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# MiR-301a is involved in adipocyte dysfunction during obesity-related inflammation via suppression of PPARγ

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The present study explored the involvement and role of miR-301a in the adipose tissues. For the first time we identified the expression of miR-301a in the white adipose tissues of mice. A decreased level of miR-301a was correlated to increased chronic inflammation in the 3T3-L1 cells and circulation in an obese mouse model. Mechanistically, we demonstrated that miR-301a attenuated saturated free fatty acid-induced activation of peroxisome proliferator-activated receptor gamma (PPARγ) and production of proinflammatory cytokines in 3T3-L1 cells. Target gene reporter assays showed that miR-301a directly targeted the 3'-untranslated region (3'UTR) of PPARγ, resulting in a decrease of PPARγ protein expression. The miR-301a inhibition of adipocyte differentiation was reversed by PPARγ overexpression.

## 1. Introduction

Inflammation is a key characteristic of metabolic syndromes such as obesity and type II diabetes. Adipocytes from obese subjects produce several chemokines and cytokines such as tumornecrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6. These proinflammatory cytokines facilitate the differentiation of macrophages by recruiting monocytes into the adipose tissue; this process is linked to adipocyte dysfunction (Coppack 2001). Although critical factors related to adipocyte dysfunction in the obese have not been fully determined, the prevailing hypothesis is that cytokines generated by adipocytes are responsible for the recruitment of immune cells to obese adipose tissue and for the promotion of monocyte differentiation to M1 macrophages to initiate systemic inflammation (Hirai et al. 2007). Inflammatory responses in the adipose tissue could be induced by extracellular and intracellular stimuli from overnutrition, increased endoplasmic reticulum (ER) stress, levels of free fatty acids, and apoptosis of adipocytes. The canonical inflammatory signaling pathway to activate nuclear factor kappa-B (NF-κB) is also responsible for adipocyte dysfunction in obese subjects (Alnaeeli and Noguchi 2015). There have also been reported that peroxisome proliferator activator receptor gamma (PPARγ) is closely linked to adipocyte dysfunction (Kubota et al. 1999). Some studies showed that adipocyte differentiation was not mediated by other transcription factors when PPARγ was knocked out (Park et al. 2010). MicroRNAs (miRNAs) are a family of small noncoding RNAs (∼21-23 nucleotides in length) that interact with target mRNAs by forming incomplete base pairing and they negatively regulate the expression of target mRNAs by inhibiting translation or by destabilizing mRNAs (Ortega et al. 2010). Several reports have indicated that miRNAs have been shown critical roles in the regulation of adipogenesis and fat cell metabolism (Mcgregor and Choi 2011). Recently, some miRNAs regulated by adiponectin were identified as novel targets for controlling adipose tissue inflammation.

Here, we reported the molecular evidence for PPARγ regulation by miR-301a during adipocyte dysfunction. We provided a potential mechanism linking obesity to the production of proinflammation cytokines in 3T3-L1 preadipocytes.

## 2. Investigations and results

### 2.1. Decreased miR-301a level and increased chronic inflammation in obesity

To observe the role of miR-301a in obesity associated 3T3-L1, we set up an obese mouse model by feeding the male C57/B6 mice with a standard high fat diet (HFD) for 8 weeks. The body weight and the miR-301a levels in inguinal white adipose tissues from CD or HFD mice were determined. As shown in Fig. 1, RT-qPCR results showed that HFD suppressed the expression of miR-301a. The regulatory mechanism between miR-301a and those cytokines in chondrocytes is still obscure.

Furthermore, the HFD group versus chow diet (CD) group, plasma proinflammatory cytokines including IL-6, TNF-α and IL-1β increased notably. Those results indicated that obesity was accompanied by systemic chronic inflammation.

