/

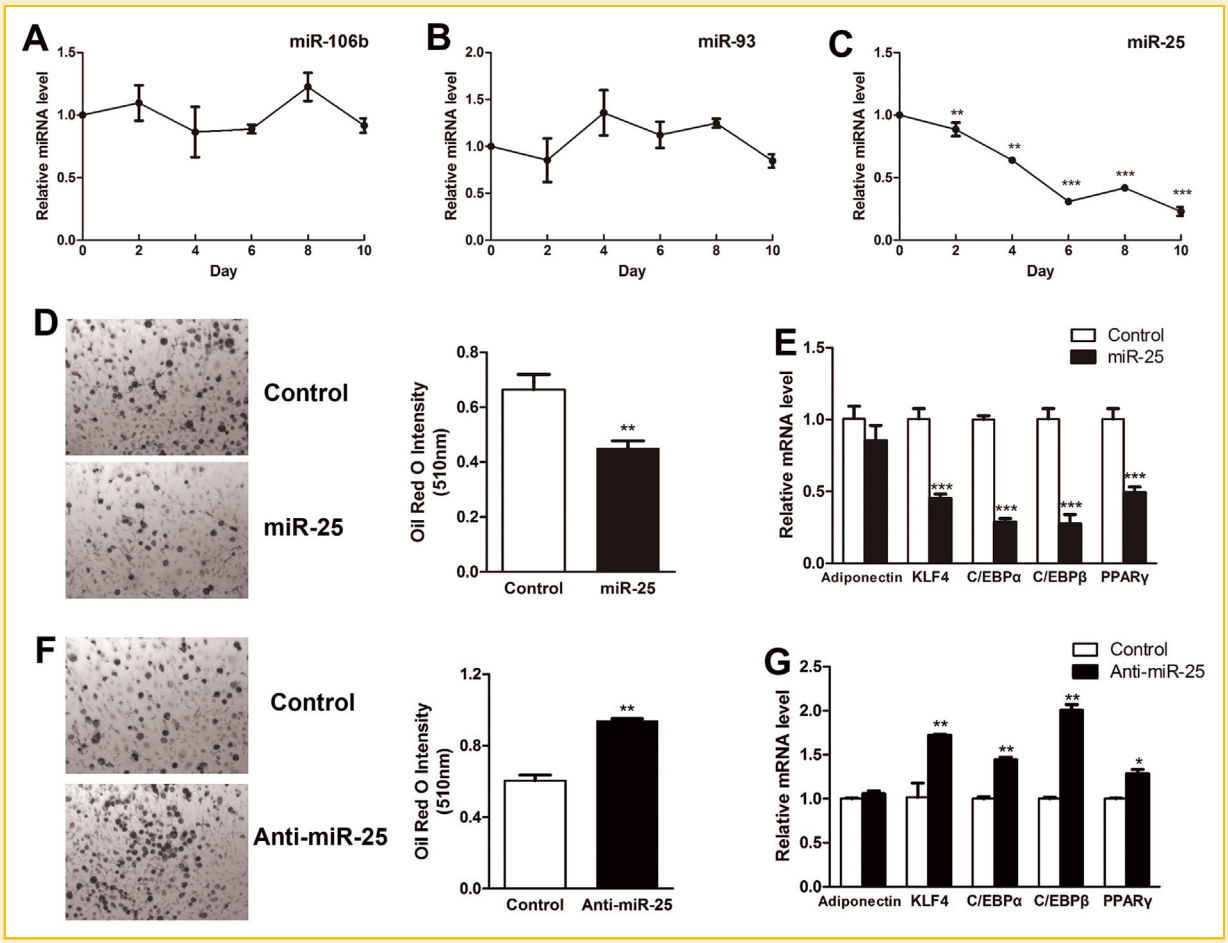

Fig. 1. Reintroduction of miR-25 suppressed 3T3-L1 adipogenesis. (A–C) Expression profiles of miR-106b-25 cluster during 3T3-L1 adipogenic differentiation were monitored using RT-PCR. Significant reduction of miR-25 was observed during 3T3-L1 adipogenesis. (D) Representative images of intracellular lipid accumulation were taken during 3T3-L1 adipocyte differentiation. The lipid droplets accumulation was quantified by eluting Oil Red O with isopropanol. Overexpression of miR-25 impaired 3T3-L1 adipogenesis. (E) RT-PCR analysis showed that overexpression of miR-25 downregulated serial adipogenic marker genes such as C/EBPα, C/EBPβ, PPARγ, and KLF4 at Day 3 after hormonal induction. (F) The lipid droplets accumulation status was captured and quantified. Overexpression of anti-miR-25 oligonucleotides promoted 3T3-L1 adipogenesis. (G) RT-PCR analysis showed that overexpression of miR-25 inhibitor reduced the mRNA levels of serial adipogenic marker genes such as C/EBPα and KLF4 after hormonal induction. ($n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

EBPβ, and PPARγ, displayed significant decrease after overexpression of miR-25 (Fig. 1E). However, Adiponectin, a regulator of fatty acid metabolism and glucose regulation, did not display a significant change in response to miR-25 overexpression (Fig. 1E). In addition, we examined the effect of miR-25 treatment on expression of transcription factors C/EBPβ and Egr1. From western blotting results, miR-25 attenuated the expression of adipogenic promoter C/EBPβ but potentiated the expression of adipogenic suppressor Egr1 (Supplementary Fig. 1).

