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ORIGINAL ARTICLE

The role and possible mechanism of lncRNA U90926 in modulating 3T3-L1 preadipocyte differentiation

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BACKGROUND: Obesity is a risk factor for metabolic diseases, while preadipocyte differentiation or adipogenesis is closely related to obesity occurrence. Long noncoding RNAs (lncRNAs) are a unique class of transcripts in regulation of a variety of biological processes. Using cDNA microarray, we found lncRNA U90926 is negatively correlated with 3T3-L1 preadipocyte differentiation.
OBJECTIVE: The aim of this study was to explore the role of lncRNA U90926 (lnc-U90926) in adipogenesis and the underlying mechanisms.
METHODS: Quantitative real-time PCR (qPCR) was performed to determine lnc-U90926 expression in 3T3-L1 preadipocytes, differentiated adipocytes, and in adipose tissues form mice. RNA fluorescent *in situ* hybridization (FISH) was performed to determine the localization of lnc-U90926 in 3T3-L1 preadipocytes. The effects of lnc-U90926 on 3T3-L1 adipogenesis were analyzed with lentivirus-mediated gain- and loss-of-function experiments. Lipid accumulation was evaluated by oil red O staining; several adipogenesis makers were analyzed by qPCR and western blotting. Dual luciferase assay was applied to explore the transactivation of target genes modulated by lnc-U90926. All measurements were performed at least for three times.
RESULTS: Lnc-U90926 expression decreased along the differentiation of 3T3-L1 preadipocytes. In mice, lnc-U90926 is predominantly expressed in adipose tissue. Obese mice have lower lnc-U90926 expression in subcutaneous and visceral adipose tissue than non-obese mice. FISH results showed that lnc-U90926 was mainly located in the cytoplasm. Overexpression lnc-U90926 attenuated 3T3-L1 adipocyte differentiation as evidenced by its ability to inhibit lipid accumulation, to decrease the mRNA levels of peroxisome proliferator-activated receptor gamma 2 (PPARγ2), fatty acid binding protein 4 (FABP4) and adiponectin (AdipoQ) as well as to reduce the protein levels of PPARγ and FABP4 ($P < 0.05$). Knockdown of lnc-U90926 showed opposite effects, which increased mRNA expression of PPARγ2, FABP4, CCAAT/enhancer-binding proteinα (C/EBPα) and AdipoQ.
CONCLUSION: Lnc-U90926 attenuates 3T3-L1 adipocyte differentiation via inhibiting the transactivation of PPARγ2 or PPARγ.

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INTRODUCTION

Obesity, characterized by excess fat accumulation, has become a global epidemic that raises the risk of many diseases, such as type 2 diabetes, cardiovascular disease and certain cancers.[1,2] Adipocyte development is closely related with obesity. Generally, obesity occurs when the number or size of adipocyte increases.[3] The process from preadipocyte to mature adipocyte is known as adipogenesis, which is a critical process for adipose tissue mass accumulation and development of obesity. Researches on adipogenesis modulation have drawn more and more attention to decipher the mechanisms of obesity.

The mechanism of adipogenesis is complex. Although the precise mechanisms of adipogenensis still remains unclear, it is generally accepted that peroxisome proliferator-activated receptor gamma (PPARγ), and CCAAT/enhancer-binding protein α (C/EBPα) are two critical factors or markers for adipogenesis.[4–7] As a transcription regulator, PPARγ is able to enhance the expression of many genes related to lipid synthesis or accumulation such as FASN,[8] PPARγ also cooperates with C/EBPα to enhance the expression of other differentiation relating genes, such as fatty acid binding protein 4 (FABP4) and hormone-sensitive lipase.[9]

Mouse 3T3-L1 preadipocytes is a well-established cell line commonly used to study adipocyte differentiation *in vitro*.[6,10,11] We exploited this system to analyse the adipocyte differentiation regulating genes by cDNA microarray, and demonstrated that a long noncoding RNA (lncRNA), U90926, (referred as lnc-U90926 hereafter) is negatively correlated with adipocyte maturation or lipid accumulation.

LncRNAs are a unique class of transcripts that have more than 200 nucleotides, often polyadenylated and lack functional open reading frames.[12–14] Previous studies have indicated that lncRNAs have regulatory roles in a variety of biological processes, such as X chromosomal inactivation,[15] chromatin remodeling,[16,17] transcriptional repression,[18] cancer metastasis.[19] Recent studies showed that lncRNAs, including steroid receptor RNA activator and lncRNA-n-AP, also have a role in white adipocyte differentiation.[20–22]

U90926 was first reported by a genome exploration research group as a long noncoding RNA.[23] However, the function of lnc-U90926 is unknown. We found that lnc-U90926 expression was decreased in mature adipocytes by microarray analysis. We therefore hypothesized that lnc-U90926 may be possibly involved in adipogenesis.

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To test our hypothesis, we applied different obesity animal models to examine the expression of lnc-U90926 expression in different adipose tissues and exploited 3T3-L1 preadipocyte to explore the mechanisms of lnc-U90926 in modulation of adipogenesis.

MATERIALS AND METHODS

Materials

The mouse embryo fibroblasts 3T3-L1 and human epithelial carcinoma cells (HeLa) were from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium was from Macgene Technology (Beijing, China), bovine serum, fetal bovine serum and puromycin were from GIBCO (Invitrogen, New York, NY, USA). Dexamethasone, 3-isobutyl-1-methylxanthie (IBMX) and insulin were purchased from Sigma (St Louis, MO, USA). LncRNA FISH (fluorescent *in situ* hybridization) kit (C10910), mouse-18S FISH Probe Mix (Lnc110104), lnc-U90926 FISH Probe Mix (C10920) and mouse-U6 FISH Probe Mix (Lnc110103) were synthesized by Guangzhou RiboBio (Guangzhou, China). All the restriction endonucleases were purchased from New England BioLabs (Beijing, China). Lnc-U90926 overexpression lentivirus was constructed and produced by Shanghai GenePharma (Shanghai, China) and lnc-U90926 knockdown lentivirus was constructed and produced by Shanghai Genechem (Shanghai, China). Anti-PPARγ (ab41928, 1:1000) and anti-FABP4 (ab81605, 1:1000) antibodies were Abcam (Cambridge, UK) products. Anti-beta Actin (TA-09, 1:1000) was obtained from Zhongshan Golden bridge-Biotechnology (Beijing, China). Secondary antibodies conjugated to HRP (1:5000) were purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China). The Ultra SYBR mixture was obtained from Beijing ComWin Biotech in China. All PCR primers used in this article were synthesized in Genomics Institute of Sangon Biotech in Shanghai, China. Lipofectamine 2000 and lipofectamine 3000 were from Invitrogen. Dual luciferase assay kit and antifade mounting medium were from Beyotime Biotechnology (Beijing, China).