### 2.2. MiR-301a suppresses saturated free fatty acid (FFA)-induced inflammatory cytokine production in 3T3-L1

To explore the regulatory role of miR-301a in obesity-related cytokine production in 3T3-L1, we employed saturated FFA (18:0) to mimic the condition of HFD or obesity as previously

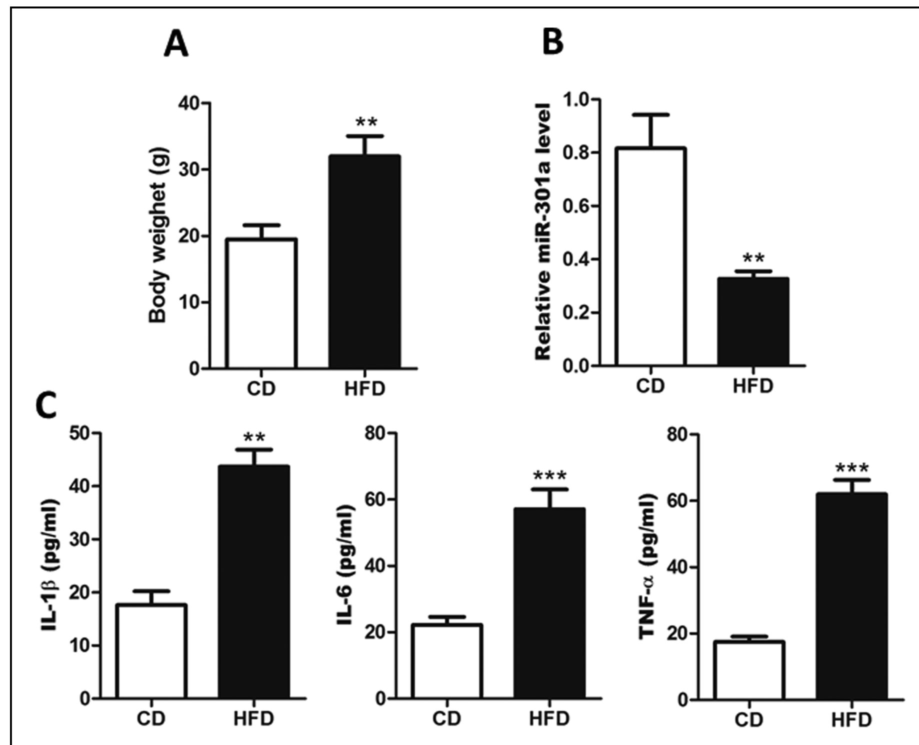


Fig. 1: Body weight of the male C57BL/6 mice fed with a standard chow diet or high fat diet for 8 weeks (A). Relative mRNA levels of miR-301a in the primary chondrocytes isolated from the mice (B). Plasma cytokine (IL-6, TNF-α and IL-1β) levels of male were determined with Elisa kits (C). Data represent the means ± SEM in each group (n = 8), $^{**}p < 0.01$ and $^{***}p < 0.001$.

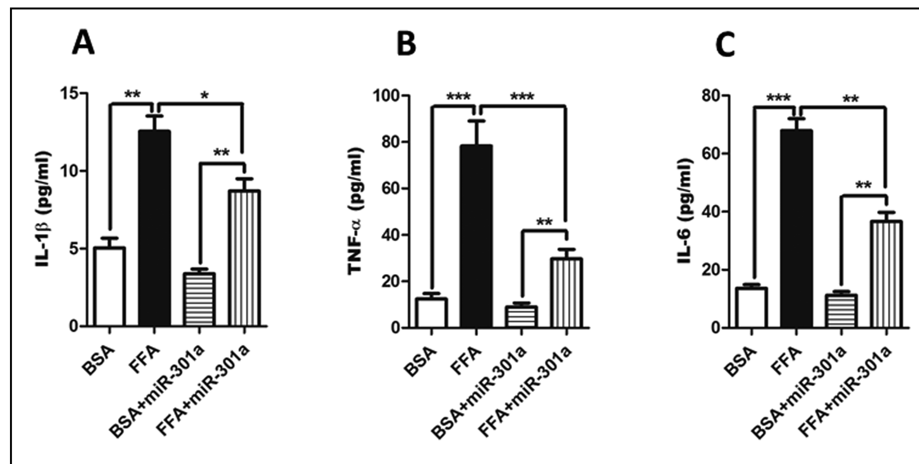


Fig. 2: 3T3-L1 cells were transfected with miR-301a or scramble miRNA as control for 24 h, and then treated with FFA (200 μM) or BSA (5%) as control for 30 min.The levels of supernatant cytokines (IL-6, TNF-α and IL-1β) were measured by ELISA kits. Data represent the means ± SEM in each group (n = 8), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

described (Xie et al. 2015). The miR-301a transfection significantly diminished the aforementioned effects of saturated FFA. Furthermore, the profiles of supernatant cytokines were measured and identical results were obtained.

