ARTICLE
Journal of Cellular Biochemistry 114:2500–2512 (2013)

Journal of Cellular Biochemistry

Knockdown of PU.1 AS lncRNA Inhibits Adipogenesis Through Enhancing PU.1 mRNA Translation

Wei-Jun Pang,[1,2]* Li-Gen Lin,[2] Yan Xiong,[1] Ning Wei,[1] Yu Wang,[1] Qing-Wu Shen[1] and Gong-She Yang[1]

[1]*Laboratory of Animal Fat Deposition and Muscle Development, College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China*

[2]*Children's Nutrition Research Center, Baylor College of Medicine, Houston, Texas, 77030*

ABSTRACT

PU.1 is an Ets family transcription factor involved in the myelo-lymphoid differentiation. We have previously demonstrated that PU.1 is also expressed in the adipocyte lineage. However, the expression levels of PU.1 mRNA and protein in preadipocytes do not match the levels in mature adipocytes. PU.1 mRNA level is higher in preadipocytes, whereas its protein is expressed in the adipocytes but not in the preadipocytes. The underlying mechanism remains elusive. Here, we find that miR-155 knockdown or overexpression has no effect on the levels of PU.1 mRNA and protein in preadipocytes or adipocytes. MiR-155 regulates adipogenesis not through PU.1, but via C/EBPβ which is another target of miR-155. We also checked the expression levels of PU.1 mRNA and antisense long non-coding RNA (AS lncRNA). Interestingly, compared with the level of PU.1 mRNA, the level of PU.1 AS lncRNA is much higher in preadipocytes, whereas it is opposite in the adipocytes. We further discover that PU.1 AS lncRNA binds to its mRNA forming an mRNA/AS lncRNA compound. The knockdown of PU.1 AS by siRNA inhibits adipogenesis and promotes PU.1 protein expression in both preadipocytes and adipocytes. Furthermore, the repression of PU.1 AS decreases the expression and secretion of adiponectin. We also find that the effect of retroviral-mediated PU.1 AS knockdown on adipogenesis is consistent with that of PU.1 AS knockdown by siRNA. Taken together, our results suggest that PU.1 AS lncRNA promotes adipogenesis through preventing PU.1 mRNA translation via binding to PU.1 mRNA to form mRNA/AS lncRNA duplex in preadipocytes. J. Cell. Biochem. 114: 2500–2512, 2013.

KEY WORDS: PU.1; miR-155; ANTISENSE lncRNA; ADIPOCYTE; ADIPOGENESIS

Adipocytes, which derive from differentiation of preadipocytes, occupy a majority among white adipose tissue (WAT). Adipocytes are specialized cells that store excess energy in the form of triglycerides and secret adipokines that influence systemic energy homeostasis [Steppan et al., 2001]. Either too little or too much WAT contributes to metabolic abnormalities as hyperlipidemia, insulin resistance and type 2 diabetes [Dutchak et al., 2012]. Thus, maintaining the appropriate amount of WAT is crucial for the optimal health. The formation of adipocytes is dependent on PPARγ, C/EBPα, and other transcription factors.

The transcription factor PU.1 is a hematopoietic lineage-specific Ets family member that is absolutely required for normal hematopoiesis [Tenen, 2003]. The expression level of PU.1 is critical for specifying cell fate, and, if perturbed, even the modest decreases in PU.1 can lead to lymphomas [Rosenbauer et al., 2006]. However, our previous studies have found that PU.1 overexpression inhibits the differentiation of 3T3-L1 preadipocytes [Wang and Tong, 2008], indicating that PU.1 may also play a role in adipogenesis. Interestingly, the PU.1 mRNA and protein exhibit an opposite expression pattern in 3T3-L1 preadipocyte and adipocytes [Wang and Tong, 2008], with high mRNA level in preadipocyte but high protein level in mature adipocyte. It has been documented that PU.1 gene also expresses antisense long non-coding RNA (AS lncRNA) that antagonizes the expression of PU.1 protein [Ebralidze et al., 2008]. In addition, miR-155 has been reported to

The authors declare that there are no conflicts of interest.

Grant sponsor: National Key Basic Research Program of China; Grant number: 2012CB124705; Grant sponsor: Program of National Natural Science Foundation of China; Grant number: 30600437; Grant sponsor: Chinese Universities Scientific Fund; Grant number: QN2009021.

*Correspondence to: Wei-Jun Pang, PhD., Laboratory of Animal Fat Deposition and Muscle Development, College of Animal Science and Technology, Northwest A&F University, 22 Xinong Road, Yangling, Shaanxi Province 712100, China. E-mail: pwj1209@gmail.com, pwj1226@nwsuaf.edu.cn

Manuscript Received: 2 September 2012; Manuscript Accepted: 14 May 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 7 June 2013

DOI 10.1002/jcb.24595 • © 2013 Wiley Periodicals, Inc.

suppress PU.1 expression [Thompson et al., 2011]. Therefore, PU.1 AS lncRNA and miR-155 may be implicated in the regulation of the opposite expression pattern of PU.1 mRNA and protein.

MicroRNAs (miRNAS) are abundant ~22 nt regulatory RNAs, deriving from endogenous short hairpin transcripts that collectively play a key role in diverse developmental and physiological process in most eukaryotes [Flynt and Lai, 2008]. MiRNAs serve as antisense guides to identify regulatory targets [Czech and Hannon, 2011]. PU.1 is a direct target gene of miR-155 and it is also suppressed by miR-155 in B cell [Vigorito et al., 2007], bone marrow cells [Hu et al., 2010], and B-lymphoma cell lines [Thompson et al., 2011]. However, further study about whether miR-155 inhibits PU.1 expression during preadipocyte differentiation needs to be done.

Recent studies have elucidated the mechanistic control of gene expression by the modulation of ncRNA [Mercer et al., 2009; Wilusz et al., 2009; Misteli, 2010], suggesting ncRNA as a possible mechanism for controlling gene expression. LncRNAs frequently originate from intronic regions and are independently transcribed [Louro et al., 2009]. LncRNAs can mediate epigenetic changes by recruiting chromatin-remodeling complexes to specific genomic loci. Other lncRNAs have been shown to regulate transcription, whereas a few lncRNAs are AS transcripts, which may regulate mRNA dynamics at a post-transcriptional level [Mercer et al., 2009; Ørom et al., 2010]. AS lncRNAs at numerous genes loci act to silence sense transcription by affecting histone acetylation and methylation states [Camblong et al., 2007; van Dijk et al., 2011]. At present, the regulatory mechanism of AS lncRNA remains unclear, although there is evidence for the regulation by similar mechanisms as for protein coding genes [Li K and Ramchandran R, 2010].

Based on the above analysis, to testify our hypothesis which the involvement of miR-155 and PU.1 AS lncRNA in PU.1 mRNA and protein exhibited an opposite expression pattern in 3T3-L1 preadipocytes and adipocytes, we explored the expression and function of miR-155 and PU.1 AS lncRNA during adipogenesis. Here, our data indicate that miR-155 does not affect either PU.1 mRNA or protein levels in both preadipocytes and adipocytes, but the knockdown of PU.1 AS lncRNA promotes PU.1 protein expression and inhibits adipogenesis through attenuating PU.1 AS lncRNA binding to its mRNA to form mRNA/AS lncRNA duplex. The findings provide novel insight into the understanding of the biological functions of AS lncRNAs.

MATERIALS AND METHODS

MAIN REAGENTS

DMEM/F12, collagenase (type I), and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA). Insulin, dexamethasone, isobutylmethylxanthine (IBMX), Oil red O, and RNase A were obtained from Sigma (USA). Bodipy, TRIzol, SuperScript III reverse transcriptase Kit, Taq DNA Polymerase, dNTP, and Lipofectamine 2000 were purchased from Invitrogen (USA). NanoJuice transfection reagent kit was from Novagen (Germany). Triglyceride G Test Kit was purchased from Wako (Japan). RNase-free DNase I was obtained from Roche (Switzerland). BCA Protein Assay Kit was purchased from Thermo Scientific (USA). Mouse Adiponectin ELISA Kit was obtained from Bogoo (Shanghai, China). The antibodies employed in the analysis were as follow: PU.1 antibody (Signalway Antibody, USA), PPARγ, C/EBPα, and C/EBPβ antibodies (Santa Cruz, USA), GAPDH antibody (EarthOx, USA), adiponectin antibody (Affinity BioReagents, USA).

ANIMAL CARE

All experiments were made to minimize animal suffering and to reduce the number of mice used, in accordance with animal protection law of Lab Animal Center in Northwest A&F University.

