

Coordinated regulation of miR-27 by insulin/CREB/Hippo contributes to insulin resistance

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ABSTRACT

MicroRNA-27 is a critical non-coding metabolic gene that is often aberrantly overexpressed in non-alcoholic fatty livers (NAFLD). However, the pathogenic role of miR-27 in NAFLD remains unknown. In this study, we attempted to identify the mechanism by which miR-27 was regulated in the context of insulin resistance, a predisposed metabolic disorder in NAFLD. Our data from cell culture and animal studies showed that insulin, CREB, and Hippo signalings coordinately regulated miR-27. First, miR-27 was upregulated in palmitate-treated cells and high fat diet-fed mouse livers, which exhibited insulin resistance and CREB overexpression. Second, miR-27 peaked in the mouse liver at the post-absorptive phase when CREB activity was increased. Also, miR-27 was increased rapidly in cell lines when CREB was deactivated by insulin treatment. Third, miR-27 was decreased in cultured cells when CREB was downregulated by siRNA or metformin treatment. In contrast, Forskolin-mediated activation of CREB promoted miR-27 expression. Fourth, Hippo signaling repressed miR-27 in a CREB-independent manner: miR-27 was reduced in cells at full confluence but was inhibited in cells transfected with siRNA against Lats2 and Nf2, which were two positive regulators of Hippo signaling. Lastly, bioinformatics and luciferase assay showed that miR-27 inhibited Akt phosphorylation by targeting *Pdk1* and *Pik3r1*. Overexpression of miR-27 impaired Akt phosphorylation in cell lines and primary mouse hepatocytes upon insulin stimulation. In conclusion, our data suggest that insulin, CREB, and Hippo signalings contribute to aberrant miR-27 overexpression and eventually lead to insulin resistance in NAFLD.

List of abbreviations

3'UTR	3' untranslated region
AKT	serine/threonine-protein kinase
CREB	cyclic AMP-response element binding protein
FAME	functional assignment of miRNAs via enrichment
FOXO1	forkhead box protein O1
HFD	high-fat-diet
IR	insulin resistance
miR or miRNA	microRNA
NAFLD	nonalcoholic fatty liver disease
PA	palmitic acid
PDPK1	3-phosphoinositide dependent protein kinase 1
PIK3R1	phosphoinositide 3-kinase regulatory subunit alpha, p85
PIK3CA	phosphoinositide 3-kinase catalytic subunit alpha, p110
PLK2	polo-like kinase 2

1. Introduction

Among the various miRNAs involved in metabolism, miR-27 was determined to be a critical regulator of lipid metabolism [1]. MiR-27 is expressed in the forms of miR-27a and miR-27b, which are from two different clusters miR-23a ~ 27a ~ 24-2 and miR-23b ~ 27b ~ 24-1 located on chromosomes 8 and 13, respectively, in mice. miR-27a overexpression impairs adipocyte differentiation by targeting peroxisome proliferator-activated receptor gamma (PPAR γ) and CCAAT/enhancer binding protein alpha (CEBPA) [2]. miR-27b is aberrantly upregulated in the liver of mice with plasma hyperlipidemia and it serves as a critical regulator of lipid metabolism in mouse livers [3]. However, the role of miR-27 in glucose homeostasis remains to be elucidated. In our previous study, we profiled the microRNA expression pattern in postnatal liver development [4]. In mice, 60 miRNAs were

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found to reach the highest level in the second postnatal week and gradually decreased after the third week. Among these 60 miRNAs, miR-27 exhibited a circadian rhythm; its level was high during food intake and low during fasting [5]. However, the mechanisms by which miR-27 is regulated remain to be determined.

Nonalcoholic fatty liver disease (NAFLD) is a chronic metabolic disorder and a risk factor for hepatocellular carcinoma [6]. NAFLD is characterized by hepatic steatosis, non-alcoholic fatty liver, and non-alcoholic steatohepatitis (NASH). Multiple risk factors are reportedly associated with NAFLD, including obesity and dyslipidemia. Insulin resistance resulting from abnormal glucose homeostasis is one of the major causes of NAFLD. There are three signaling pathways that participate in NAFLD pathogenesis: insulin- [7], CREB- [8], and Hippo [9] signalings. Specifically, patients and mice with NAFLD often exhibit insulin insensitivity, CREB overexpression [8], and decreased Hippo activity [10].

Taken together, we hypothesized that these signaling pathways together regulate miR-27 expression in the liver and aberration of miR-27 may contribute to insulin insensitivity and associated metabolic disorders. Both cell- and animal-based experiments, such chemical treatment, transient transfection of siRNA/miRNA, and luciferase reporter assay, were designed and conducted to address this hypothesis. Collectively, our data suggest that abnormal miR-27 overexpression due to abnormal insulin/CREB/Hippo signaling may contribute to insulin insensitivity, and miR-27 can be a potential therapeutic target against NAFLD.