To further elucidate the function of miR-25, we also utilized the inhibitor against miR-25 and monitored the effect of miR-25 inhibitor in modulating adipogenesis. Consistently, overexpression of anti-miR-25 oligonucleotides significantly promoted lipid droplet accumulation during 3T3-L1 adipocyte differentiation (Fig. 1F). Furthermore, most of the adipogenic marker genes presented dramatic increases after ectopic expression of anti-miR-25 oligonucleotides (Fig. 1G). Taken together, these results indicated that miR-25 might act as a putative negative regulator of 3T3-L1 adipogenesis.

MIR-25 DID NOT AFFECT 3T3-L1 CELL PROLIFERATION

Given that clonal expansion is one of the essential cellular events in early adipogenesis, we decided to address the effect of miR-25 in cell proliferation and cell cycle progression. The XTT assays displayed that ectopic expression of miR-25 did not influence cell proliferation (Fig. 2A). Furthermore, inhibition of endogenous miR-25 by synthetic miRNA inhibitors did not affect cell growth (Fig. 2A). Given that previous studies demonstrated that miR-106b-25 cluster could disrupt the G1 to S phase transition through targeting p21 and E2F1 [Petrocca et al., 2008; Kan et al., 2009], we next determined the function of miR-25 in controlling G1/S phase transition. However,