ISOLATION OF SVF AND ADIPOCYTES FROM MOUSE WHITE ADIPOSE TISSUE

Epididymal fat pads from three 6-month-old C57BL/6 male mice were minced in Krebs–Ringer phosphate buffer and digested with 1 mg/ml collagenase type I at 37°C for 1 h as described in the literature [Wang and Tong, 2008]. Digested tissue was filtered through a nylon mesh and centrifuged at 500 rpm for 10 min. The top layer (adipocyte fraction) was collected. The remaining was centrifuged again at 1,500 rpm for 10 min, and the pellet (stromal-vascular fraction, SVF) was collected. Protein and RNA were extracted from both fractions. Briefly, the samples were homogenized in TRIZOL reagent and the total RNAs were extracted according to the manufacturer's instructions. The total RNAs were incubated with RNase-free DNase I to eliminate contaminated genomic DNA before being reversely transcribed into cDNA using RevertAid^{TM} First Stand cDNA Synthesis Kit. Adipocytes and SVF lysed in lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P (NP)-40, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM PMSF), respectively. Samples centrifuged at 8,000 rpm and 4°C for 15 min, collected protein supernatant.

CELL CULTURE

3T3-L1 cells were cultured to confluence in DMEM supplemented with 10% (v/v) calf serum. At 2 days post-confluence (designated day 0), the cells were induced to differentiate with DMEM supplemented with 10% (v/v) FBS, 1 μM dexamethasone, 0.5 mM IBMX and 1 μg/ml insulin for 2 days. Then every 2 days, media was changed with DMEM supplemented with 10% (v/v) FBS and 1 μg/ml insulin.

OVEREXPRESSION AND KNOCKDOWN OF miR-155 IN 3T3-L1 PREADIPOCYTES

The overexpression and knockdown of miR-155 constructs were provided by T. Deng (Methodist Hospital, TX). Scrambled shRNA (miR-155 shRNA, empty vector and miR-155 overexpression vector, respectively), packaging plasmid, and envelope protein plasmid were contransfected into HEK293T packaging cells (2×10^5 cells/well) by using Lipofectamine 2000 according to the manufacturer's instruction. After transfection (48 h), the supernatant containing viral particles was collected and passed through a 0.45 μm filter to remove cellular debris. 3T3-L1 preadipocytes were seeded at 1×10^5 cells per well and cultured in DMEM/F12 medium containing 10% FBS. On reaching 70–80% confluence, the viral suspensions containing 6 μg/ml polybrene were added. After infection, cells were induced using above method and were harvested for quantitative real time RT-PCR (qRT-PCR) and Western blot analysis at differentiation day 0 and day 7.

RNA ISOLATION AND qRT-PCR ANALYSIS

Total RNAs of different treatment cells were isolated using Trizol reagent. 1.5 μg RNAs were reverse-transcribed using SuperScript III reverse transcriptase Kit according to the manufacturer's instructions. cDNA was analyzed by ABI Prism 7700 qRT-PCR (Sequence Detection System, Applied Biosystems), using SYBR Green PCR Master Mix Reagent Kit (TaKaRa). Levels of gene expression were determined by using a standard curve. 18S rRNA was utilized as an internal control for cDNA normalization. Primers used for qRT-PCR are listed in Table I. The threshold cycle (CT) value was determined using the manual setting on the ABI Prism 7700 Sequence Detection System and exported into a Microsoft Excel Sheet for subsequent data analyses where the relative expression ratios of target genes were calculated by $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001].

RNA PROTECTION ASSAY

3T3-L1 preadipocytes and preadipocytes induced to 7 days were used to prepare total RNA. Total RNA preparations were then digested with RNase A, which digests single-stranded RNA and not duplex RNA, plus DNase I and DNase I alone at 37°C for 2 h [Li et al., 2010]. The treated RNAs were reverse-transcribed using SuperScript III reverse transcriptase Kit. And then, the protected duplex RNA fragments were detected using RT-PCR with the specific primers to amplify the overlapping region of PU.1 RNA (P3 and P4 in Fig. 4A). Amplification was initiated by degeneration at 95°C for 5 min and then following by 30 cycles: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min (10 min in the last cycle) in 25 μl reaction system including 16.5 μl sterile water, 5 μl 10 × PCR buffer $(NH)_2SO_4$, 2.5 μl 2 mM dNTP, 0.5 μl 25 μM forward primer, 0.5 μl 25 μM reverse primer, 1 μl 25 mM $MgCl_2$, 0.5 μl 0.5 U/μl Taq DNA Polymerase, and 1 μl cDNA. Primers used for RNA protection assay are listed in Table II.

PU.1 AS siRNA TRANSFECTION

PU.1 AS siRNA transfection was performed as described [Papetti and Skoultchi, 2007]. A 21bp double stranded RNA oligonucleotide targeting PU.1 AS siRNA (5-AAUCGUAAGUAACCAAGUCAU-3) and a control siRNA (5-AAGAGGAUAGGGAAGAGCUAU-3) were obtained from Qiagen. All siRNA oligos contained 3′dTdT overhangs. 3T3-L1 preadipocytes were seeded in 6-well plates at the concentration of 1×10^5 cells per well. Cells at 70–80% confluence in serum-containing medium without antibiotics were transfected with PU.1 AS siRNA 24 h later using NanoJuice transfection reagents according to the manufacturer's protocol. Transfected cells were then cultured in serum-containing medium for an additional 3 days before induction of differentiation under standard differentiation conditions [Luo et al., 2012]. At differentiation days 0, 1, 3, 5, and 7, cells were harvested for subsequent analysis.

TABLE II. Sequence of Oligonucleotides Used for RNase Protection Assay

Name	Primer sequences (5′ → 3′)	Product size (bp)
PU.1 AS		
P1	CCCAGCCCCAGTTTCCTCTGGGC	
P2	AAGGGCCTGCCACTGGGAGATAG	172
PU.1 S/AS		
P3	CTGACCCACGACCGTCCAGT	
P4	TCGCTGCCCACGAAGGAGT	230
PU.1 S		
P5	GTGGGTGGACAAGGACAAAG	
P6	GGCGACGGGTTAATGCTAT	303
β-actin		
Forward	TGCTGTCCCTGTATGCCTCTG	
Reverse	TTGATGTCACGCACGATTTCC	223

ADIPONECTIN SECRETION

Freshly isolated mature adipocytes were incubated with PU.1 AS siRNA and scrambled RNA for 48 h. Supernatants were collected to determine adiponectin concentrations using ELISA according to the manufacturer's instructions.

Establishment of retroviral-mediated PU.1 AS knockdown 3T3-L1 cells is shown in experimental procedures of our previous study [Lin et al., 2012]. Briefly, HEK293T cells were transfected with 20 μg of MSCV-LMP plasmids expressing PU.1 AS shRNA or scrambled shRNA using lipofectamine 2000 reagent in 10 cm plates. Medium was changed on the second day. Two days later, packaged retroviral particles in the supernatant were collected and filtrated. Viral suspension (0.75 ml) was then mixed with 0.25 ml of culture medium containing 4 μg of polybrene to infect 3T3-L1 cells in 6-well plates. Three hours after infection, medium was changed to fresh DMEM. At 2 days post-confluence, cells were induced to differentiate. At day 5

TABLE I. Sequence of Oligonucleotides Used for qRT-PCR

Name	Primer sequences (5′ → 3′)
miR-155	
Stem-loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCCCT
Forward	CGGCGGTTTAATGCTAATTGTGAT
Reverse	CCAGTGCAGGGTCCGAGGTA
PU.1 AS	
Forward	CCCAGCCCCAGTTTCCTCTGGGC
Reverse	AAGGGCCTGCCACTGGGAGATAG
PU.1 S	
Forward	ACCTTCCAGTTCTCGTCCAA
Reverse	CCTGTCTTGCCGTAGTTGC
18S rRNA	
Forward	AACGAGACTCTGGCATGCTAACTAG
Reverse	CGCCACTTGTCCCTCTAAGAA

after induction, fresh DMEM was changed for 48 h. Supernatants were collected for ELISA.

OIL RED O AND BODIPY STAINING

Cells were washed three times with PBS at differentiation day 7, fixed with 10% formalin in PBS, stained with 0.5% Oil Red O for 30 min or Bodipy for 15 min at room temperature [Melo et al., 2011], and photographed to measure total lipid accumulation.

OIL RED O EXTRACTION

Cells at differentiation days 0, 1, 3, 5, and 7 were washed three times with PBS and fixed with 10% formaldehyde for 30 min at room temperature. After washing, the cells were stained with 1% filtered Oil red O for 40 min at room temperature. Then Oil red O solution was removed. Intracellular triglyceride levels in the cells were agitatedly extracted with 100% isopropanol solution of 2,000 μl for 15 min in Shaker. Finally, a hole with PBS was used to adjust zero and OD value of each hole was detected in 500 nm using type of UV-2102 PC ultraviolet spectrophotometer (Unico Instrument Co., Ltd, Shanghai, China).

TRIGLYCERIDE CONTENT ASSAY

Triglyceride (TG) content analysis was conducted according to the previous method [Nakajima et al., 2003]. Briefly, cultured cells on six-well plates were washed twice with PBS, scraped off into 0.4 ml of 25 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, and then homogenized. TG in the cell lysate was extracted with the same volume of chloroform–methanol (2:1, v/v) and quantified enzymatically using a TG Test Kit.