2. Materials and methods

2.1. Animals

Male wild-type C57BL/6 mice, aged 10 to 24 weeks, were bred and maintained at the University Laboratory Animal Center at National Taiwan University College of Medicine. Normally fed mice ($n = 3$) were fed *ad libitum* with a chow diet, whereas high-fat-diet (HFD) mice ($n = 3$) were fed a diet containing 60% fat for 13 weeks since they were 5 weeks old. Livers of adult mice ($n = 16$), all aged 10–12 weeks, were collected at different phases of the feeding cycle (0, 6, 12, 18 Zeitgeber Time [h]).

2.2. Cell culture experiments

HepaRG (Life Technologies), Hepa1-6 (ATCC), AML12 (ATCC), and NIH3T3 (ATCC) cells were cultured according to the manufacturer's instructions. For the palmitate (PA, Sigma)-induced IR insulin resistance model, AML12 cells were cultured in a medium containing bovine serum albumin (BSA)-conjugated PA at 0, 0.15, and 0.3 mM for 2 d. The molar ratio of PA:BSA was 6:1. For *in vitro* transfection, transfection of miRNA mimics or siRNA was performed using Opti-MEM (1×) (Gibco), Lipofectamine RNAiMAX Reagent (Life Technologies), miRIDIAN mimic (negative control mimic and *mmu-miR-27a-3p*; Dharmacon), or Stealth RNAi siRNA (negative control siRNA and designated siRNA; Life Technologies). For *in vitro* insulin stimulation, cells were first grown to 80% confluence, and cultured in a serum-free medium to reduce basal levels of CREB phosphorylation. After overnight starvation, cells were treated with 100 nM insulin (Gibco) for 1 h and then harvested. For cells transfected with CREB siRNA and negative control siRNA, cells were transfected for 48 h and cultured in a serum-free low glucose (5 mM) medium for overnight starvation. Then, cells were treated with 25 mM glucose for 30 min to mock the hyperglycemic condition. To induce Hippo signaling, AML12 cells were grown in a normal culture medium and harvested after the cells reached 70%, 90%, and 100% confluence. For *in vitro* metformin treatment, metformin hydrochloride (Cayman) was added to the culture medium at 0, 5, and 10 mM for 48 h before harvesting and further analysis.

2.3. Isolation and culture of mouse hepatocytes

Primary hepatocytes from 10- to 12-week-old mice were isolated and cultured in Dulbecco's Modified Eagle Medium F-12 (Hyclone) according to our published protocol [4].

2.4. RNA isolation and quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from liver tissues using Trizol (Life Technologies) and subjected to qPCR to quantify the expression of miRNAs and protein-coding genes. Rnu6b and mature miR-27a and miR-27b were amplified and quantified using Taqman miRNA probes (Life Technologies) and the SensiFAST Probe qPCR kit (Bioline). Mature miR-16 was amplified and quantified using self-designed primers according to published protocols [11]. For protein-coding genes such as CREB and GAPDH, total RNA was first reverse-transcribed into complementary DNA using M-MLV (Life Technologies) and then qPCR was performed using the SensiFAST SYBR qPCR kit (Bioline) and the QuantStudio 3 System (Thermo Fisher). Relative expression was calculated using the $\Delta\Delta CT$ method. All Taqman probe IDs, primer sequences, and self-designed primers are listed in Supplementary Table 1.

2.5. Western blot analysis

Proteins were extracted from liver tissue or cells using sodium dodecyl sulfate (SDS)-based lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.1% SDS) containing protease/phosphatase inhibitors. The subsequent procedures were performed as previously described [4]. The antibodies used in this study were used in the supplemental Table 1.

2.6. Reporter plasmid and luciferase assays

The full-length (FL) or partial length (DEL) 3' untranslated region (UTR) of *Pdpk1* and *Pik3r1* from C57BL/6 tail DNA were PCR amplified with primers (Supplementary Table 1) and constructed into psiCHECK2 (Promega) vectors containing the luciferase reporter gene. The miR-27a mimics (Dharmacon) and newly constructed plasmids containing FL or DEL 3'UTR were then co-transfected into NIH3T3 cells (ATCC) at 50 nM and 200 ng per well, respectively. Forty-eight hours after transfection, cytoplasmic luciferase activities were determined using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

3. Results

3.1. miR-27 was upregulated in both cellular and animal models of obesity-induced insulin resistance