### *2.3. MiR-301a suppresses saturated FFA-induced PPARγ activity in 3T3-L1*

As shown in Fig. 3, those results showed that saturated FFA-induced PPARγ were largely attenuated by miR-301a transfection. To further observe the role of miR-301a in PPARγ activity, miR-301a and a report gene of SV40 promoter containing a standard PPARγ binding element were co-transfected into the 3T3-L1 cells. Results demonstrated that saturated FFA-induced PPARγ activity was attenuated by miR-301a overexpression or potentiated by miR-301a inhibition, respectively.

### *2.4. MiR-301a specifically inhibits PPARγ protein expression by targeting 3'-UTR of PPARγ mRNA*

To identify the molecular mechanisms underlying miR-301a regulation of adipogenic differentiation, we searched for miR-301a targets using a computational miRNA target prediction analysis by miRanda. In order to confirm whether miR-301a directly targeted the 3'-UTR of PPARγ mRNA, pmirGLO 3'-UTR luciferase reporter plasmids containing putative binding sequences for miR-301a or mutant PPARγ-3'-UTR were synthesized. Consistent with the bioinformatic prediction, the co-transfection of miR-301a with a wild type reporter (pmirGLO-PPARγ-3'-UTR-WT) resulted in a highly significant decrease in luciferase activity in 3T3-L1 cells, compared with the control group (Fig. 4A). There was no decrease in luciferase activity after miR-301a co-transfected with the empty vector or mutant reporter (pmirGLO-PPARγ-3'-UTRMut). Those results

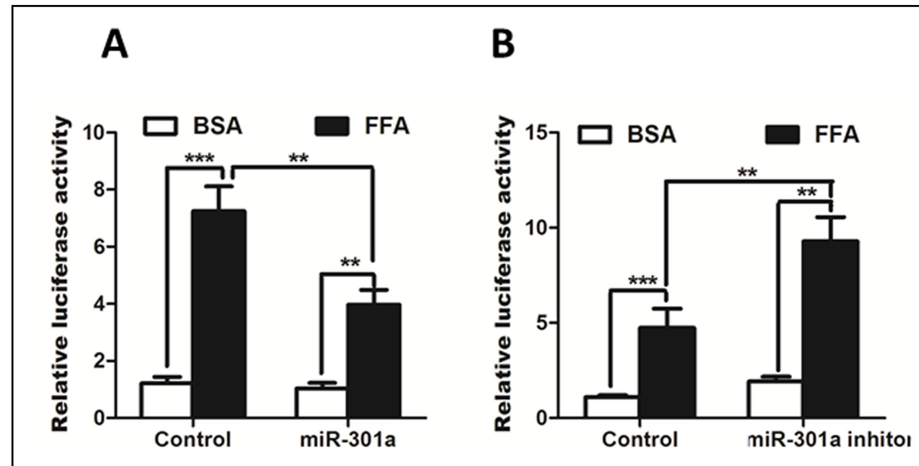


Fig. 3: 3T3-L1 cells were co-transfected with miR-301a (20 n M) and a SV40 reporter construct (0.4 µg/ml) containing a PPARγ binding site for 24 h, and then treated with FFA (200 µM) for 24 h. Relative luciferase activity was measured. Data represent the means ± SEM in each group (n = 3), $^{**}p < 0.01$ and $^{***}p < 0.001$.