WESTERN BOLT ANALYSIS

Cells were harvested and lysed in lysis buffer. Samples centrifuged at 8,000 rpm and 4°C for 15 min, and protein supernatant was collected. Protein concentrations were determined using BCA assay. 30 μg of total protein were separated by SDS-PAGE using a 10% polyacrylamide separation gel and a 5% polyacrylamide stacking gel. The proteins were transferred to nitrocellulose membranes. The membranes were blocked in TTBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% of Tween 20) containing 5% skim milk powder for 1 h and then probed with anti-PU.1 (1:500), PPARγ (1:1,000), C/EBPα (1:1,000), C/EBPβ (1:1,000), and adiponectin (1:1,000) polyclonal antibodies and anti-GAPDH monoclonal antibody (1:2,000) in TTBS with 5% milk powder in cold room at 4°C overnight. After three washings with TTBS for 10 min each, the membranes were incubated with either anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) diluted to 1:1,000 in TTBS with 5% milk for 1 h. Finally, the targeted protein was detected by ECL in dark room.

STATISTICAL ANALYSIS

All data were obtained from at least three independent experiments. Quantitative data are expressed as the means ± SEM. Statistics are calculated with SPSS statistics v13.0 software. Student's *t*-test is used for individual comparisons. Multiple comparisons are assessed by one-way ANOVA followed by Dunnett's tests. Difference between groups are considered statistically significant if $P < 0.05$.

RESULTS

EXPRESSION OF PU.1 AND miR-155 DURING PREADIPOCYTE DIFFERENTIATION

The 43–50 nucleotides of mouse PU.1 (NM_011355) 3′ UTR are predicted consequential pairing with the seed sequence of miR-155, which includes 8 nucleotides using TargetScan Release 6.2 (Fig. 1A; Fig. S1A, S1B). The level of miR-155 reaches the top at day 1 during preadipocyte differentiation and then declines, whereas its levels almost do not change from day 0 to day 7 under non-induced condition (Fig. 1B). The level of PU.1 mRNA is the highest at day 0 and the lowest at day 3 under the induced condition, but its levels have no significant difference from day 0 to day 7 under non-induced condition (Fig. 1C). Interestingly, the levels of PU.1 mRNA and protein have an inverse relationship in both 3T3-L1 preadipocytes and adipocytes (Fig. 1C,D).

OVEREXPRESSION OR KNOCKDOWN OF miR-155 DOSE NOT AFFECT THE EXPRESSION LEVELS OF PU.1 mRNA AND PROTEIN

To determine whether miR-155 regulates expression of PU.1 mRNA and protein, either overexpression or knockdown of miR-155 were performed in preadipocytes. The results indicate that overexpression of miR-155 markedly increases its expression, and knockdown of miR-155 significantly decreases its expression in both preadipocytes and adipocytes (Fig. 2A). Unexpectedly, at the pointed time levels of PU.1 mRNA and protein have no significant difference in either preadipocyte at day 0 or at day 7 after induction (Fig. 2B,C). Moreover, overexpression of miR-155 inhibits adipogenesis and knockdown of miR-155 promotes adipogenesis according to the results of Oil Red O staining (Fig. 2D), Oil red O extraction (Fig. 2E) and TG kit assay (Fig. 2F). We further find that miR-155 regulates adipogenic differentiation through mediation of C/EBPα, C/EBPβ, and PPARγ expression (Fig. 2G). Using TargetScan Release 6.2, the 454–461 nucleotides of C/EBPβ (NM_009883) 3′ UTR of C/EBPβ mRNA are the direct target of miR-155 (Fig. 2H; Fig. S2A, S2B).

DIFFERENTIAL EXPRESSION OF PU.1 mRNA AND AS lncRNA

PU.1 can simultaneously transcribe PU.1 mRNA and AS lncRNA which may potentially bind to form RNA–RNA duplex based on principle of base complementrity (Fig. 3A). To explore expression of PU.1 AS lncRNA during preadipocyte differentiation, qRT-PCR primers (Table I) were designed to detect the expression. The results show that level of AS lncRNA is significantly higher than that of mRNA in both 3T3-L1 preadipocytes and SVF extracted from the mouse adipose tissue, whereas there is an opposite expression pattern in adipocytes (Fig. 3B,C). The novel findings suggest that PU.1 AS lncRNA may be implicated in the regulation of adipogenesis.

IDENTIFICATION OF PU.1 AS lncRNA/PU.1 mRNA DUPLEX IN 3T3-L1 PREADIPOCYTES AND ADIPOCYTES

To detect PU.1 AS lncRNA binding to PU.1 mRNA in preadipocytes and adipocytes, RNase protection assay was carried out. Position of primers (Table II) used to check PU.1 AS lncRNA, RNA/RNA duplex and mRNA is shown in Fig. 4A. The results indicate that PU.1 AS lncRNA binds mainly to PU.1 mRNA in preadipocytes and less in

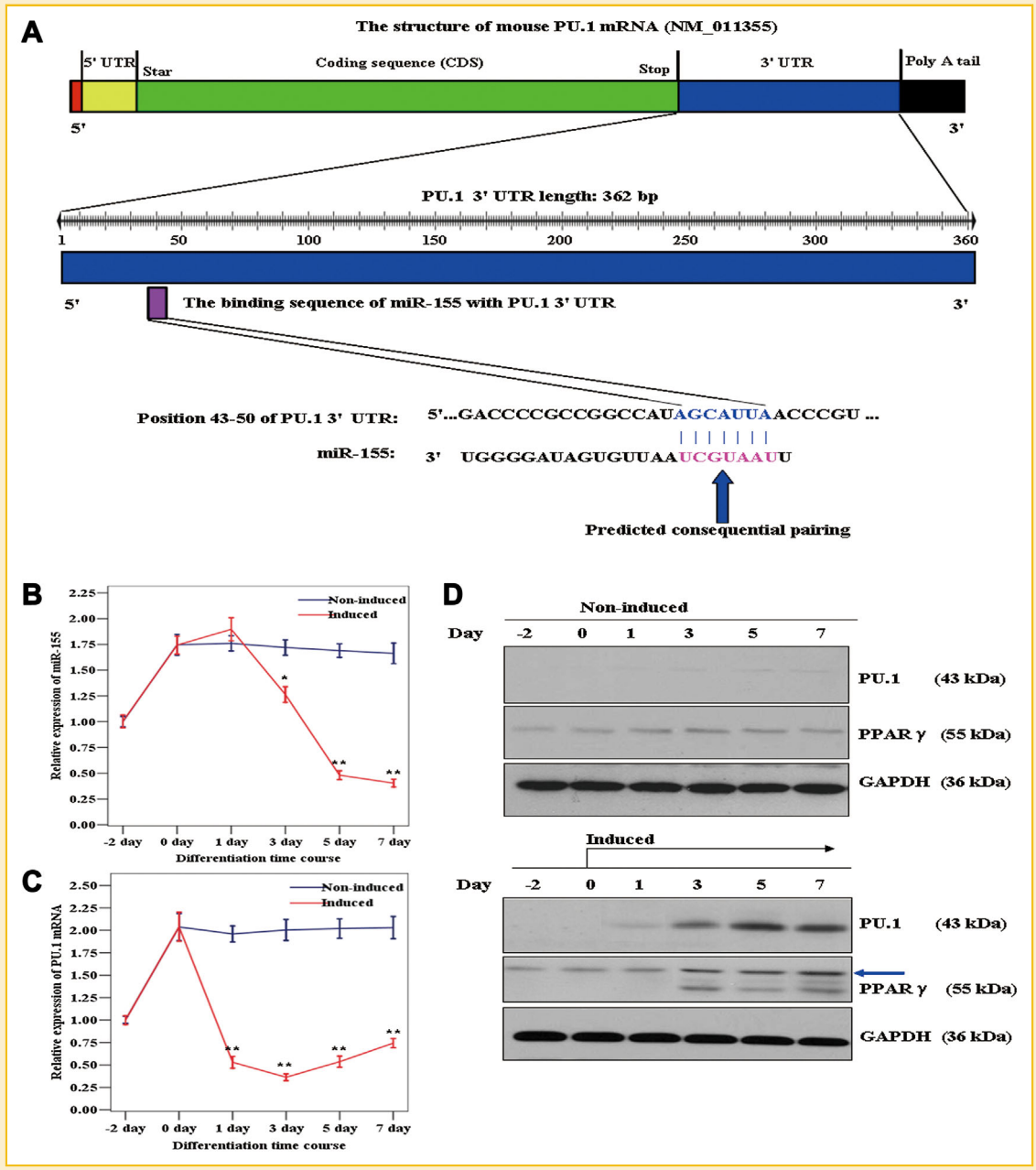

Fig. 1. Expression of PU.1 and miRNA-155 during 3T3-L1 preadipocyte differentiation. A: The predicted consequential pairing between PU.1 mRNA and miRNA-155. B,C: Time course expression of miRNA-155 and PU.1 mRNA during differentiation. Values represent the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$. The results are representative of three independent experiments. D: Time course expression of PU.1 protein in 3T3-L1 preadipocytes under non-induced (upper) and induced conditions (lower).

adipocytes (Fig. 4B). This binding may result in blocking translation of PU.1 mRNA in preadipocytes. It is an important reason why the levels of PU.1 mRNA and protein do not match in both 3T3-L1 preadipocytes and adipocytes. The model for underlying regulatory mechanism of PU.1 AS lncRNA in preadipocytes and adipocytes is shown in Fig. 4C.