Cell culture and cell chemical staining

3T3-L1 cells culture, differentiation and oil red O staining for lipid accumulation were performed as previously described.[24] HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C in a humidified atmosphere (95% air and 5% CO_2).

Microarray assay

Total RNA was isolated from 3T3-L1 cells with Trizol at indicated time point (day 0, 2, 4 and 13) in the process of differentiation. Global transcription profiling including coding and noncoding genes was performed in Beijing Capitalbio (Beijing, China). In order to find the differentially expressed genes, statistical analyses exploiting Bayesian analysis of variance for microarrays (BAMarray; http://www.bamarray.com) were accomplished by comparing day 2, 4 and 13 versus day 0, the undifferentiated preadipocytes.

RNA extraction and quantitative real-time PCR assay

Mature adipocytes were isolated from adipose tissues,[25] then RNA extraction and qPCR analysis were performed as described before.[26] PCR primers were designed by Primer 5.0 Software (Quebec, Canada) (Table 1). All experiments were conducted at least three times. Relative gene expression was calculated by the $2^{-\Delta\Delta\ Cq}$ method. Data were normalized to18S rRNA expression.

Animal model and analyses

Six-week old male C57BL/6 mice, *ob/ob* mice and *db/db* mice were purchased from the Beijing HFK Bioscience (Beijing, China). Animal experiments were performed under the guidance of the Principles of Laboratory Animal Care established by the National Institutes of Health and approved by the Animal Care and Use Committee at Shandong University. Mice were fed adaptively (C57BL/6 mice were fed with basal diet containing 5% fat, *ob/ob* mice and *db/db* mice high-fat diet containing 20% fat) in laboratory cages in a temperature-controlled room under a 12 h light–dark cycle with free access to water and food for a week. Then, C57BL/6 mice were divided into 3 groups randomly, the mice in one group were fed with a basal diet and the other 2 groups were fed with a high-fat diet (HFD). One group fed with HFD was conducted as a control for *ob/ob* mice and *db/db* mice. These *ob/ob* mice and *db/db* mice were kept feeding with HFD all the time until the body weights of these animals were 30% greater than that of control mice (C57BL/6 mice fed with HFD), then these transgenic obese mice and the control mice were analyzed.

Although another high-fat diet group (designated as HFD) were kept feeding in parallel with the group of C57BL/6 mice with basal diet (designated as BD) until the difference of body weight between HFD and BD reached 30%, then these obese mice (HFD) and the control mice (BD) were analyzed.

Table 1. The sequences of primers for PCR

Genes	*Sequence*
For qRT-PCR	
18S rRNA (+)	CGCGGTTCTATTTTGTTGGT
18S rRNA (−)	AGTCGGCATCGTTTATGGTC
lnc-U90926 (+)	TTCTCTGACTGGGGGTCCTC
lnc-U90926 (−)	AGCTGGAAGCATGATCCGAC
PPARγ (+)	TGGTGACTTTATGGAGCCTAA
PPARγ (−)	GGCGAACAGCTGAGAGGACTCTG
FABP4 (+)	AAGGTGAAGAGCATCATAACCCT
FABP4 (−)	TCACGCCTTTCATAACACATTCC
C/EBPα (+)	GCGGGAACGCAACAACATC
C/EBPα (−)	GCGGGAACGCAACAACATC
AdipoQ (+)	GCAGGGTGAGTCCAGTGATT
AdipoQ (−)	GTCCCACTCAAGGAGGACAG
For DNA molecular cloning	
pLVX-U90926 (+)	CCGCTCGAGCACACACACACACACACACACAC
pLVX-U90926 (−)	CGGGATCCTTTTTTTTTTTTTTTTTTTTAATGTAAGCTTTTTATTGACAC
pGL3-PPARγ2 −2000 bp (+)	CCCGGTACCCTCTTCCTCTATCCCGATGGTT
pGL3-PPARγ2 − 1500 bp (+)	CCCCGGTACCGACTTTAATGATTAATTTAG
pGL3-PPARγ2 − 1000 bp (+)	CCCCGGTACCACAAGTCACTGAATTATATTAG
pGL3-PPARγ2 − 500 bp (+)	CCCCGGTACCAATATTCTTCAGATGTGTGA
pGL3-PPARγ2 (−)	CCCCCTCGAGTCTATGTCTTGCAAAAGATTGGTTG
pGL3-C/EBPα −2000 bp (+)	CCCCGGTACCCTTATCCCACTTTCTTTGTTGATGACTG
pGL3-C/EBPα − 2000 bp (−)	CCCCCTCGAGGAGTTAGAGTTCTCCCGGCAT

Abbreviations: C/EBPα, CCAAT/enhancer-binding protein α; FABP4, fatty acid binding protein 4; PPARγ2, proliferator-activated receptor gamma 2.

The animals were killed following deep anesthesia with ether. Subcutaneous and visceral adipose tissues were collected and snap frozen in liquid nitrogen.