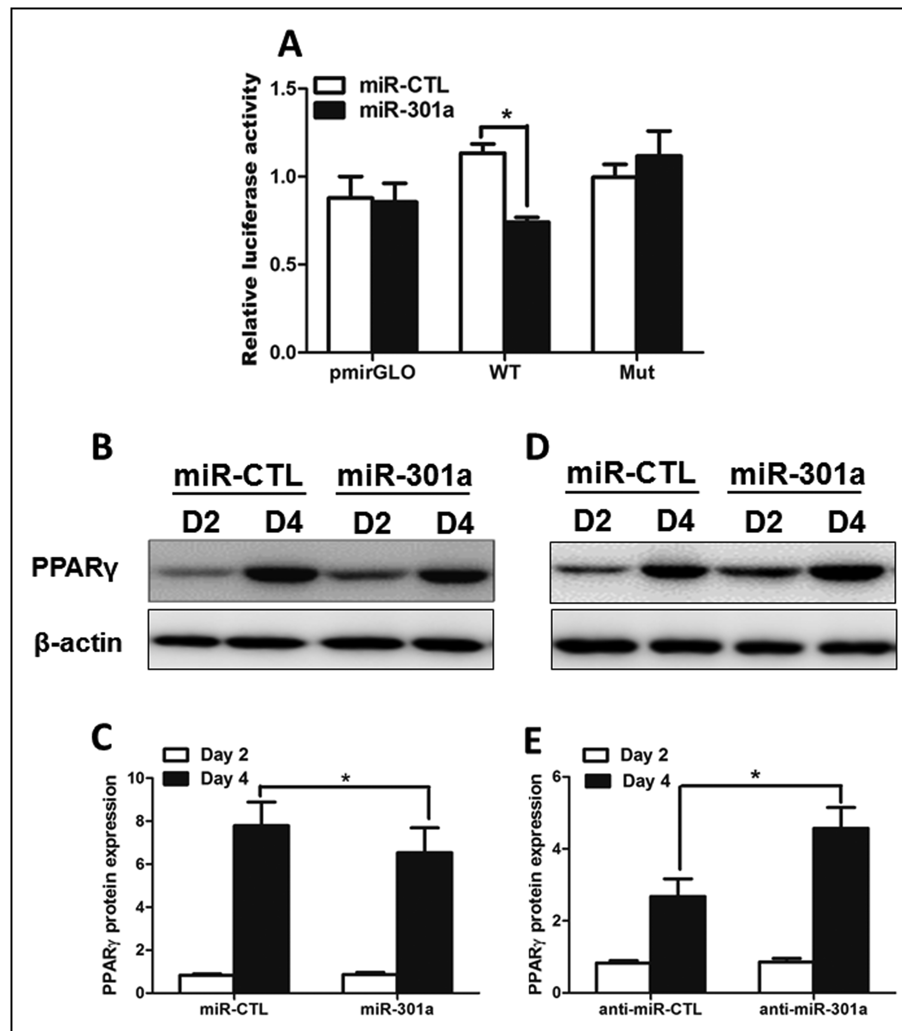


Fig. 4: MiR-302a inhibits PPARγ protein expression by targeting 3'-UTR of PPARγ mRNA. (A) The pmirGLO, pmirGLO PPARγ-3'-UTR-WT or pmirGLO PPARγ-3'-UTR-Mut vector was co-transfected with miR-301a or miR-CTL into 3T3-L1 cells, and the luciferase assay was performed. (B, C) 3T3-L1 cells were transfected with transfected with miR-301a or miR-CTL for 24 h and then treated with adipogenic medium for an additional 4 days. Western blotting analysis (B) and densitometric analysis (C) were performed to examine PPARγ protein levels. The levels were normalized to β-actin loading control. The values represent mean ± SEM (n = 3). (D, E) 3T3-L1 cells were transfected with anti-miR-301a or anti-miR-CTL for 24 h, and then treated with adipogenic medium for additional 4 days. The expression of PPARγ protein was evaluated by Western blotting analysis (D) and densitometric analysis (E). PPARγ protein levels were normalized to β-actin loading control.

indicated that the predicted site is a direct target of miR-301a. To further confirm whether or not the miR-301a could regulate the expression of PPARγ, the PPARγ protein level was determined using Western blotting in the 3T3-L1 cells transiently transfected with miR-301a or anti-miR-301a. Overexpression of miR-301a significantly reduced PPARγ protein levels at day 4 in the 3T3-L1 cells (Fig. 4B), while endogenous inhibition of miR-301a elevated the protein level of PPARγ (Fig. 4C). These results suggested that miR-301a could negatively regulate PPARγ expression through direct binding to the 3'-UTR of PPARγ.

## 3. Discussion

In the present study, we identified miR-301a as a novel negative regulator of adipocyte differentiation whose effect is mediated, at least in part, by its potent repression of PPARγ. Interestingly, miR-301a could also suppress proinflammatory cytokine production via inactivating PPARγ. In this study, we identified the enhancement of proinflammatory cytokine production and suppression of miR-301a in 3T3-L1 pre-adipocytes after FFA stimulation.