KNOCKDOWN OF PU.1 AS lncRNA INHIBITS ADIPOGENNSIS

To further investigate the function of PU.1 AS lncRNA in adipogennsis, knockdown of PU.1 AS lncRNA by RNAi was performed in 3T3-L1 preadipocytes. The results indicate that knockdown of PU.1 AS lncRNA inhibits adipogenesis using Oil Red O staining (Fig. 5A left), Bodipy staining (Fig. 5A right), Oil red O extraction (Fig. 5B), and TG kit assay (Fig. 5C). PU.1 AS lncRNA siRNA significantly represses its level, resulting in increase of PU.1 mRNA level in both preadipocytes and adipocytes (Fig. 5D). PU.1 protein level is increased (Fig. 5E) owing to the increase of single strand PU.1 mRNA and the decrease of RNA-RNA duplex, leading to inhibiting preadipocyte differentiation through downregulation of PPARγ and C/EBPα expression level.

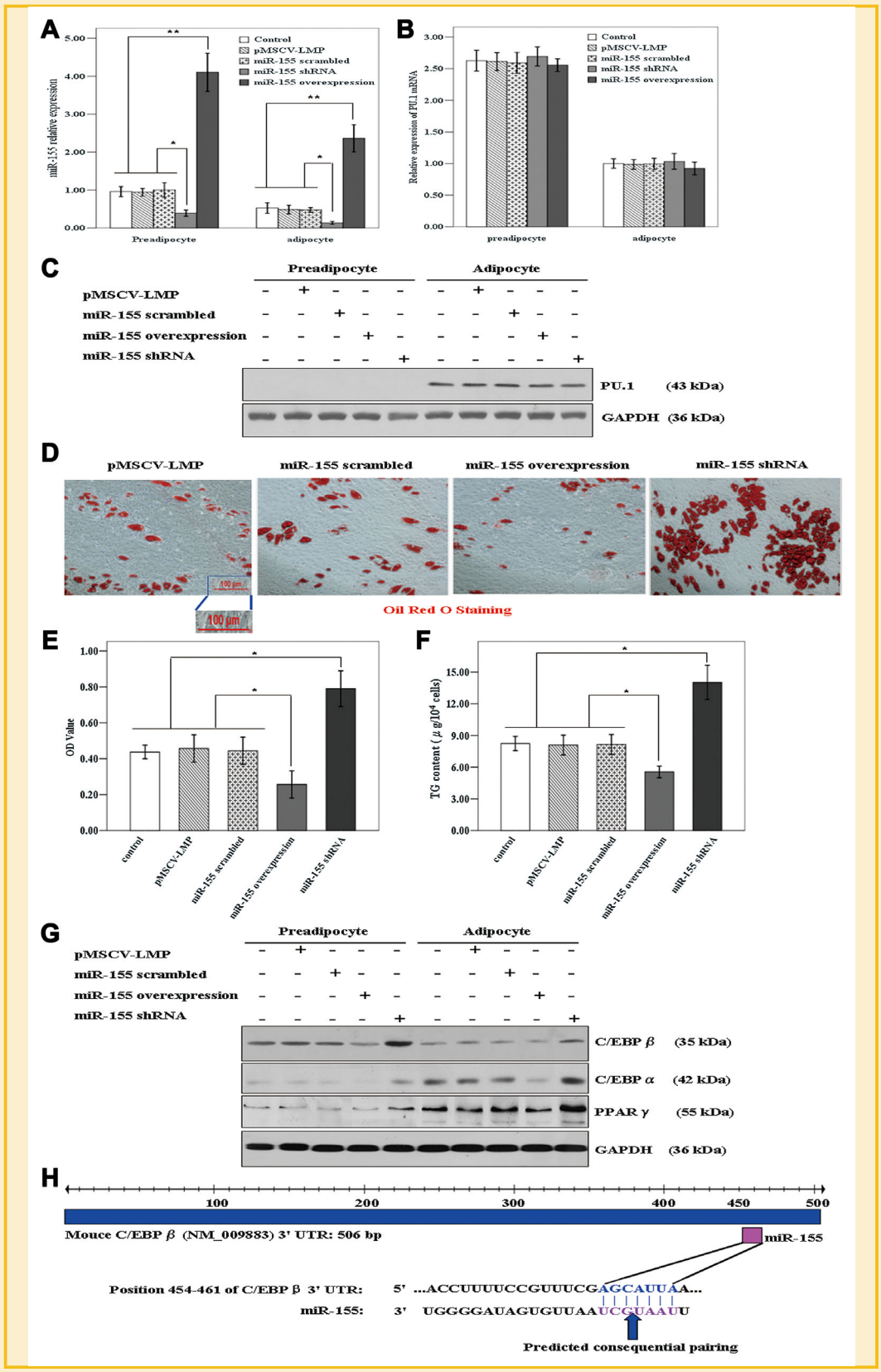

Fig. 2. Overexpression or knockdown of miRNA-155 does not affect the levels of PU.1 mRNA and protein in preadipocytes and adipocytes. Preadipocytes were infected with pMSCV-LMP empty vector, pre-miR-155, scrambled shRNA, and miR-155 shRNA, respectively. At differentiation day 0 and day 7, total RNA and protein were extracted for qRT-PCR and Western blot analysis. A: miRNA-155 expression. B: PU.1 mRNA expression. C: PU.1 protein expression. D: Oil Red O Staining at day 7. E: Analysis of Oil Red O extraction at day 7. F: Analysis of TG contents at day 7. G. C/EBPα, C/EBPβ, and PPARγ protein expression. H. The predicted consequential pairing between C/EBPβ mRNA and miRNA-155. Each bar indicates the mean ± SEM from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