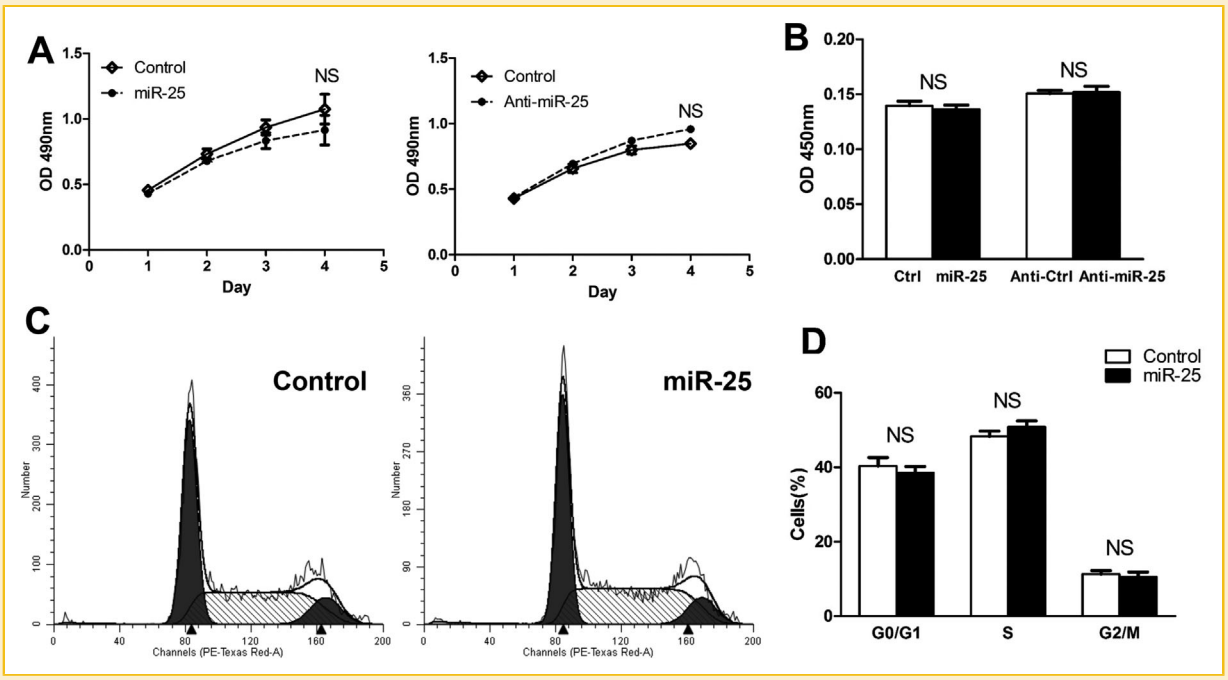

Fig. 2. Ectopic presence of miR-25 did not affect 3T3-L1 cell proliferation. (A) After transfection with miR-25, the effect of miR-25 in regulating cell proliferation was examined by XTT assay. XTT assay showed that neither overexpression of miR-25 nor inhibition of miR-25 affected cell growth. (B) After transfection with miR-25, the effect of miR-25 in modulating cell cycle transition was examined by BrdU incorporation assay. BrdU incorporation assay showed that miR-25 did not influence the G1/S phase transition. (C,D) Flow cytometry analysis showed that ectopic expression of miR-25 did not display significant changes on cell cycle progression. ($n = 3$; *, $P < 0.05$; **, $P < 0.01$).

unexpectedly, according to the results from BrdU assays, overexpression of miR-25 did not produce any significant alterations in S phase (Fig. 2B). In order to further evaluate the function of miR-25 in the regulation of cell cycle progression, flow cytometry was utilized. Similarly, enhanced expression of miR-25 by oligonucleotide transfection did not alter the cell cycle progression, although we noticed limited but not statistically significant repression on G1/S phase transition (Fig. 2C,D). In accordance with our results, similar results were achieved in a previous study [Petrocca et al., 2008], suggesting that miR-25 function is not essential for the proliferation of 3T3-L1 cells.

KLF4 AND C/EBPA WERE TWO BONA FIDE TARGETS FOR MIR-25

To identify the potential targets regulated by miR-25, we utilized the algorithm TargetScan, which makes use of thermodynamics-based model to mimic miRNA:mRNA duplex binding and thus predicts potential miRNA targets [Lewis et al., 2005]. Among the candidate genes, KLF4 and C/EBPα are of great interest due to their functional significance in the modulation of 3T3-L1 adipogenesis [Christy et al., 1989; Linhart et al., 2001; Birsoy et al., 2008]. According to sequence alignments, the target sequences for miR-25 within the 3′UTR regions of KLF4 and C/EBPα are highly conserved across a variety of species, including mouse, human, chimpanzee, rat and horse (Fig. 3A,B). RT-PCR results demonstrated that overexpression of miR-25 significantly suppressed the mRNA levels of KLF4 and C/EBPα genes (Fig. 3C). We also examined KLF4 and C/EBPα at several specific time points after hormone cocktails′ induction and the expression patterns of KLF4 and C/EBPα were showed (Supplementary Fig. 2), which presented that reintroduction of miR-25 significantly downregulated the adipogenic marker genes KLF4 and C/EBPα after hormonal induction. Furthermore, western blot results demonstrated that overexpression of miR-25 significantly suppressed the protein levels of KLF4 and C/EBPα (Fig. 3D), which is partially consistent with a previous study that miR-25 modulated airway remodeling through reducing KLF4 expression level in human airway smooth muscle cells [Kuhn et al., 2010].