FISH assay

FISH assay was carried out according to previous studies.[27,28] Briefly, cells were rinsed briefly in PBS and then fixed in 4% formaldehyde for 10 min at room temperature. Cells were permeabilized in PBS containing 0.5% Triton X-100 for 5 min at 4 °C, then washed in PBS 3× 5 min. Hybridization was carried out with a FISH probe in a moist chamber at 37 °C in the dark overnight according to the protocol provided by the manufacturer. After RNA FISH, all images were obtained with a Zeiss LSM800 confocal microscope (Microscopy Core Facility of Shandong University).

lnc-U90926 overexpression or knockdown

For overexpression analysis, lnc-U90926 sequence was cloned into lentivirual expression vector LV5 (Shanghai GenePharma), then lentiviruses ($1x10^9$ TU ml^{-1}) were generated with Lentivector Expression Systems. 3T3-L1 preadipocytes were seeded at a density of 5×10^3 per well in 96-well plates. After 24 h incubation, the cells were infected with lentivirus (10 μl recombinant lnc-U90926 lentiviral stocks or control lentiviral stocks plus 90 μl complete medium). Twelve hour post-infection, the medium was replaced with fresh complete medium. Three to four days later, the infection rate of the cells could be directly observed with green fluorescent protein under fluorescent microscopy. The infected cells were removed from the wells and seeded into one 100 mm cell culture dish, then cultured with a drug pressure of 10 μg ml^{-1} puromycin for 2 weeks till stable clones became obvious. Stable monoclonal was transferred to 24-well plates and cultured in the medium with 5 μg ml^{-1} puromycin to amplify. qPCR analysis was used to select lnc-U90926 overexpression cell lines (designated as OV). And control cell lines (designated as NC) were generated in the same way described above with a control lentivirus. Eventually, we chose 3 pair NC and OV cell clones to conduct our experiments and the results were similar, so we showed only one pair in this article.

In order to knockdown the expression of lnc-U90926, the target sequences of lnc-U90926, were 5′-CCACTGAGCAGAAGAACTA-3′ for RNAi (2-1), 5′-TATCAAGATTCCCTGGCAA-3′ for RNAi(3-2) and 5′-ACACAG TGCCCATGCTACA-3′ for RNAi(4-1); the scrambled control sequence was 5′-TTCTCCGAACGTGTCACGT-3′. And these sequences were cloned into lentivirual shRNA expression vector GV118 (Shanghai Genechem) to generate lentivirus ($1x10^8$ TU ml^{-1}). First, we tested these three kinds of lentivirus with different target sequences, and found RNAi(3-2) was more effective to knockdown lnc-90926 expression, therefore, we chose the lentivirus with these target sequence for further experiments. 3T3-L1 preadipocytes were seeded at a density of 5×10^4 per well in 12-well plates. After 24 h incubation, the cells were infected with lentivirus (40 μl lnc-U90926 RNAi(3-2) lentiviral stocks or control lentiviral stocks plus 360 μl complete medium). Twelve hours post-infection, the medium was replaced with fresh complete medium. Three to four days later, the infection rate of the cells could be directly observed with green fluorescent protein under fluorescent microscopy. We could not isolate cell clones with drug selection for vector GV118 had no selection gene in mammalian cells. So we applied a mixed population to perform our experiments. The knockdown effect of lnc-U90926 was checked by qPCR. Lnc-U90926 knockdown cell pool was designated as KD whereas the scramble control cell pool was designated as CON.

Plasmids construction

The PPARγ2 and C/EBPα promoter (−2000 bp) constructs were generated as previously described, which were named as pGL3-PPARγ2 and pGL3-C/EBPα respectively.[24,29] The PPARγ2 promoter reporters of different lengths (−1500 bp, −1000 bp, −500 bp) were prepared in the same way as mentioned above. Lnc-U90926 expression vector was constructed by PCR-based amplification from cDNA of mouse 3T3-L1 preadipocytes and then subcloned into pLVX-IRES-ZsGreen1 Vector. The primers were listed in Table 1. All sequences of the new constructs were confirmed by DNA sequencing with the technical support from Sangon Biotech (Shanghai, China).

Luciferase reporter assay

Transfection was performed with Turbofect transfection reagent (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instruction as previously described.[24] MIX (10 μg ml^{-1} insulin, 0.5 mM IBMX and 1 μM dexamethasone), a reagent for adipogenesis, and TNF-α were served as positive and negative control for PPARγ2 transcativition in 3T3-L1 preadipocyes, respectively.[30,31] 3T3-L1 cells were co-transfected with 800 ng luciferase reporter plasmid (pGL3- PPARγ2 promoter or pGL3-basic) and 100 ng pRL-TK (a Renilla luciferase plasmid). For each transfection, 2 μl of lipofectamine 3000 transfection reagent was used. After 12 h, MIX or 50 ng ml^{-1} TNF-α were added to 3T3-L1 preadipocyes. Twenty-four hours after transfection, cells were lysed for luciferase assay. Luciferase activity was measured by a Dual Luciferase reporter assay kit from Beyotime Institute of Biotechnology (Shanghai, China) according to the manufacturer's instructions. Luciferase activity was normalized to Renilla luciferase activity. The luciferase assay of lnc-U90926 on C/EBPα promoter was similar to described above. The experiments were performed at least three times.

Western blotting

Protein samples were prepared and protein concentration was measured as previously described.[24] Samples were subjected to electrophoresis (SDS-polyacrylamide gel electrophoresis) with equal amounts of protein (30 μg). The primary antibodies used were as follows: PPARγ, FABP4 and Actin. Secondary antibodies conjugated to HRP from ZSGB-BIO (Beijing, China) were used for detecting the specific bands. All measurements were performed in triplicate. Relative densitometry was measured with Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data are presented as mean ± s.e.m. Analyses were performed using Graph Pad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). The statistical significance of comparisons between two groups was determined with Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Lnc-U90926 expression decreases during adipogenesis

LncRNAs expression during preadipocytes differentiation was evaluated by transcriptome microarray analysis. Adipocyte differentiation was confirmed by oil red O staining (Figure 1a). Microarray analysis showed that a lncRNA, lnc-U90926 (ENSMUSG00000029409, chr5:93285096-93290608), was apparently reduced during 3T3-L1 preadipocytes differentiation (Figures 1b and c), which was further confirmed by qPCR analysis. As a differentiation maker, PPARγ2 expression increased along preadipocyte differentiation (Figure 1d).