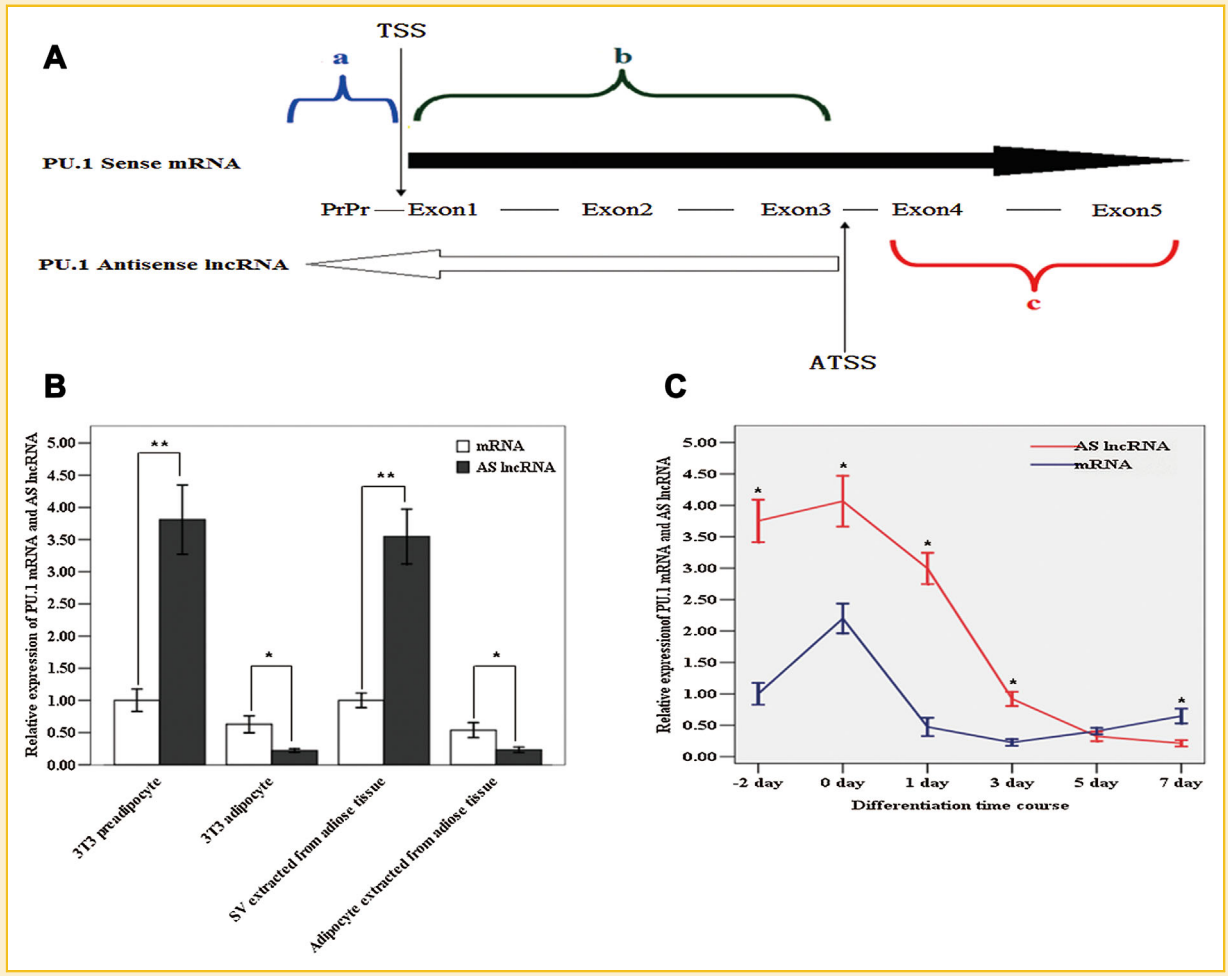

Fig. 3. The differential expression of PU.1 mRNA and AS lncRNA. A: Transcription diagram of PU.1 AS lncRNA and mRNA. PrPr, PU.1 gene proximal promoter; (a) non-overlap sequence of AS; (b) overlap sequence of AS lncRNA and mRNA; (c) non-overlap sequence of mRNA; TSS, sense transcriptional start site; ATSS, AS transcriptional start site. B: Expression of PU.1 mRNA and AS lncRNA in preadipocytes, SV and adipocytes. 18S rRNA was utilized as an internal control for cDNA normalization. C: Time course expressions of PU.1 mRNA and AS lncRNA during differentiation. Each bar indicates the mean ± SEM from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

KNOCKDOWN OF PU.1 AS lncRNA DECREASES EXPRESSION AND SECRETION OF ADIPONECTIN

Adiponectin, which is secreted from adipocytes, promotes preadipocyte differentiation and lipid accumulation [Fu et al., 2005]. Level of adiponectin protein reaches saturation by differentiation day 5 (Fig. S3). Since the level of adiponectin increases during 3T3-L1 preadipocyte maturation, it is thought that adiponectin could be used as adipocyte maturation marker [Ikeda et al., 2011]. To identify the effect of knockdown of PU.1 AS lncRNA on expression and secretion of adiponectin, we detected the expression level of adiponectin using Western blot during differentiation of PU.1 AS lncRNA deficient cells, and determined secretion of adiponectin by ELSA in mature adipocytes incubated with PU.1 siRNA. The results indicate that the level of adiponectin protein is significantly downregulated at differentiation days 3 and 7 in PU.1 AS deficient cells (Fig. 6A). Knockdown of PU.1 AS lncRNA also decreases secretion of adiponectin in mature adipocytes (Fig. 6B). Furthermore, we find that knockdown of PU.1 AS lncRNA by retrovirus-mediated shRNA significantly inhibits adipogenesis (Fig. 7A,B,C) and reduces expression and secretion of adiponectin in adipocytes (Fig. 7C,D).

DISCUSSION

The deposition of excess adipose in domestic animals and the human obesity epidemic has focused on adipose tissue and the development of preadipocytes, which are known as adipogenesis [Lefterova and Lazar, 2009]. PU.1 is an ETS family transcription factor involved in myelo-lymphoid differentiation [Choe et al., 2010; Takemoto et al., 2010]. Its role in controlling preadipocyte differentiation has been reported recently [Wang and Tong, 2008]. It is found that overexpression of PU.1 in murine 3T3-L1 cells inhibits preadipocyte differentiation [Wang and Tong, 2008], whereas knockdown of retroviral-mediated PU.1 AS in 3T3-L1 cells facilitates cellular differentiation [Lin et al., 2012]. Interestingly, the PU.1 mRNA and protein exhibits an opposite expression pattern in 3T3-L1

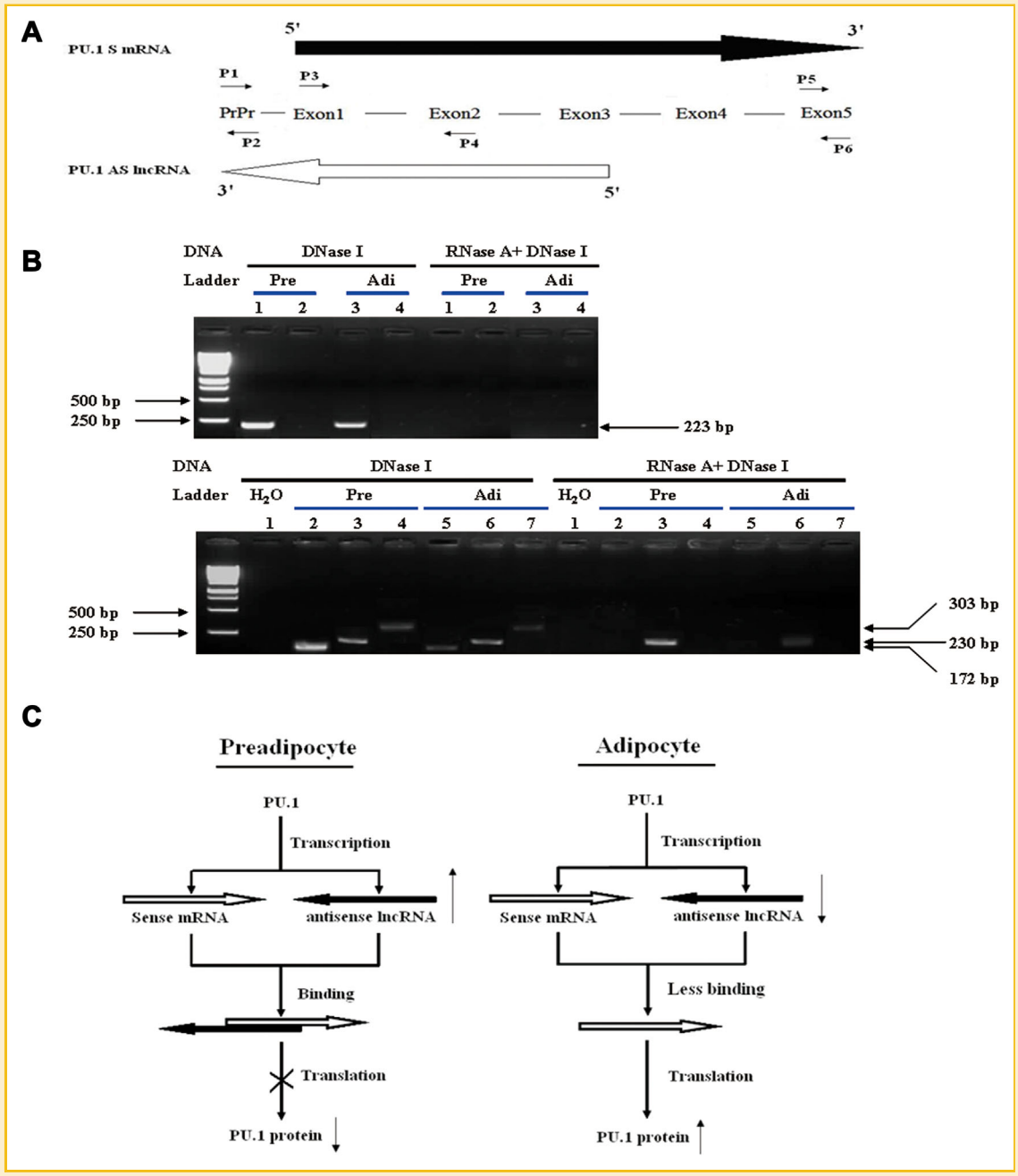

Fig. 4. Underlying regulatory mechanism of PU.1 mRNA and AS lncRNA. A: The position of primers used for detecting PU.1 mRNA, AS lncRNA, and mRNA/AS lncRNA. B: RNA protection assay. Total RNA extracted from 3T3 preadipocytes and adipocytes at differentiation day 7 were then digested with DNaseI alone, RNaseA (It digests single-stranded RNA, but not duplex RNA) plus DNaseI. 1–4 lanes are PCR products of β-actin which are the positive control (upper). 1 lane is the negative control (using H_2O instead of cDNA in RT-PCR mixture including β-actin primers); 2 and 5 lanes are PCR products of PU.1 S using primer P5 and P6 in Table II; 3 and 6 lanes are PCR products of S/AS using primer P3 and P4; 4 and 7 lanes are PCR products of AS using primer P1 and P2 (lower). Pre: preadipocyte, Adi: adipocye. C: The model for underlying regulatory mechanism of PU.1 mRNA and AS lncRNA in preadipocytes and adipocytes.

preadipocyte and adipocytes [Wang and Tong, 2008], with higher mRNA level in preadipocyte but higher protein level in mature adipocyte. Similar phenomenon has been reported on other genes. Conrads et al. (2005) investigated RNA and protein correlation by large-scale combined proteomic and microarray methods and found that many genes were positively correlated, but there were also a number of genes that showed reverse correlation. To date, the molecular mechanism controls for this opposite pattern of mRNA and protein expression is still unclear. As for the expressional pattern of PU.1 during adipogenesis, we think that microRNAs or AS lncRNA may be implicated in regulation of this phenomenon.