Two experimental models of insulin resistance were developed to elucidate the biological role of miR-27. First, AML12 cells were treated with PA (0, 0.15, and 0.3 mM) for 48 h to induce insulin resistance. As shown in Fig. 1A, PA-treated AML12 cells exhibited prominent lipid accumulation in the cytoplasm compared to vehicle-treated AML12 cells. Next, insulin resistance was verified by stimulating cells with insulin (100 nM) for 10 min. Western blot analysis revealed diminished phosphorylation of AKT^{S473} (Fig. 1B) in AML12 cells treated with an increased dose of PA, clearly demonstrating insulin insensitivity. Moreover, qPCR analysis showed that miR-27a and miR-27b levels significantly increased in a dose-dependent manner in PA-treated cells (Fig. 1C).

Next, we sought to verify the expression of miR-27a and miR-27b in HFD-fed C57BL/6 mice. As shown in Fig. 1D, HFD-fed mouse livers exhibited severe steatosis compared to matched control mouse livers. In addition, western blot analysis clearly showed diminished phosphorylation of AKT^{S473}, but not AKT^{T308} in HFD-fed mouse liver upon refeeding for 2 h after fasting for 16 h (Fig. 1E), which was similar to

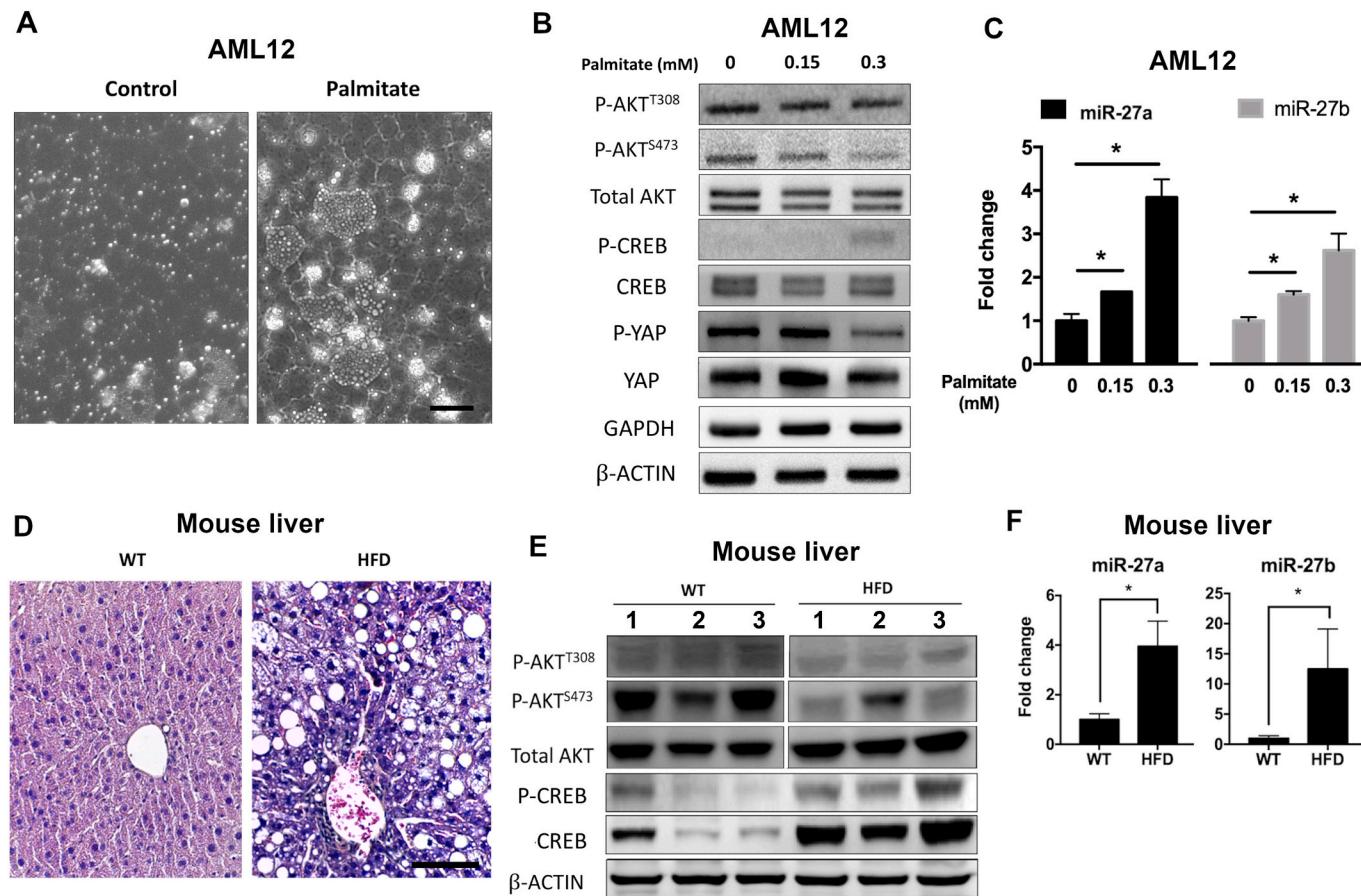


Fig. 1. Aberrant miR-27 expression in palmitate-treated AML12 cells and high-fat-diet (HFD)-fed mice that developed insulin resistance. (A) light microscopic (LM) images of AML12 cells treated with control and palmitate for 48 h. Palmitate-treated cells exhibited lipid accumulation (arrows). Scale bar = 100 μ m. (B) Western blot analysis determined the basal phosphorylation level of AKT, CREB, and YAP. (C) QPCR analysis showing miR-27a and miR-27b expression in cells treated with palmitate (0, 0.15, and 0.3 mM). (D) LM images of normal and steatosis livers of wild-type (WT) and HFD mice, respectively. Scale bar = 100 μ m. (E) Western blot analysis was performed to determine the basal phosphorylation level of AKT and CREB in the livers of WT and HFD mice. Each lane represents sample obtained from individual mouse ($n = 3$ mice per group). (F) QPCR was performed to measure miR-27a and miR-27b levels in WT and HFD-fed mouse livers. GAPDH or β -actin protein expression were used as loading controls for western blot. Expression values of miRNAs were normalized to those of *Rnub6*. * $P < 0.05$. Graphs show mean \pm SEM.