To verify whether miR-25 downregulates KLF4 and C/EBPα by binding to their 3′UTR regions, the luciferase reporter plasmids harboring 3′UTR regions were constructed for experimental verification. The miR-25 predicted target sequences from mouse KLF4 or C/EBPα cDNA were inserted into the downstream 3′UTR region of the firefly luciferase gene. The luciferase reporters harboring 3′UTR regions were cotransfected with miR-25 mimics or negative control. The luciferase reporter assays presented significant repression in the luciferase activity after overexpression of miR-25 when compared with control groups (Fig. 3E). Further experiments showed that the deletion of miR-25 binding sites within the luciferase reporters completely abolished the aforementioned suppressive effect, suggesting that miR-25 specifically interacts with these binding sites within KLF4 and C/EBPα genes (Fig. 3E). Taken together, these results demonstrated that KLF4 and C/EBPα, two key modulators of adipocyte differentiation, were two direct targets of miR-25.

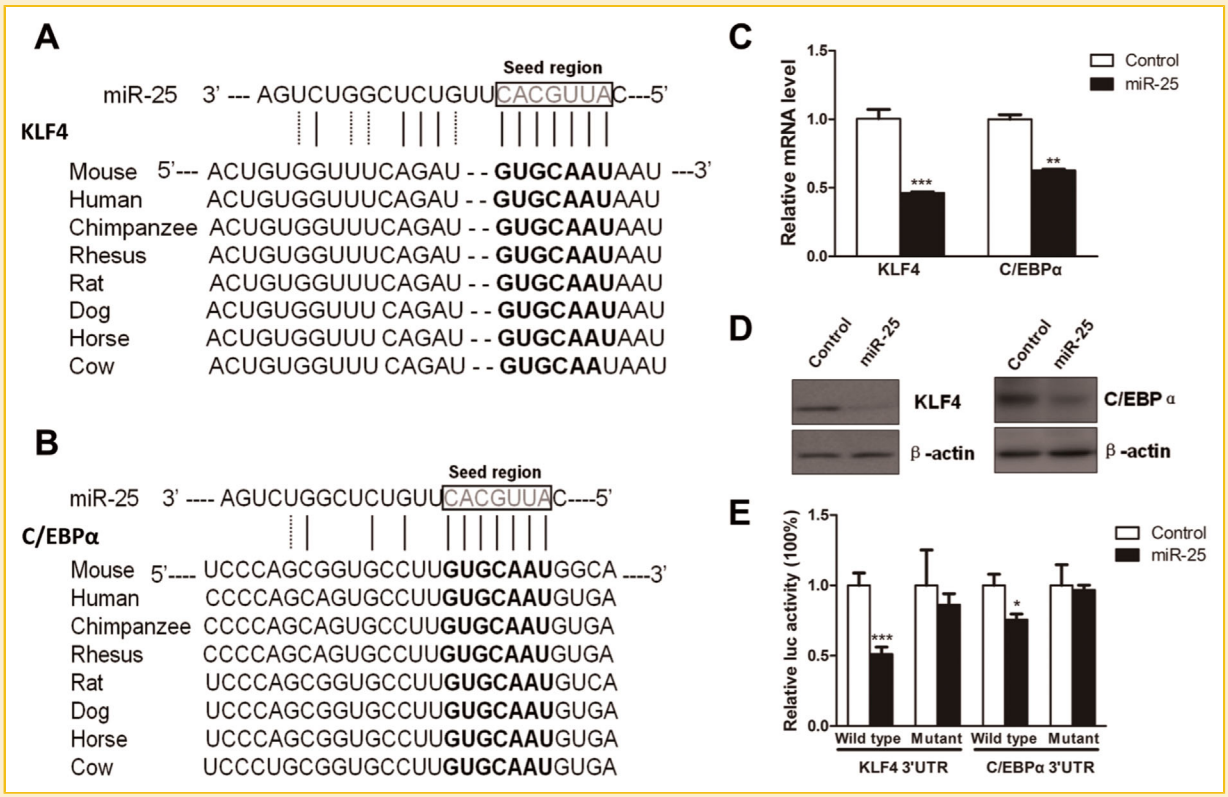

Fig. 3. MiR-25 directly targeted adipogenic markers KLF4 and C/EBPα. (A,B) The 3′-UTR region of either KLF4 or C/EBPα harbors a putative miR-25 binding site that is highly conserved in multiple species. (C) Overexpression of miR-25 dramatically reduced the mRNA levels of KLF4 and C/EBPα in the 3T3-L1 cells without hormonal induction. (D) Given the undetectable KLF4 and C/EBPα protein levels at day 0, the protein samples were collected at day 2 and subjected to Western blot analysis. At 48 h post-stimulation, enhanced expression of miR-25 repressed KLF4 and C/EBPα. (E) The interaction of miR-25 with 3′UTR region of either KLF4 or C/EBPα was confirmed by luciferase reporter assays in HEK293 cells. ($n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

EXOGENOUS EXPRESSION OF KLF4 OR C/EBPA INHIBITED THE FUNCTION OF MIR-25