Lnc-U90926 is predominantly expressed in adipose tissue and localized in cytosol

To determine lnc-U90926 expression in other tissues, we examined its expression in seven other tissues by qRT-PCR besides epididymal adipose tissue (Figure 2a). Results showed that lnc-U90926 was predominantly expressed in white adipose tissue. Furthermore, we demonstrated that lnc-U90926 was mainly localized in the cytosol of 3T3-L1 preadipocytes by using RNA FISH assay (Figure 2b).

Lnc-U90926 expression in adipose tissue is reduced in obese mice

To explore the relationship between lnc-U90926 expression and obesity, we collected subcutaneous, perirenal and epididymal adipose tissues from C57BL/6 mice and obese mice including HFD obese mice, *ob/ob* mice and *db/db* mice. Lnc-U90926 expression was evaluated by qPCR. Results showed that lnc-U90926 levels were lower in adipose tissues from obese mice when compared to non-obese control mice (Figures 2c–e). The expression of PPARγ2, FABP4 and AdipoQ in mice epididymal adipose tissues was also measured by qPCR. Our data showed that these genes were

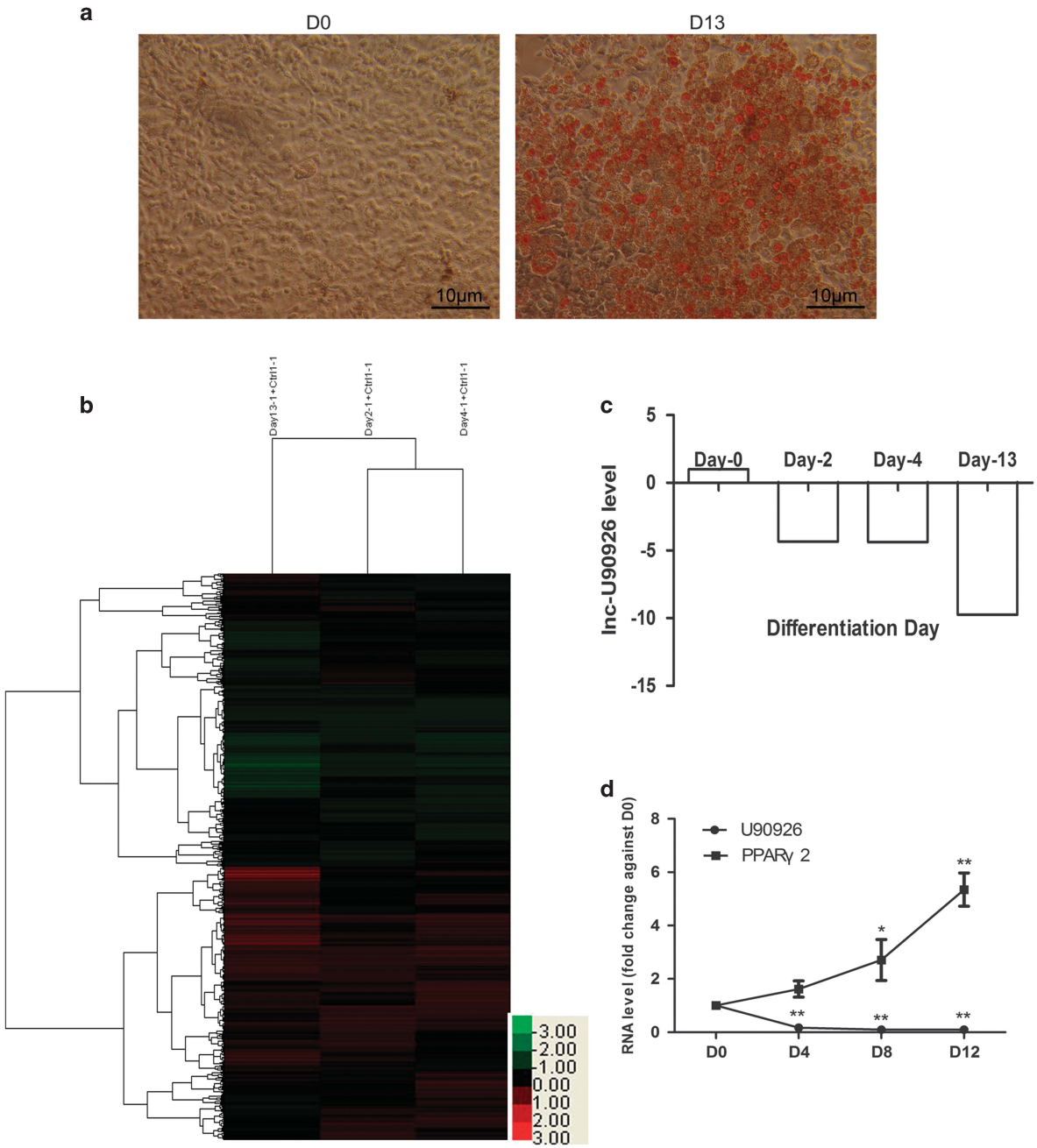

Figure 1. The expression of lncRNA U90926 during 3T3-L1 adipogenesis. (**a**) Lipid accumulation was observed with oil red O staining under light microscopy. Magnification 200×. (**b**) Global transcription profiling from our transcriptome microarray analysis on day 0, 2, 4 and 13 during 3T3-L1 preadipocytes differentiation. (**c**) lnc-U90926 expression was calculated with Bayesian analysis of variance for microarrays (BAMarray; http://www.bamarray.com) by comparing day 2, 4 and 13 versus day 0 undifferentiated preadipocytes. (**d**) The lnc-U90926 expression was normalized to the level of 18S rRNA with PPARγ2 as a positive control of adipogenesis. Black circles stands for U90926, black squares for PPARγ2. Results are shown as mean ± s.e.m. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus D0.

significantly increased in obese mice compared with non-obese control mice (Figures 2f and g).