finding made by previous study [12]. Moreover, miR-27a and miR-27b levels were significantly higher in HFD-fed mouse liver than in the control liver (Fig. 1F). Taken together, miR-27a was significantly elevated in both cell lines and animals with insulin resistance. Next, we explored the upstream mechanism by which miR-27 is regulated.

3.2. Insulin suppressed miR-27 in human differentiated hepatic cell line

We hypothesized that miR-27 is regulated by insulin. To validate this finding, we collected the chow-diet-fed C57BL/6 mouse livers at Zeitgeber times (ZT) 0, 6, 12, and 18 and determined the expression of miR-27. MiR-27a and miR-27b were expressed in a rhythmic pattern (Fig. 2A). Specifically, miR-27 expression was repressed in the post-prandial state and peaked in the post-absorptive state. In addition, miR-27 is induced by overnight fasting and repressed by refeeding [5], which strongly suggests that miR-27 is controlled by insulin. To further test this, human HepaRG cells and mouse AML12 cells, both of which are differentiated hepatic cell lines [12], were treated with 100 nM insulin. Western blot analysis showed that AKT^{S473} phosphorylation in both cell types was significantly increased after insulin treatment (Fig. 2B and D). Interestingly, compared to the control group, miR-27 levels were significantly reduced after insulin induced AKT phosphorylation (Fig. 2C and E). These data suggest that insulin negatively regulates miR-27 expression in both human and mouse liver cells.

3.3. CREB positively regulated miR-27 expression

Our data revealed that CREB activity (i.e. phosphorylation level) was upregulated when miR-27 was increased in the context of insulin resistance (Fig. 1B and E). Moreover, miR-27 was decreased when CREB was deactivated in insulin-treated AML12 cells (Fig. 3A), and mice liver during fasting state (i.e. ZT 12–18 h) (Fig. 3B). We hypothesized that CREB is a critical transcription activator of miR-27. To test this hypothesis, we transfected both human HepaRG and mouse AML12 cells with CREB siRNA (si-CREB) and negative control siRNA (si-NC). CREB was downregulated at the protein level by si-CREB (Fig. 3C and E). As expected, miR-27 levels were significantly lower in CREB-depleted cells than in control cells (Fig. 3D and F). To further verify the CREB-mediated upregulation of miR-27, AML12 cells were treated with forskolin to phosphorylate and activate CREB (Fig. 3G). As expected, miR-27 was upregulated by forskolin-induced CREB activation (Fig. 3H). These results suggest that CREB is a positive regulator of miR-27 in both human- and mouse-originated cells.