We then performed rescue experiments to further validate whether ectopic expression of KLF4 or C/EBPα could attenuate the function of miR-25 in 3T3-L1 cells. The expression vectors of mouse KLF4 or C/EBPα genes were used in the experiments. According to the western blotting results, as expected, exogenous expression of KLF4 or C/EBPα could significantly upregulated the expression of KLF4 and C/EBPα, respectively (Fig. 4A,B). In order to further elucidate the effect of KLF4 and C/EBPα in regulating adipogenesis, 3T3-L1 cells overexpressing miR-25 were transfected with the KLF4 or C/EBPα expression vector plus their corresponding empty vectors. To evaluate the biological importance of KLF4 and C/EBPα in the modulation of 3T3-L1 adipogenesis, 3T3-L1 cells overexpressing miR-25 were transfected with mouse KLF4 or C/EBPα expression vector simultaneously. Thereafter, the adipocyte differentiation of 3T3-L1 cells was subsequently initiated by hormonal induction. The 3T3-L1 cells were fixed and the lipid droplets were quantified by Oil Red O staining at Day 7 post-induction. From Oil Red O staining, enhanced accumulation of lipid droplets was observed after overexpression of either KLF4 (Fig. 4C) or C/EBPα (Fig. 4D). The alterations in mRNA levels of several adipogenic marker genes after ectopic expression of KLF4 or C/EBPα were verified by RT-PCR (Fig. 4E,F). According to the RT-PCR results, it was showed that ectopic expression of KLF4 or C/EBPα can block the suppressive effect of miR-25 on adipogenic marker genes. Taken together, we successfully demonstrated that exogenous expression of KLF4 or C/EBPα significantly attenuated the function of reintroduced miR-25 mimics.

DISCUSSION

In this study, we found that miR-25, a member of miR-106b-25 cluster, was significantly downregulated during the differentiation from 3T3-L1 preadipocytes towards mature adipocytes. Overexpression of miR-25 dramatically suppressed 3T3-L1 adipogenesis and downregulated a number of adipogenesis-related genes. Further experiments demonstrated that KLF4 and C/EBPα were two novel direct targets of miR-25. Taken together, our experimental data showed that miR-25 exerted its anti-adipogenic effect partially through downregulation of two adipogenic promoters KLF4 and C/EBPα.