Overexpression of lnc-U90926 attenuates adipogenesis in preadipocytes

To investigate the role of lnc-U90926 in the process of 3T3-L1 preadipocyte differentiation, we generated lnc-U90926 stably over-expressing 3T3-L1 preadipocyte cell line (OV) and control cell lines (NC) by infection with lentivirus encoding lnc-U90926 or with control lentivirus. qRT-PCR analysis showed an 160-fold increase in lnc-U90926 expression in OV cells over NC (Figure 3b). Overexpression of lnc-U90926 significantly prevented adipocyte differentiation, as evidenced by oil red O staining (Figure 3a). In addition, we also examined the expression PPARγ2, FABP4 and AdipoQ, markers of adipocyte differentiation, by qRT-PCR at d0 and d12 during adipogenesis. Results showed that lnc-U90926 overexpression significantly reduced the mRNA levels of PPARγ2, FABP4 and AdipoQ at day 12 as compared with NC group. Meanwhile, PPARγ2 and FABP4 were obviously decreased

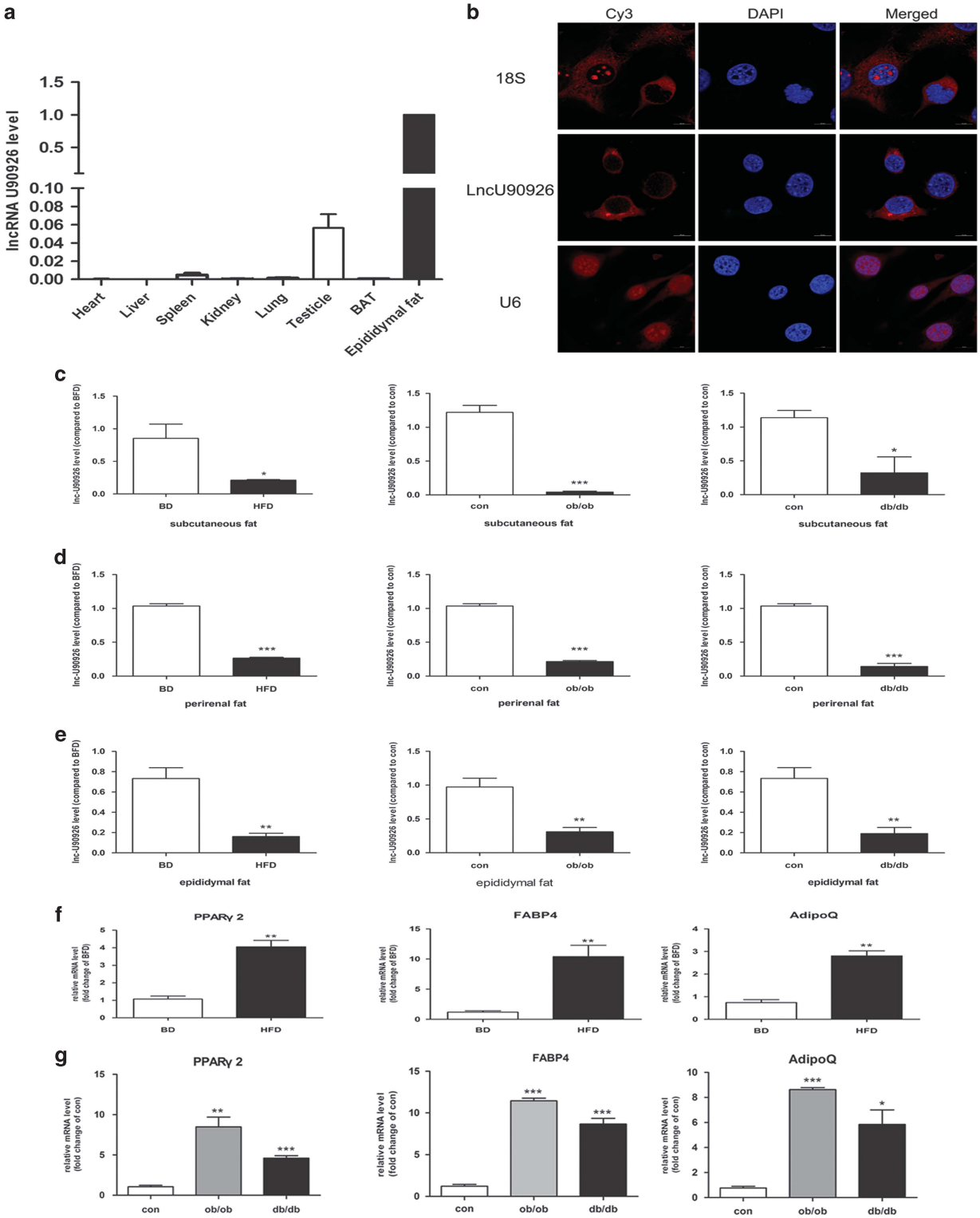

Figure 2. Lnc-U90926 expression, cellular localization and correlation with obesity. (**a**) Lnc-U90926 expression in different tissues from C57BL/6 mice compared with WAT. BAT stands for brown adipose tissue, WAT stands for white adipose tissue from epididymal fat, ($n = 6$). (**b**) Cellular localization analysis: RNA FISH assay of lnc-U90926 in 3T3-L1 preadipocytes. Scale bars, 10 μm. DAPI is 4′, 6-diamidino-2-phenylindole. Lnc-U90926 expression in (**c**) subcutaneous fat tissue, (**d**) perirenal fat tissue, (**e**) epididymal fat tissue extracted from obese mice including high-fat diet induced C57BL/6 obese mice, *ob/ob* obese mice and *db/db* obese mice or their parallel control mice. PPARγ2, FABP4 and AdipoQ expression in epididymal fat tissue from (**f**) HFD obese mice and (**g**) *ob/ob* obese mice and *db/db* obese mice compared with their controls ($n \geqslant 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control. In panels **d**–**f**, HFD stands for high-fat diet induced obese mice; whereas BD stands for mice fed with basal diet, which were used as control for HFD. The control mice for *ob/ob* obese mice and *db/db* obese mice were C57BL/6 mice fed with the same high-fat diet with these *ob/ob* mice and *db/db* mice.