3.4. Hippo signaling suppressed miR-27 expression in the context of increased cell-cell contact

Next, we speculated that Hippo signaling suppresses miR-27 expression because it is found to be downregulated during NAFLD and

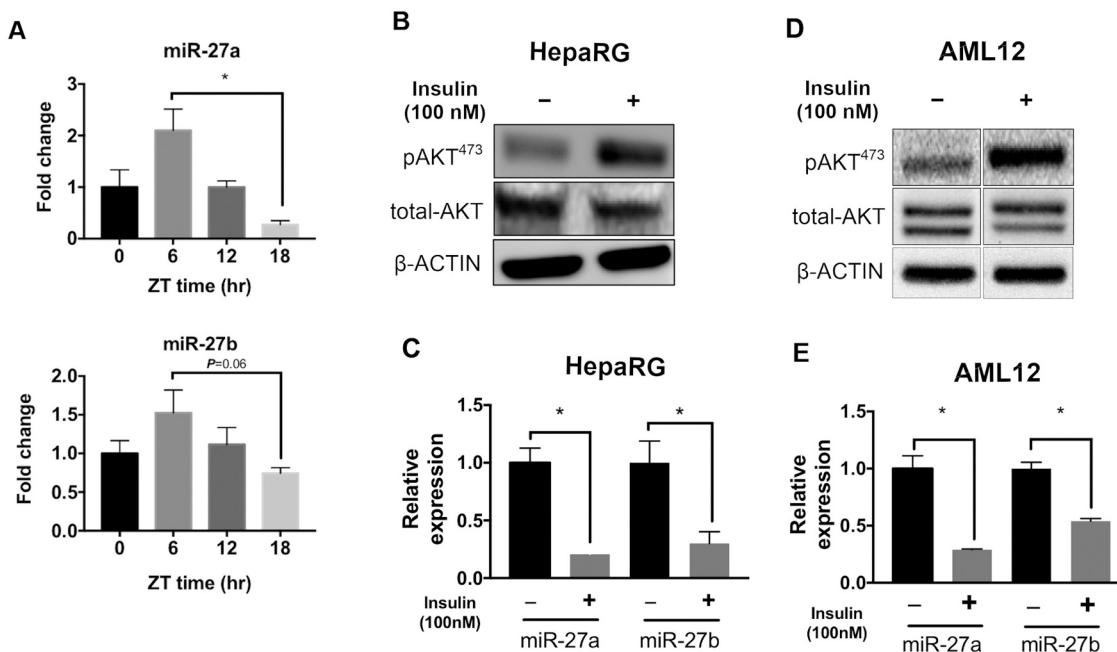


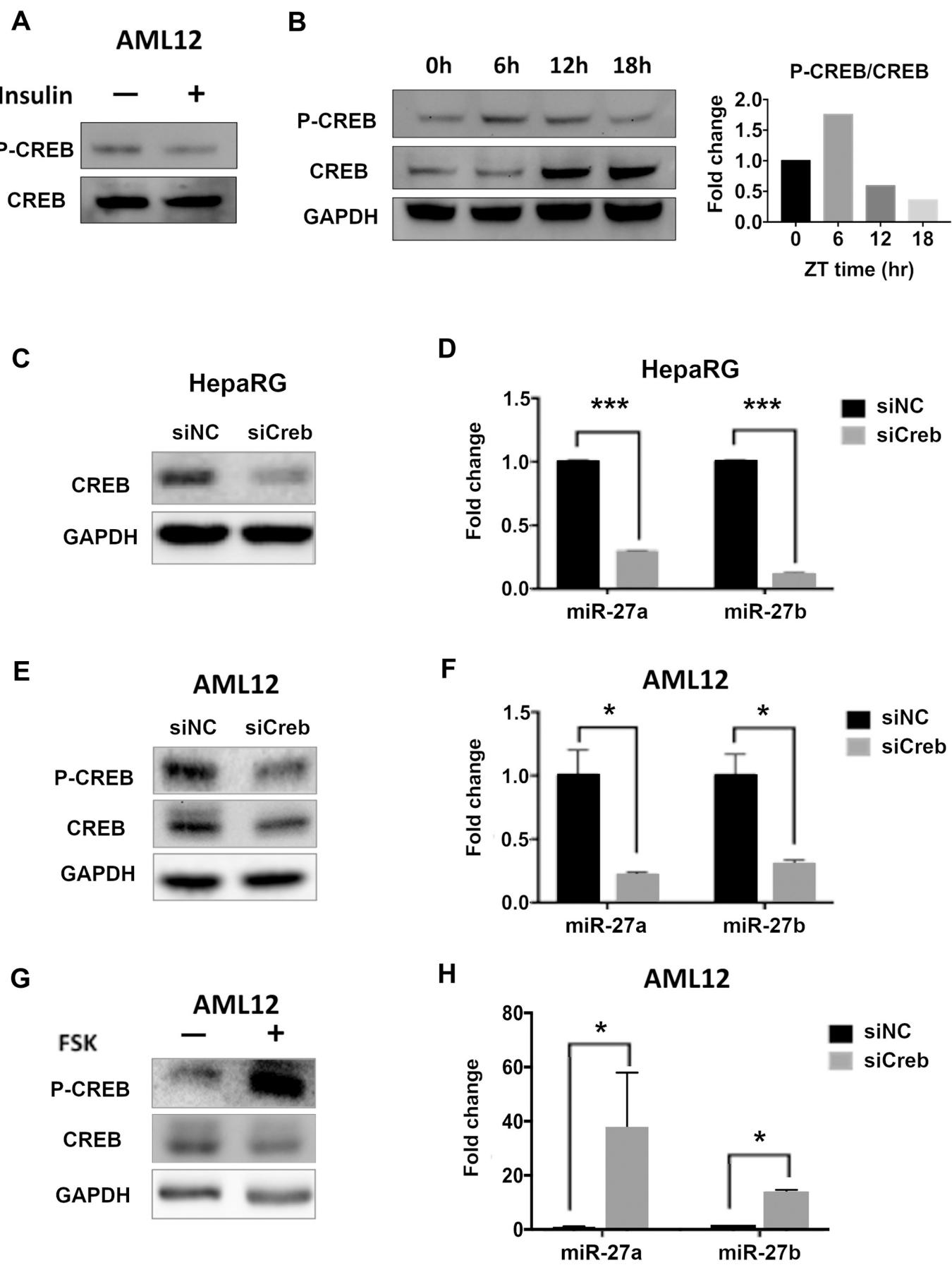
Fig. 2. miR-27a expression exhibited rhythmic pattern in mouse livers and was repressed by insulin stimulation *in vitro*. (A) QPCR analysis showing miR-27a and miR-27b levels in liver at different Zeitgeber (ZT) times (0, 6, 12, and 18). (B-E) After overnight nutrient deprivation in glucose-deficient medium, HepaRG (B-C) and AML12 (D-E) cells were treated with or without insulin for 1 h or 10 min, respectively. Western blot analysis was performed to assess AKT levels in HepaRG cells. β-actin protein expression was used as a loading control. QPCR was performed to determine miR-27a and miR-27b levels. Expression values of miRNAs were normalized to those of *Rnu6b*. *P < 0.05. Graphs show mean ± SEM. All experiments were repeated at least twice and similar trend was observed.