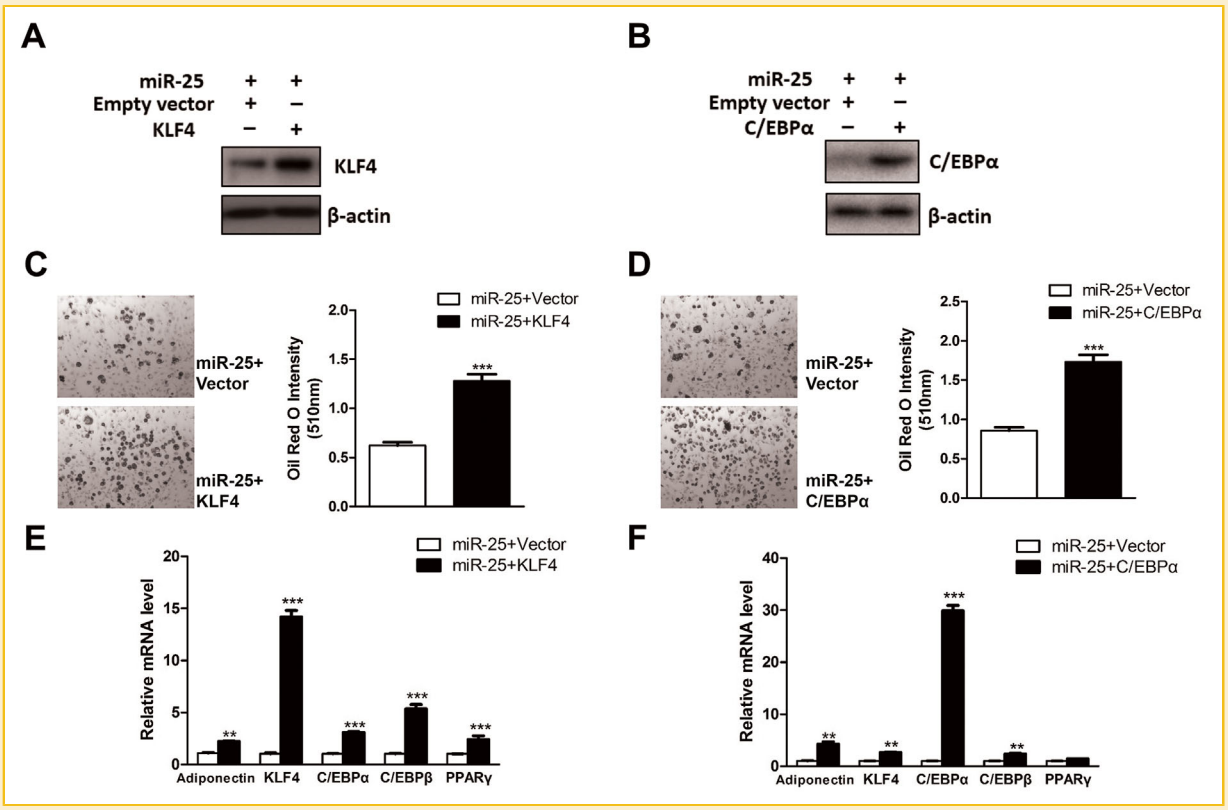

Fig. 4. Exogenous expression of KLF4 or C/EBPα inhibited the function of miR-25. (A) 3T3-L1 cells overexpressing miR-25 were transfected with mouse KLF4 expression vector simultaneously. The protein levels of KLF4 were monitored by Western blotting. (B) 3T3-L1 cells overexpressing miR-25 were transfected with mouse C/EBPα expression vector simultaneously. The protein levels of C/EBPα were monitored by Western blotting. (C) 3T3-L1 cells overexpressing miR-25 were transfected with mouse KLF4 expression vector. After transfection, the 3T3-L1 adipogenesis was initiated by hormonal induction. The effect of KLF4 in antagonizing the function of miR-25 was evaluated by Oil Red O staining. (D) 3T3-L1 cells overexpressing miR-25 were transfected with mouse C/EBPα expression vector. After transfection, the 3T3-L1 adipogenesis was triggered by hormone cocktails. The effect of C/EBPα in blocking the function of miR-25 was evaluated by Oil Red O staining. (E,F) Total RNA after overexpression of miR-25 plus KLF4 (E) or C/EBPα (F) in 3T3-L1 cells was prepared and mRNA alteration of serial adipogenic markers after hormonal induction was analyzed by RT-PCR. ($n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