MicroRNAs play important roles in diverse physiological processes and are potential therapeutic agents. It was reported that miR-155 binds to the 3′-untranslated region of PU.1 mRNA to negatively

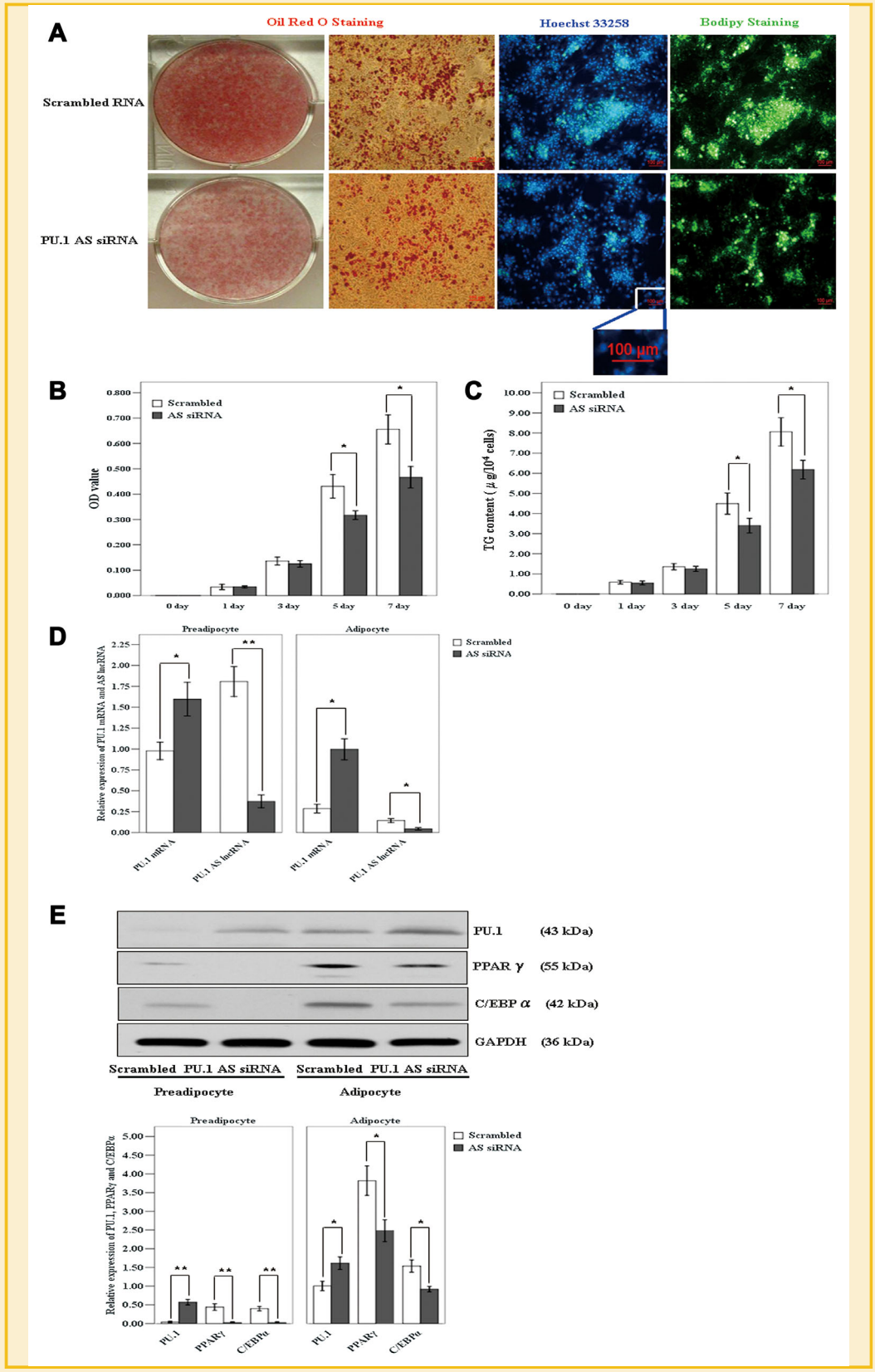

Fig. 5. Knockdown of PU.1 AS lncRNA inhibits adipogenesis. A: Oil Red O and Bodipy staining of adipocytes. Preadipocytes were transfected with PU.1 AS siRNA and scrambled RNA, respectively. At differentiation day 7, cells were stained using Oil Red O and Bodipy. B and C: Oil Red O extraction and TG contents during preadipocyte differentiation at the pointed times. D: Expression of PU.1 mRNA and AS lncRNA in preadipocytes and adipocytes. At day 0 and day 7 after induction, expression of PU.1 mRNA and AS lncRNA was checked by qRT-PCR. E: PU.1 protein levels are upregulated in preadipocytes and adipocytes transfected with PU.1 AS siRNA. Each bar indicates the mean ± SEM from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

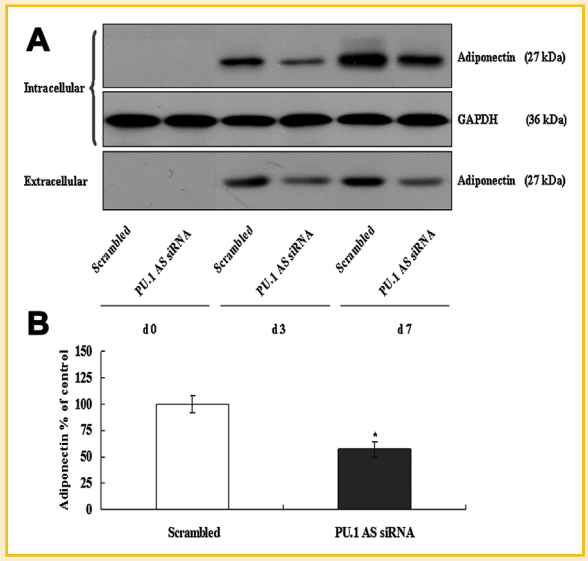

Fig. 6. PU.1 AS knockdown by siRNA decreases expression and secretion of adiponectin. A: Knockdown of PU.1 AS inhibits expression of adiponectin during 3T3-L1 maturation. 3T3-L1 preadipocytes were tranfected with PU.1 AS siRNA or scrambled RNA. At day 2, differentiation days 1 and 5, the cells were then cultured in fresh DMEM for 48 h, at which time the medium and total cell layer were harvested for Western blot analysis of adiponectin of intra- and extracellular proteins. B: Secretion of adiponectin is decreased in PU.1 AS deficient fat cells at differentiation day 7. Data represent the mean ± SEM of six independent experiments in duplicates. $^{*}P < 0.05$.

regulated PU.1 protein expression [Vigorito et al., 2007]. Moreover, overexpression of miR-155 in the THP1 monocytic cell line decreases PU.1 protein levels [Rocio et al., 2009]. Increased expression of the NF-κB target micro-RNA miR-155 is correlated with reduced expression of transcription factor PU.1 and CD10 in several B-lymphoma cell lines [Thompson et al., 2011]. Based on these findings, miR-155 may suppress PU.1 protein expression from PU.1 mRNA in preadipocytes. To our surprise, we find that neither miR-155 overexpression nor miR-155 knockdown affects the expression levels of PU.1 mRNA and protein. Therefore, miR-155 is unlikely to play a key role in the regulation of PU.1 mRNA and protein expression in preadipocyte and adipocytes. Interestingly, we find that miR-155 inhibits adipogenic differentiation through decrease of C/EBPβ expression causing downregulation of C/EBPα and PPARγ. In addition, besides miR-155 other predicted miRNAs (Fig. S1A) target PU.1 mRNA 3′ UTR, so it could not been ruled out these miRNAs may be involved in the difference observed between preadipocyte and adipocyte. At present, RNA-mediated translational interference is not restricted to microRNAs, but can also be mediated by AS lncRNAs [Ebralidze et al., 2008].