insulin resistance [8,9]. To assess this, AML12 cells were grown at low, medium, and high confluence to activate Hippo signaling at different levels. Expectedly, activation of Hippo signaling increased as cell confluence increased. In particular, the increased activation of Hippo signaling was verified by Yap phosphorylation and Akt dephosphorylation (Fig. 4A). Moreover, miR-27a and miR-27b were downregulated in cells at full confluence compared to those at low and medium confluence (Fig. 4B). Furthermore, both qPCR and western blot analysis indicated that the expression of several reported miR-27 targets, including FOXO1 [13] and PLK2 [14] is elevated at the protein level (Fig. 4A). However, phosphorylation level of CREB was similar among cells grown at different confluence (Fig. 4A), which suggested that Hippo signaling downregulated miR-27 in a CREB-independent manner. To determine if this upregulation was due to Hippo-mediated miR-27 downregulation, AML12 cells were transfected with miR-27a mimics and grown to full confluence to test the effect of miR-27 overexpression when Hippo signaling is fully activated. QPCR results verified increased miR-27a levels after transfection (Fig. 4C). Conversely, FOXO1 and PLK2 protein expression was lower in cells transfected with miR-27a mimics than cells transfected with negative control mimics (Fig. 4D). These data suggest that Hippo signaling suppresses miR-27 expression and upregulates miR-27 target gene expression. Next, we sought to determine the effect of Hippo inactivation on miR-27 expression. AML12 was transfected with siRNA to knockdown Lats2 and/or Nf2, which are critical upstream genes in Hippo signaling. Yap was dephosphorylated when AML12 was transfected with Lats2 and Nf2 siRNA, indicating inactivation of Hippo signaling (Fig. 5A). Nf2 phosphorylates and activates Lats1 and Lats2 to further phosphorylate YAP [15]. Therefore, the transfection of Lats2 siRNA alone was not sufficient to reduce YAP phosphorylation, possibly due to the activity of the residual Lats1 and Lats2. Additionally, miR-27a and miR-27b were upregulated in cells with decreased Lats2 levels (Fig. 5B). Moreover, FOXO1, but not PLK2 was significantly downregulated in cells co-transfected with both siLats2 and siNf2 (Fig. 5A). These data indicate that Hippo signaling regulates miR-27 expression and *Foxo1* is one of the key downstream target genes

in Hippo signaling.