According to our study, we showed that neither overexpression nor downregulation of miR-25 affected 3T3-L1 cell growth. As for the role of miR-25 in regulating cell proliferation especially cell cycle progression, contradictive results have been identified in previous reports [Petrocca et al., 2008; Rodriguez-Aznar et al., 2013]. Petrocca *et al.* showed that suppression on miR-25 function induced by LNA antisense oligonucleotides did not produce remarkable alteration in the cell growth of Snu-16 cells. Conversely, Rodriguez-Aznar *et al.* found that miR-25 directly downregulated CDKN1C mRNA level, leading to cell cycle re-entry and increased number of primary neurons. Collectively, these opposite results proposed that miR-25 may regulate cell proliferation through cell type-dependent mechanism. Another alternative explanation to elucidate the controversial role of miR-25 in modulating cell growth is that miR-25 may affect cell growth in a culture condition-dependent manner. A paradigm to support this hypothesis could be found in another non-coding RNA (ncRNA) termed H19. H19 is the first identified imprinted ncRNA and it has been shown to play an important role in a variety of biological events [Matouk et al., 2013]. From gain- and loss-of-function assays, H19 did not affect cell growth in the human bladder carcinoma cell line T24P under normal culture condition [Ayesh et al., 2002]. However, under stress condition such as serum starvation, H19 conferred a significant growth advantage for T24P cells [Ayesh et al., 2002]. Taken together, it is possible that miR-25 may exert its effect on cell proliferation in a cell type-dependent or culture condition-dependent manner and that is probably why miR-25 did not influence 3T3-L1 cell proliferation.

Given the potent role of miR-25 as an anti-adipogenic factor, it would be of great interest to investigate the biological importance of miR-25 in human adipogenesis. Considering that human mesenchymal stem cells (hMSCs) possess multipotency and they could differentiate into multiple cell types such as adipocytes, osteoblasts, and chondrocytes, bone marrow-derived hMSCs were used to investigate the expression alteration of miR-25. However, on the basis of side-by-side comparison with mouse 3T3-L1 preadipocytes, a slightly upregulated expression profiles were observed during adipogenic differentiation in bone marrow-derived hMSCs from two independent donors (data not shown). In spite of the unknown molecular mechanism, these data indicate another distinct role of

miR-25 in modulating human adipogenesis. Furthermore, this observation raised an interesting but elusive question of how miR-25 biogenesis is differently controlled under various intrinsic circumstances, which ultimately contributes to the elucidation of the missing links between adipogenesis and miRNAs.

It is well established that the maintenance of osteogenesis and adipogenesis of hMSCs is essential for cellular homeostasis and the disruption of this balanced homeostasis will ultimately result in many diseases such as osteoporosis and obesity [Zhang et al., 2010; Zhang et al., 2011a; Zhang et al., 2011b; Zhang et al., 2009]. Nevertheless, the factors orchestrating cell fate decisions remain to be elucidated. A large-scale analysis of thousands of genes differentially expressed during the terminal differentiation into osteoblasts or adipocytes was performed and the experimental data revealed that gene expression repression was the most prevalent event prior to commitment in both lineages [Scheideler et al., 2008]. Further computational data indicated that this gene repression resulted from miRNA-mediated regulatory mechanism [Scheideler et al., 2008]. Recently, the association of miR-106b-25 cluster with osteogenesis has been suggested by several independent groups [Gao et al., 2011; Zhang et al., 2012]. To evaluate the functional role of miR-25 in osteogenesis, it is important to monitor the expression profiles of miR-25 during cell differentiation of hMSCs towards osteoblast. This investigation would enable us to have a better understanding of how miR-25 is involved in cell fate decision. Moreover, deciphering the missing molecular mechanisms is of great importance for understanding the role of miR-25 in cell differentiation and providing clues for therapeutic strategy against adipogenesis-related diseases.

In conclusion, this present investigation documents a previous unknown role of miR-25 in modulating adipogenesis and provides evidence suggesting that miR-25 suppresses 3T3-L1 adipocyte differentiation through targeting KLF4 and C/EBPα.

ACKNOWLEDGMENTS

We thank Prof. Faye Suk-Ying Tsang for providing the mouse KLF4 expression vector and Prof. Stephen Kwok-Wing Tsui (The Chinese University of Hong Kong, Hong Kong) for providing the antibody against mouse KLF4. This study was supported by the National Basic Research Program (973 Program) (2012CB911201), the National Natural Science Foundation (31171397 and 31271533).

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