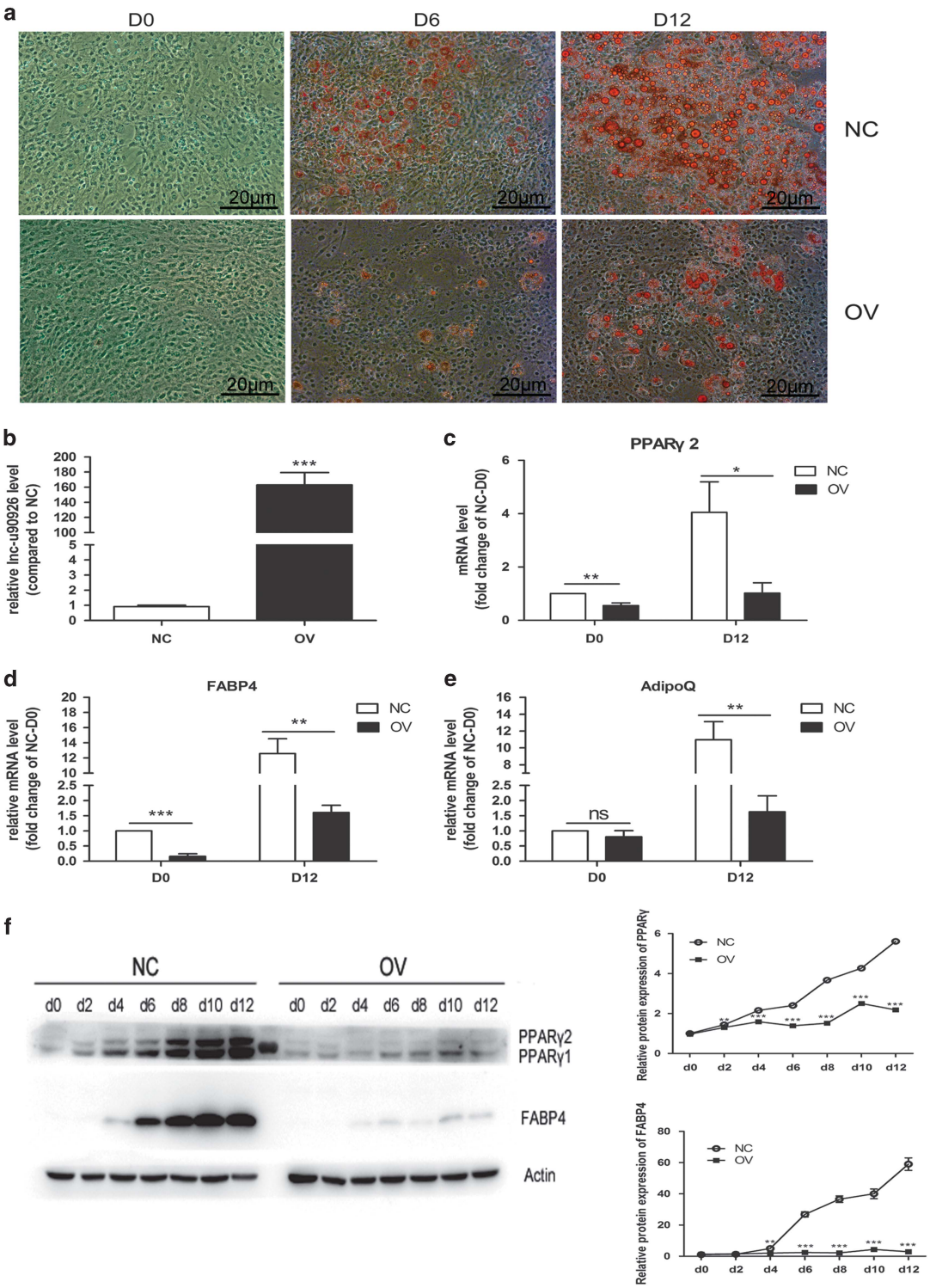

Figure 3. The effect of lnc-U90926 overexpression on adipogenesis. (**a**) Representative oil red O staining on day 0, 6, 12 from 3T3-L1 NC and OV cell clones. Magnification, 100×. (**b**) qPCR verification of 3T3-L1 cell lines stably over-expressing lnc-U90926 (designated as OV) and normal control clones (designated as NC). The expression of (**c**) PPARγ2, (**d**) FABP4, (**e**) AdipoQ on day 0 and day 12 during differentiation of 3T3-L1 NC and OV cell clones. (**f**) The protein expression level of PPARγ and FABP4 in NC and OV cells along with differentiation. The data show the means of three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus NC.

in OV compared with NC group at day 0 (Figures 3c–e). Accordingly, lnc-U90926 also markedly reduced PPARγ and FABP4 expression at protein level during adipocyte differentiation (Figure 3f).

Knockdown lnc-U90926 in 3T3-L1 cells enhances adipogenesis

To further verify a role of lnc-U90926 in adipocyte differentiation, we conducted lentivirus-mediated loss-of-function experiments. Three lentivirus containing shRNA sequence targeting lnc-U90926