3.5. miR-27 overexpression reduced insulin/Akt signaling by targeting *Pdk1* and *Pi3kr1* *in vitro*

We used the bioinformatics algorithm FAME (<http://acgt.cs.tau.ac.il/fame/>) [16] to analyze the potential signaling pathway in which miR-27 is involved. FAME annotates microRNA functions by analyzing the expression profiles of miRNAs/mRNAs using miRNA-target predictions made by TargetScan (http://www.targetscan.org/vert_72/). As a result, the algorithm predicted that miR-27 was involved in insulin signaling pathway, which was ranked fifth among the top ten KEGG pathways that were enriched with putative miR-27 targets (Table 1). Altogether, our experimental data shown in Fig. 2 and bioinformatics analysis suggest that miR-27 is negatively regulated by insulin and it may further regulate the downstream insulin signaling pathway. miR-27 is known to target PI3K to regulate Akt phosphorylation in cancer stem cells [17]. We speculated that miR-27a and miR-27b may function as upstream regulators of Akt phosphorylation. Thus, we hypothesized that miR-27 is downregulated by insulin but upregulated by CREB to decrease Akt phosphorylation. An obvious contradiction to this hypothesis is that Hippo signaling represses Akt phosphorylation according to a previous study [10]. However, a slight increase in Akt phosphorylation was observed when miR-27 was highly suppressed by Hippo signaling. In fact, miR-27 was significantly suppressed and AKT phosphorylation was higher in cells at full confluence (100%) compared to those at 95% confluence (Fig. 4A and B). A similar pattern was noted in cells transfected with siRNA against Lats2/Nf2 when compared with the control (Fig. 5A and B). To elucidate the mechanisms by which miR-27 participates in Akt phosphorylation, miR-27a was overexpressed by transfecting human HepaRG cells and primary mouse hepatocytes with miR-27a mimics. As miR-27a and miR-27b shared the same seed matching sequence, the same set of experiments with miR-27b were not repeated. After 16 h of culture in a serum-free growth medium containing low glucose (5 mM), HepaRG cells were treated with 100 nM



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Fig. 3. Differential miR-27 expression in human and mouse cell lines with various CREB expression and activity. (A) Mouse AML12 cells were treated with or without insulin for 10 min. (B) mouse liver harvested at different Zeitgeber (ZT) times (0, 6, 12, and 18). P-CREB was normalized to total CREB protein and the result was presented as fold change to ZT0. (C-D) Human HepaRG and (E-F) mouse AML12 cells were transfected with si-NC and si-CREB at 50 nM for 48 h. (G-H) AML12 cells were treated with vehicle or 100 μ M forskolin (FSK) for 1 d. Western blot analysis was performed to determine the level of CREB and related genes. QPCR was performed to measure miR-27 levels. GAPDH protein expression was used as a loading control for western blot. Expression values of miRNAs were normalized to those of *Rnu6b*. Data are presented as fold changes compared to the control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ NS: not significant. Graphs show mean \pm SEM.