PPARγ is a critical transcription factor of fat cell development and it is tightly regulated during adipogenesis (Zhang et al. 2010). There has been reported that it is expressed at the highest level in adipose tissue and adipocyte cell lines. The role of PPARγ as a critical transcription factor of adipogenesis has been supported by overwhelming evidence from both *in vivo* and *in vitro* studies. The expression of PPARγ is induced early during adipocyte differentiation (Kawai and Rosen 2010). Once expressed, it activates adipocyte-specific genes resulting in the adipocyte phenotype and ensuring maintenance of terminal differentiation.

MiRNAs can regulate adipocyte differentiation by targeting diverse signaling molecules and pathways. Previous studies have demonstrated that miRNAs, including miR-130 and miR-27, regulated adipogenesis by targeting PPARγ (Lee 2011; Zou et al. 2014). To study the molecular mechanism by which miR-301a regulates adipocyte differentiation, we analyzed the potential targets with the assistance of prediction programs. MiR-301a overexpression suppressed the cellular level of PPARγ protein, whereas the functional inhibition of miR-301a led to the elevation of PPARγ expression; these observations strongly suggest that PPARγ is regulated by miR-301a. The dual luciferase reporter assay also identified PPARγ as a direct target of miR-301a. These results suggest that miR-301a might negatively regulate adipogenic differentiation partly by targeting PPARγ mRNA. In summary, a series of findings provide evidence that miR-301a might be a novel negative regulator of PPARγ and serve as a suppressor of adipogenic differentiation. Reciprocal inhibition between miR-301a and PPARγ downstream of saturated FFA signal regulates obesity-related chronic inflammation in 3T3-L1. Our findings provide a potential mechanism linking obesity to cartilage inflammation. Therefore, miR-301a is an attractive target for new therapies aimed at reducing excess fat tissue. However, to better understand the exact role of miR-301a in adiposity, studies are needed in various pathologic animals relating to obesity (high fat diet), diabetes and insulin resistance.

## 4. Experimental

### 4.1. Animals

Male C57BL/6J mice were obtained from Shanghai Laboratory Animal Company (Shanghai, China) White adipose tissues (WATs) from C57BL/6 mice fed a chow-diet (CD) or an HFD for 8 weeks were isolated. The isolated tissues were washed with phosphate-buffered saline (PBS), quickly frozen in liquid nitrogen, and then stored at –80 °C until analysis.

### 4.2. Cell cultures

3T3-L1 pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Grand Island, NY, USA) containing 10% fetal calf serum (FCS; GIBCO-BRL) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; GIBCO-BRL). To induce adipocytic differentiation, cells were exposed to an adipogenic medium containing 0.5 μM isobutylmethylxanthine, 1 μM dexamethasone and 1 μg/ml insulin for 2 days. The medium was replaced with a fresh complete medium containing insulin, and the cells were incubated for an additional 2 days. Subsequently, media was changed every 2 days until the cells were well differentiated.

### 4.3. Enzyme-linked immunosorbent assay (ELISA)

Cytokines in cell supernatants or plasma were measured with TNF-α, IL-6 and IL-1β ELISA Kit from R&D system according to manufacturer's protocols.

### 4.4. RNA Extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted directly from the tissues or cells using Trizol reagent (*Invitrogen*). One microgram of total RNA was used for cDNA preparation and was reverse transcribed using a reverse transcriptase kit (*Invitrogen*) according to the manufacturer's instructions. Real-time quantitative PCR was performed on Applied Biosystems model 7300 and 7900 instruments. The expression levels of each mRNA were normalized to the levels of GAPDH and the relative expression levels of miR-540 were normalized to the levels of U6. The following amplimers were used for RT-PCR analyses:
PPARγ-F: 5'- CAGAGTCTGCTGATCTGCGAG-3', PPARγ-R: 5'-TGC AGG GGGGTGATATGTT -3'; GAPDH-F: 5'-GGAAAGCTGTGG CGTGAT-3', GAPDH-R: 5'-AAGGTGGAAGAATGGGAGTT-3'; miR-301a-F: 5'- ACATTGCCATCACTCTTGTACTG-3', miR-301a-R: 5'-GTGCAGG GTCCGAGGT -3'; U6-F: 5'- CGCTTCGGCAGCACATAT-ACTA -3', U6-R: 5'- CGCTTCACGAATTTGCGTGTCA -3'.