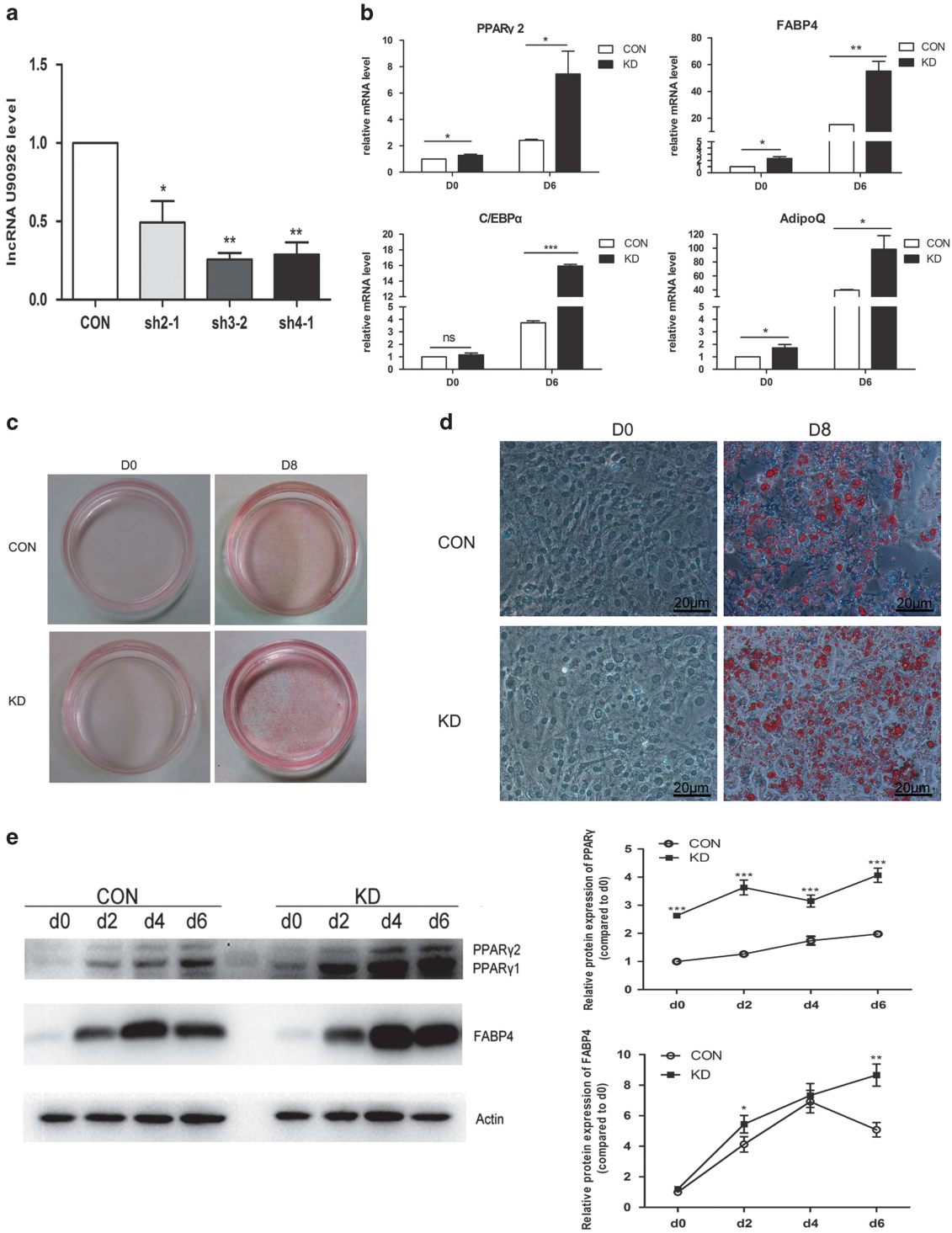

Figure 4. The effect of lnc-U90926 knockdown on adipogenesis. (**a**) qPCR verification of knockdown effect of three kinds of lentivirus encoding three different shRNA sequences targeting to lnc-U90926. (**b**) The expression of PPARγ2, FABP4, C/EBPα and AdipoQ on day 0 and day 6 during the adipogenesis of 3T3-L1 control cells (CON) and knockdown cells (KD) by qPCR. (**c**, **d**) Oil red O staining was performed to detect the lipid accumulation in CON and KD cells on day 0 and day 8. (**e**) The protein expression of PPARγ and FABP4 at different time points during the adipogenesis of CON and KD cells. The results are shown as the mean ± s.e.m. of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus CON.

and one lentivirus containing scramble control sequence were used to infect 3T3-L1 preadipocytes. After infection, the total RNA was extracted to determinate the lnc-U90926 knockdown efficiency of each lentivirus. As shown in Figure 4a, the three lentivirus encoding three shRNA sequences targeted to lnc-U90926 displayed marked knockdown efficiency. We chose lentivirus 3-2 for following experiments. Knockdown lnc-U90926 expression remarkably increased the mRNA levels of adipocyte markers including PPARγ2, CEBPα, FABP4 and AdipoQ at day 6 as evaluated by qRT-PCR. Knockdown of lnc-U90926 also increased

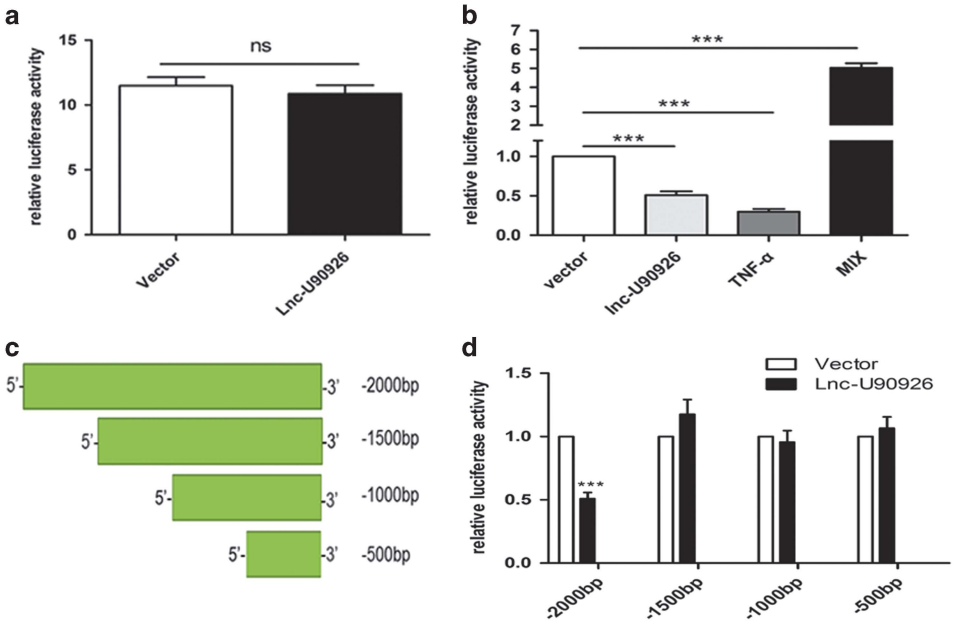

Figure 5. The effect of lnc-U90926 overexpression on the transactivation of C/EBPα or PPARγ2. (**a**) Relative luciferase activity of C/EBPα promoter in HeLa cells. (**b**) Relative luciferase activity of PPARγ2 promoter. (**c**) Diagram of PPARγ2 promoter of different lengths. (**d**) Relative luciferase activity of PPARγ2 promoters with different lengths in HeLa cells. The results are shown as the mean ± s.e.m. of three independent experiments. ***$P < 0.001$ versus Vector.