Natural AS transcripts (NATs) are RNA molecules that are transcribed from the opposite DNA strand and overlap in part with sense mRNA. AS RNA is a rather uncommon term in a physiology environment until short interfering RNAs emerged as the tool of choice to knock down the expression of specific genes [Werner and Berdal, 2005]. The mammalian genome contains large spans of AS lncRNAs and recent studies have indicated that some of these AS lncRNAs might be functional [Li et al., 2010]. They utilize several mechanisms, including DNA replication interference, chromatin remodeling, transcriptional interference, RNA masking, dsRNA-dependent mechanisms, and translation interference to mechanistically regulate gene expression [Michal and Yitzhak, 2006]. Recently, NAT levels have been identified as dysregulated in various disease states [Beltran et al., 2008; Faghihi et al., 2010]. Computational studies suggest that 15–25% of mammalian genes overlap, giving rise to pairs of sense and antisense RNAs [Katayama et al., 2005]. Many studies indicated that AS lncRNA decreases mRNA level, such as, AS lncRNAs of tie-1 [Li et al., 2010], fibroblast growth factor-2 (FGF-2) [MacFarlane and Murphy, 2010], mprF [Rubio et al., 2011]. It was reported that PU.1 gene also expressed mRNA and the AS lncRNA in HL-60 cells [Ebralidze et al., 2008]. Therefore, it is possible that PU.1 AS RNA is involved to account for the opposite pattern of PU.1 mRNA and protein expression in preadipocytes and adipocytes. At present, although it has not been reported that other lncRNAs are involved in the modulation, it cannot be excluded whether they mechanistically regulate PU.1 expression through DNA replication interference, chromatin remodeling, transcriptional interference, RNA masking and translation interference, to name a few. Based on above analysis, we suggest that PU.1 AS lncRNA might involve in translation interference of PU.1 mRNA.

To further investigate the levels of PU.1 mRNA and AS lncRNA expression in preadipocytes and adipocytes, we designed qRT-PCR primers based on PU.1 sense and AS sequences to detect their expression. Our results show that the expression level of PU.1 AS lncRNA is much higher than that of its mRNA in preadipocytes, whereas it is an opposite expressional mode in adipocytes. Therefore, PU.1 AS lncRNA plays a potential important role in regulation of PU.1 mRNA and protein levels. To explore the molecular mechanism involvement of PU.1 AS lncRNA, RNase protection assay was performed. The results indicate that a much more binding form of PU.1 AS lncRNA/mRNA duplex is found in preadipocytes than in adipocytes, hinting that translation of PU.1 mRNA is prevented in preadipocyes but not in adipocytes because there is still free PU.1 mRNA. We further find that knockdown of PU.1 AS lncRNA by siRNA or retrovirus-mediated shRNA inhibits adipogenesis through upregulating PU.1 protein level. Moreover, knockdown of PU.1 AS lncRNA represses preadipocyte differentiation via downregulating expression of the master genes (PPARγ and C/EBPα) and reducing expression and secretion of adiponectin. Further exploration is needed as to whether adiponectin is a specific or non-specific target PU.1 AS lncRNA. Therefore, the long-term or transient effects of a decrease in PU.1 AS lncRNA on adipogenesis are consistent.

Taken together, our findings uncover a novel molecular mechanism on adipogenic regulation by PU.1 AS lncRNA. Partial complementary binding of PU.1 AS lncRNA and its mRNA forms AS lncRNA/mRNA duplex interrupt translation of PU.1 mRNA, resulting in the opposite expression pattern of PU.1 mRNA and protein in adipocytes and preadipocytes. To our knowledge, this is the first report on the identification of an AS lncRNA that plays a functional regulatory role in adipogenesis. Moreover, it need further study whether PU.1 AS lncRNA affects regulation of miR-155 to PU.1 mRNA during adipogenic differentiation. The elucidation of mechanism on regulating PU.1 expression by PU.1 AS lncRNA will provide insight into novel pathways of regulatory adipogenesis.

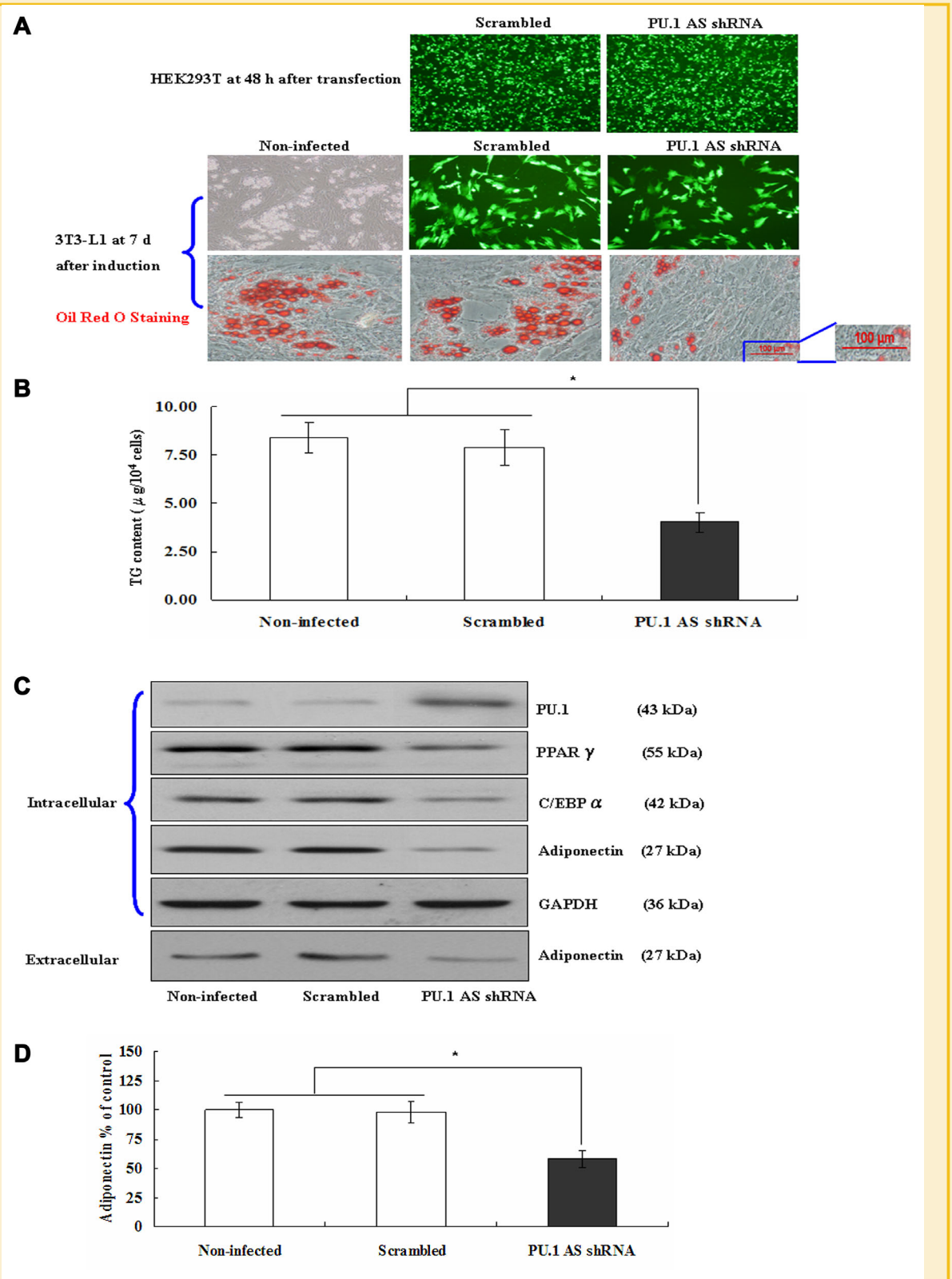

Fig. 7. PU.1 AS knockdown by shRNA inhibits adipogenesis and reduces secretion of adiponectin. A: PU.1 AS knockdown represses adipogenesis. Preadipocytes of 70–80% confluence were infected with retrovirus. Lipid droplets are stained using Oil Red O at differentiation day 7 (lower). B: PU.1 AS knockdown reduces TG content. TG content is analyzed at differentiation day 7. C: The expression levels of the adipogenic markers are down-regulated in PU.1 AS deficient fat cells. The intracellular (the medium) and extracellular (total cell layer) proteins are extracted for Western blot analysis of the indicated proteins at differentiation day 7, respectively. D: Secretion of adiponectin is decreased in PU.1 AS deficient fat cells at differentiation day 7. Data represent the mean ± SEM of six independent experiments in duplicates. $^{*}P < 0.05$ versus the non-infected and scrambled groups.

ACKNOWLEDGMENTS

We thank Dr. T. Deng (Methodist Hospital, TX) for providing miR-155 overexpression and knockdown constructs; Dr. Q. Tong (Baylor College of Medicine, TX) for helpful discussions and comments; Miss Jamie Lynn (University of Georgia, GA), and Dr. GY Chu (Northwest A&F University, Yangling, China) for correcting grammatical and spelling errors; and the reviewer's comments very much. This work was supported by the grants from National Key Basic Research Program of China (no. 2012CB124705), Program of National Natural Science Foundation of China (no.30600437) and Chinese Universities Scientific Fund (no.QN2009021).

REFERENCES

Beltran M, Puig I, Peña C, García JM, Álvarez AB, Peña R, Bonilla F, Herreros AG. 2008. A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. Genes Dev 22:756–769.