### 4.5. Western blot analysis

Cells were washed twice with ice-cold PBS, total cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer, separated by electrophoresis in SDS-containing polyacrylamide gels, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Incubation with primary antibodies to detect PPARγ (Cell Signaling Technology) was followed by incubation with the appropriate secondary antibody conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotech); Immunoreactivity was visualized using enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology) and analyzed by the Quantity One system (Bio-Rad Laboratories). The expression of GAPDH was used for normalization and all the data were acquired from three independent experiments.

### 4.6. Luciferase vector construction and reporter assay

To identify the interaction with PPARγ and miR-301a dual luciferase assays was performed with a vector containing miRNA target sequence. The 3'UTR sequence of PPAR-γ mRNA was acquired from NCBI, and the sequence of miR-301a was obtained from miRBase. The 3T3-L1 cells were seeded in a 24-well plate; 0.4 μg of miR-301a mimics or NC were co-transfected with 0.4 μg 3'UTR luciferase reporter vectors using lipofectamine 2000 (*Invitrogen*). Cell lysates were collected 24 h after transfection and their luciferase activities were determined using Dual Luciferase Reporter Assay System (Promega).

### 4.7. Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA. Differences between groups with $p < 0.05$ were considered statistically significant.

### References

Alnaeeli M, Noguchi CT (2015) Erythropoietin and obesity-induced white adipose tissue inflammation: redefining the boundaries of the immunometabolism territory. Adipocyte 4: 153–157.
Coppack SW (2001) Pro-inflammatory cytokines and adipose tissue. Proc Nutr Soc 60: 349–356.
Hirai S, Kim YI, Goto T, Kang MS, Yoshimura M, Obata A, Yu R, Kawada T (2007) Inhibitory effect of naringenin chalcone on inflammatory changes in the interaction between adipocytes and macrophages. Life Sci 81: 1272–1279.
Kawai M, Rosen CJ (2010) PPARγ: a circadian transcription factor in adipogenesis and osteogenesis. Nat Rev Endocrinol 6: 629–636.
Kubota N, Terauchi Y, Miki H, Kadowaki T (1999) PPARγ mediates high-fat diet–induced adipocyte hypertrophy and insulin resistance. Molecular Cell 4: 597–609.
Lee EK (2011) miR-130 Suppresses adipogenesis by inhibiting peroxisome proliferator-activated receptor γ expression. Mol Cell Biol 31: 626–638.
McGregor RA, Choi MS (2011) microRNAs in the regulation of adipogenesis and obesity. Curr Mol Med 11: 304–316.
Ortega FJ, Moreno-Navarrete JM, Pardo G, Sabater M, Hummel M, Ferrer A, Rodriguez-Hermosa JI, Ruiz B, Ricart W, Peral B, Fernández-Real

JM (2010) MiRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. Plos One 5: e9022.
Park KW, Waki H, Choi SP, Park KM, Tontonoz P (2010) The small molecule phenamil is a modulator of adipocyte differentiation and PPARγ expression. J Lipid Res 51: 2775–2784.
Xie Q, Wei M, Kang X, Liu D, Quan Y, Pan X, Liu X, Liao D, Liu J, Zhang B (2015) Reciprocal inhibition between miR-26a and NF-κB regulates obesity-related chronic inflammation in chondrocytes. Biosci Rep 35: e00204.
Zhang L, Su P, Xu C, Chen C, Liang A, Du K, Peng Y, Huang D (2010) Melatonin inhibits adipogenesis and enhances osteogenesis of human mesenchymal stem cells by suppressing PPARγ expression and enhancing Runx2 expression. J Pineal Res 49:364–372.
Zou B, Ge Z, Zhu W (2014) Persimmon tannin represses 3T3-L1 preadipocyte differentiation via up-regulating expression of miR-27 and down-regulating expression of peroxisome proliferator-activated receptor-γ in the early phase of adipogenesis. Eur J Nutr doi:10.1007/s00394-014-0814-9.