PPARγ2, FABP4 and AdipoQ expression at day 0 (Figure 4b), and increased lipid accumulation as evaluated by oil red O staining (Figures 4c and d).

Moreover, the protein levels of PPARγ and FABP4 were significantly elevated in the process of adipogenesis in lnc-U90926 knockdown cells compared with control cells (Figure 4e).

Lnc-U90926 inhibits PPARγ2 promoter transactivation

To explore the possible mechanisms underlying the effects of lnc-U90926 on adipogenesis, we examined whether lnc-U90926 influences the transactivation of certain essential factors such as CEBPα and PPARγ. Our data showed that lnc-U90926 overexpression did not change C/EBPα transactivation (Figure 5a) while obviously inhibited PPARγ2 promoter transactivation; TNF-α and MIX were exploited as negative and positive controls (Figure 5b). To narrow down the specific sequence of PPARγ2 promoter that is closely related to lnc-U90926 regulation, we constructed three truncated PPARγ2 promoters (−1500 bp, −1000 bp, −500 bp) and cloned them into pGL3 (Figure 5c). The luciferase activity of full length PPARγ2 promoter (−2000 bp) was much lower in lnc-U90926 overexpression cells than in vector control cells, but the other three shorter PPARγ2 reporters (−1500 bp, −1000 bp and −500 bp) displayed almost the same luciferase activity in lnc-U90926 overexpression cells as in control cells (Figure 5d). These results indicated that lnc-U90926 was able to inhibit the transactivation of PPARγ2 promoter probably through −2000 bp to −1500 bp.

DISCUSSION

Obesity has become a global epidemic that affects more than 500 million people worldwide, which increases the risks of type 2 diabetes, cardiovascular disorders, hypertension, thrombosis, hypertension, hyperinsulinemia and cancers.[1,2,32,33] Adipogenesis is defined as a process including adipocyte differentiation and lipid accumulation. Excess adipogenesis is closely related to body fat mass accumulation and obesity. However, precise mechanisms modulating adipogenesis have not been fully understood.

It is estimated that less than 2% of the mammalian genomes are ultimately translated into proteins while approximately half to two-thirds of genomic DNA is pervasively transcribed.[34–36] The ENCODE (Encyclopedia of DNA Elements) project to catalog human DNA elements revealed that 75% of the human genome is transcribed into noncoding RNA.[37] However, ncRNAs have been poorly conserved during evolution. ncRNAs lack open reading frames; their expression is significantly lower than mRNAs and usually is cell-type specific.[38] LncRNAs are a novel class of transcripts that contain more than 200 nucleotides. They were originally described as transcriptional noise, but recent studies indicated that lncRNAs may have critical roles in variety of biological process, including cell differentiation,[39] epigenetic gene regulation[16,40] and carcinogenesis.[19] There is evidence showing that lncRNAs may regulate adipocyte differentiation.[20–22,41]

Lnc-U90926 was first reported by a genome exploration research group as a long noncoding RNA.[23] However, its biological function is unknown. In our preliminary study, we found that lnc-U90926 level was significantly lower in mature 3T3-L1 adipocytes than preadipocytes by microarray analysis. Therefore, we proposed that lnc-U90926 might have a regulatory role in adipogenesis.

Using qPCR, we demonstrated that the RNA level of lnc-U90926 decreased along 3T3-L1 differentiation, which substantiated the findings by microarray. As previous studies showed that the presence of most lncRNAs is tissue specific,[19,22,42–44] we therefore examined the expression of lnc-U90926 in multiple tissues of mice. As expected, lnc-U90926 was predominantly in white adipose tissue. Moreover, we demonstrated that lnc-U90926 is mainly located in the cytoplasm while little is nucleus localized in 3T3-L1 preadipocytes.

To investigate the relationship between lnc-U90926 expression and obesity, we used three animal models including C57BL/6 mice fed with high-fat diet or basal diet, obesity mice (*ob/ob* and *db/db*). We determined the expression levels of lnc-U90926 in adipose tissues from different part of the body by qPCR. Results showed

that lnc-U90926 expression was significantly lower in adipose tissues from obese mice than those of control mice, revealing a negative correlation of lnc-U90926 expression with obesity.

To determine whether lnc-U90926 regulates adipogenesis, we generated lnc-U90926 overexpression and knockdown cells from 3T3-L1 preadipocytes by lentivirus infection. Results showed that lnc-U90926 overexpression apparently attenuated adipocyte differentiation, as evidenced by lipid accumulation and decreased expression of differentiation markers including PPARγ2, FABP4 or AdipoQ. In contrast, lnc-U90926 knockdown showed opposite effects. These data indicated that lnc-U90926 was a negative regulator of adipogenesis.

It has been reported that another lncRNA steroid receptor RNA activator exerted its effect on adipogenesis through associating with PPARγ and co-activating PPARγ dependent-reporter gene expression.[20,21] In this study, we examined whether lnc-U90926 influences the transactivation of PPARγ and CEBPα, two critical factors or markers for adipogenesis. We constructed luciferase reporter plasmids both for PPARγ2 and C/EBPα by ligasing the 2000 regulatory promoter region into luciferase reporter vector (pRL-luc).The results showed that lnc-U90926 was able to reduce the transactivation of PPARγ2 promoter but not C/EBPα. As the majority of lnc-U90926 was localized in cytoplasm, while PPARγ2 is nuclei-localized, it seems unlikely that lnc-U90926 directly interacts with PPARγ2 promoter. Further study is needed to address the details. In mice, PPARγ1 and PPARγ2 are two alternative splicing products of PPARγ. PPARγ2 is the full length of PPARγ; PPARγ1 is the shorter form of PPARγ. Actually, PPARγ1 shares the same promoter with PPARγ2. So the transactivation of PPARγ2 stands for total PPARγ.

Taken together, in this study we demonstrated for the first time that lnc-U90926 has an inhibitory role in adipogenesis in 3T3-L1 preadipocytes, and revealed a negative correlation of lnc-U90926 expression in adipose tissue with obesity in mice. One mechanism is possibly through suppressing the transcriptional activity of PPARγ.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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