Camblong J, Iglesias N, Fickentscher C, Dieppois G, Stutz F. 2007. Antisense RNA stabilization induces transcriptional gene silencing via histone deacetylation in *S. cerevisiae*. Cell 131:706–717.

Choe KS, Ujhelly O, Wontakal SN, Skoultchi AI. 2010. PU.1 directly regulates cdk6 gene expression, linking the cell proliferation, and differentiation programs in erythroid cells. J Biol Chem 285:3044–3052.

Conrads KA, Yi M, Simpson KA, Lucas DA, Camalier CE, Yu LR, Veenstra TD, Stephens RM, Conrads TP, Beck GR, Jr. 2005. A combined proteome and microarray investigation of inorganic phosphate-induced pre-osteoblast cells. Mol Cell Proteomics 4:1284–1296.

Czech B, Hannon GJ. 2011. Small RNA sorting: Matchmaking for Argonautes. Nat Rev Genet 12:19–31.

Dutchak PA, Katafuchi T, Bookout AL, Choi JH, Yu RT, Mangelsdorf DJ, Kliewer SA. 2012. Fibroblast growth factor-21 regulates PPARγ activity and the antidiabetic actions of thiazolidinediones. Cell 148:556–567.

Ebralidze AK, Guibal FC, Steidl U, Zhang P, Lee S, Bartholdy B, Jorda MA, Petkova V, Rosenbauer F, Huang G, Dayaram T, Klupp J, O'Brien KB, Will B, Hoogenkamp M, Borden KL, Bonifer C, Tenen DG. 2008. PU.1 expression is modulated by the balance of functional sense and antisense RNAs regulated by a shared cis-regulatory element. Genes Dev 22:2085–2092.

Faghihi MA, Kocerha J, Modarresi F, Engström PG, Chalk AM, Brothers SP, Koesema E, St Laurent G, Wahlestedt C. 2010. RNAi screen indicates widespread biological function for human natural antisense transcripts. PLoS ONE 5:e13177.

Flynt AS, Lai EC. 2008. Biological principles of microRNA-mediated regulation: Shared themes amid diversity. Nat Rev Genet 9:831–842.

Fu Y, Luo N, Klein RL, Garvey WT. 2005. Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. J Lipid Res 46:1369–1379.

Hu YL, Fong S, Largman C, Shen WF. 2010. HOXA9 regulates miR-155 in hematopoietic cells. Nucleic Acids Res 38:5472–5478.

Ikeda Y, Hama S, Kajimoto K, Okuno T, Tsuchiya H, Kogure K. 2011. Quantitative comparison of adipocytokine gene expression during adipocyte maturation in non-obese and obese rats. Biol Pharm Bull 34:865–870.

Katayama S, Tomaru Y, Kasukawa T, RIKEN Genome Exploration Research Group, Genome Science Group (Genome Network Project Core Group), FANTOM Consortium. 2005. Antisense transcription in the mammalian transcriptome. Science 309:1564–1566.

Lefterova MI, Lazar MA. 2009. New developments in adipogenesis. Trends Endocrinol Metab 20:107–114.

Li K, Blum Y, Verma A, Liu Z, Pramanik K, Leigh NR, Chun CZ, Samant GV, Zhao B, Garnaas MK, Horswill MA, Stanhope SA, North PE, Miao RQ, Wilkinson GA, Affolter M, Ramchandran R. 2010. A noncoding antisense RNA in tie-1 locus regulates tie-1 function in vivo. Blood 115:133–139.

Li K, Ramchandran R. 2010. Natural antisense transcript: A concomitant engagement with protein-coding transcript. Oncotarget 1:447–452.

Lin LG, Pang WJ, Chen KY, Wang F, Gengler J, Sun YX, Tong Q. 2012. Adipocyte expression of PU.1 transcription factor causes insulin resistance through upregulation of inflammatory cytokine gene expression and ROS production. Am J Physiol Endocrinol Metab 302:E1550–E1559.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408.

Louro R, Smirnova AS, Verjovski-Almeida S. 2009. Long intronic noncoding RNA transcription: Expression noise or expression choice? Genomics 93:291–298.

Luo X, Hutley LJ, Webster JA, Kim YH, Liu DF, Newell FS, Widberg CH, Bachmann A, Turner N, Schmitz-Peiffer C, Prins JB, Yang GS, Whitehead JP. 2012. Identification of BMP and activin membrane-bound inhibitor (BAMBI) as a potent negative regulator of adipogenesis and modulator of autocrine/paracrine adipogenic factors. Diabetes 61:124–136.

MacFarlane LA, Murphy PR. 2010. Regulation of FGF-2 by an endogenous antisense RNA: Effects on cell adhesion and cell-cycle progression. Mol Carcinog 49:1031–1044.

Melo RC, D'Ávila H, Bozza PT, Weller PF. 2011. Imaging lipid bodies within leukocytes with different light microscopy techniques. Methods Mol Biol 689:149–161.

Mercer TR, Dinger ME, Mattick JS. 2009. Long non-coding RNAs: Insights into functions. Nat Rev Genet 10:155–159.

Michal L, Yitzhak P. 2006. Genome-wide natural antisense transcription: Coupling its regulation to its different regulatory mechanisms. EMBO Rep 7:1216–1222.

Misteli T. 2010. Higher-order genome organization in human disease. Cold Spring Harb Perspect Biol 2 a000794.

Nakajima I, Muroya S, Chikuni K. 2003. Growth arrest by octanoate is required for porcine preadipocyte differentiation. Biochem Biophys Res Commun 309:702–708.

Ørom UA, Derrien T, Guigo R, Shiekhattar R. 2010. Long noncoding RNAs as enhancers of gene expression. Cold Spring Harb Symp Quant Biol 75:325–331.

Papetti M, Skoultchi AI. 2007. Reprogramming leukemia cells to terminal differentiation and growth arrest by RNA interference of PU.1. Mol Cancer Res 5:1053–1062.

Rocio TM, Fethi L, Peter SF, Tilman S. 2009. MicroRNA-155 modulates the pathogen binding ability of dendritic cells (DCs) by down-regulation of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). J Biol Chem 284:16334–16342.

Rosenbauer F, Owens BM, Yu L, Tumang JR, Steidl U, Kutok JL, Clayton LK, Wagner K, Scheller M, Iwasaki H, Liu C, Hackanson B, Akashi K, Leutz A, Rothstein TL, Plass C, Tenen DG. 2006. Lymphoid cell growth and transformation are suppressed by a key regulatory element of the gene encoding PU.1. Nat Genet 38:27–37.

Rubio A, Conrad M, Haselbeck RJ, Brown-Driver V, Finn J, Silverman JA. 2011. Regulation of mprF by antisense RNA restores daptomycin susceptibility to daptomycin-resistant isolates of Staphylococcus aureus. Antimicrob Agents and Chemother 55:364–367.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. 2001. The hormone resistin links obesity to diabetes. Nature 409:307–312.

Takemoto CM, Brandal S, Jegga AG, Lee YN, Shahlaee A, Ying Y, Dekoter R, McDevitt MA. 2010. PU.1 Positively regulates GATA-1 expression in mast cells. J Immunol 184:4349–4361.

Tenen DG. 2003. Disruption of differentiation in human cancer: AML shows the way. Nat Rev Cancer 3:89–101.

Thompson RC, Herscovitch M, Zhao I, Ford TJ, Gilmore TD. 2011. NF-kappaB down-regulates expression of the B-lymphoma marker CD10 through a miR-155/PU.1 pathway. J Biol Chem 286:1675–1682.

van Dijk EL, Chen CL, d'Aubenton-Carafa Y, Gourvennec S, Kwapisz M, Roche V, Bertrand C, Silvain M, Legoix-Né P, Loeillet S, Nicolas A, Thermes C, Morillon A. 2011. XUTs are a class of Xrn1-sensitive antisense regulatory non-coding RNA in yeast. Nature 475:114–117.

Vigorito E, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, Das PP, Miska EA, Rodriguez A, Bradley A, Smith KG, Rada C, Enright AJ, Toellner KM, Maclennan IC, Turner M. 2007. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. Immunity 27:847–859.

Wang F, Tong Q. 2008. Transcription factor PU.1 is expressed in white adipose and inhibits adipocyte differentiation. Am J Physiol Cell Physiol 295:C213–C220.

Werner A, Berdal A. 2005. Natural antisense transcripts: Sound or silence? Physiol Genomics 23:125–131.

Wilusz JE, Sunwoo H, Spector DL. 2009. Long noncoding RNAs: Functional surprises from the RNA world. Genes Dev 23:1494–1504.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. A: The predicted miRNAs target PU.1 mRNA using TargetScan Release 6.2. B: the statistic information of predicted consequential pairing between PU.1 mRNA and miRNA-155.
Fig. S2. A: The predicted miRNAs target C/EBPβ mRNA using TargetScan Release 6.2. B: the statistic information of predicted consequential pairing between C/EBPβ mRNA and miRNA-155.
Fig. S3. Time-course expression of adiponectin during preadipocyte differentiation.