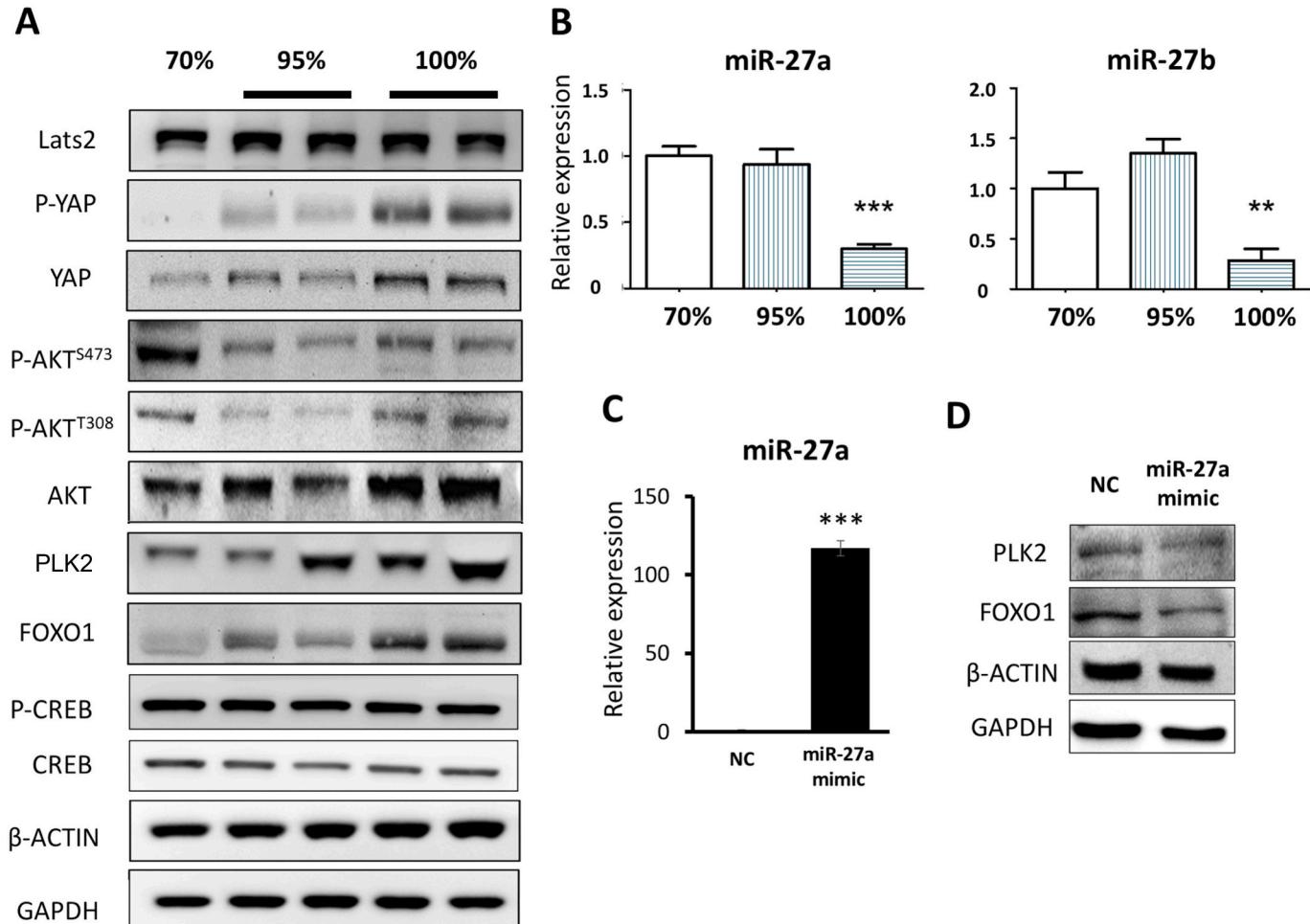


Fig. 4. Activation of Hippo signaling downregulated miR-27 and upregulated target genes of miR-27 in high-density cell cultures. (A) Western blot analysis revealed the protein expression of genes involved in Hippo, AKT, CREB signaling, and (B) qPCR analysis determined miR-27 levels in cells grown at various cell densities. (C-D) AML12 cells were transfected with miR-27a mimics (50 nM). (C) qPCR was performed to determine miR-27a levels. (D) Western blot analysis was performed to examine miR-27 target gene expression. GAPDH or β -actin protein expression were used as a loading control. Expression values of miRNAs were normalized to those of *Rnu6b*. Data are presented as fold changes compared to the control group. ** $P < 0.01$, *** $P < 0.001$. Graphs show mean \pm SEM.

insulin for 1 h to activate AKT signaling. RNA and protein were extracted to analyze miR-27 and AKT levels, respectively. At 48 h after transfection, miR-27a levels were significantly higher in HepaRG cells and primary mouse hepatocytes than matching cells that were transfected with negative control mimics (Fig. 6A). The expression of PIK3R1, PDPK1, and p-AKT^{S473} was decreased in all miR-27a-overexpressing cells as indicated by western blot analysis (Fig. 6B-C). These results supported the prediction made by the bioinformatics algorithms. Furthermore, we performed a luciferase reporter assay to verify whether PDPK1 and PIK3R1 were direct targets of miR-27a. *Pdk1* or *Pik3r1* 3'UTR sequences with or without miR-27 seed matching sequence were generated by PCR and cloned into the psiCHECK2 reporter vector to construct FL or DEL reporter constructs (Fig. 6D). Together with miR-27a or negative control mimic, these reporter constructs were co-transfected into NIH3T3 cells for 48 h before detection of luciferase

reporter activity. As a result, compared to cells receiving negative control, miR-27a mimics repressed luciferase activity of psiCHECK2 carrying FL *Pdk1* 3'UTR and *Pik3r1* 3'UTR to ~80% and ~60%, respectively; however, this miR-27a-mediated repression was abolished in cells transfected with the DEL construct (Fig. 6E). These data suggest that miR-27 suppresses the expression of *Pdk1* and *Pik3r1* by directly binding to the miR-27 binding site at 3'UTR. In other words, *Pdk1* and *Pik3r1* are direct targets of miR-27.

3.6. Metformin decreased miR-27 expression in human and mouse liver cell lines

Metformin, an old but effective medicine for the treatment of diabetes, is a negative regulator of CREB expression [18]. Therefore, metformin is speculated to serve as a miR-27 regulator by repressing CREB.

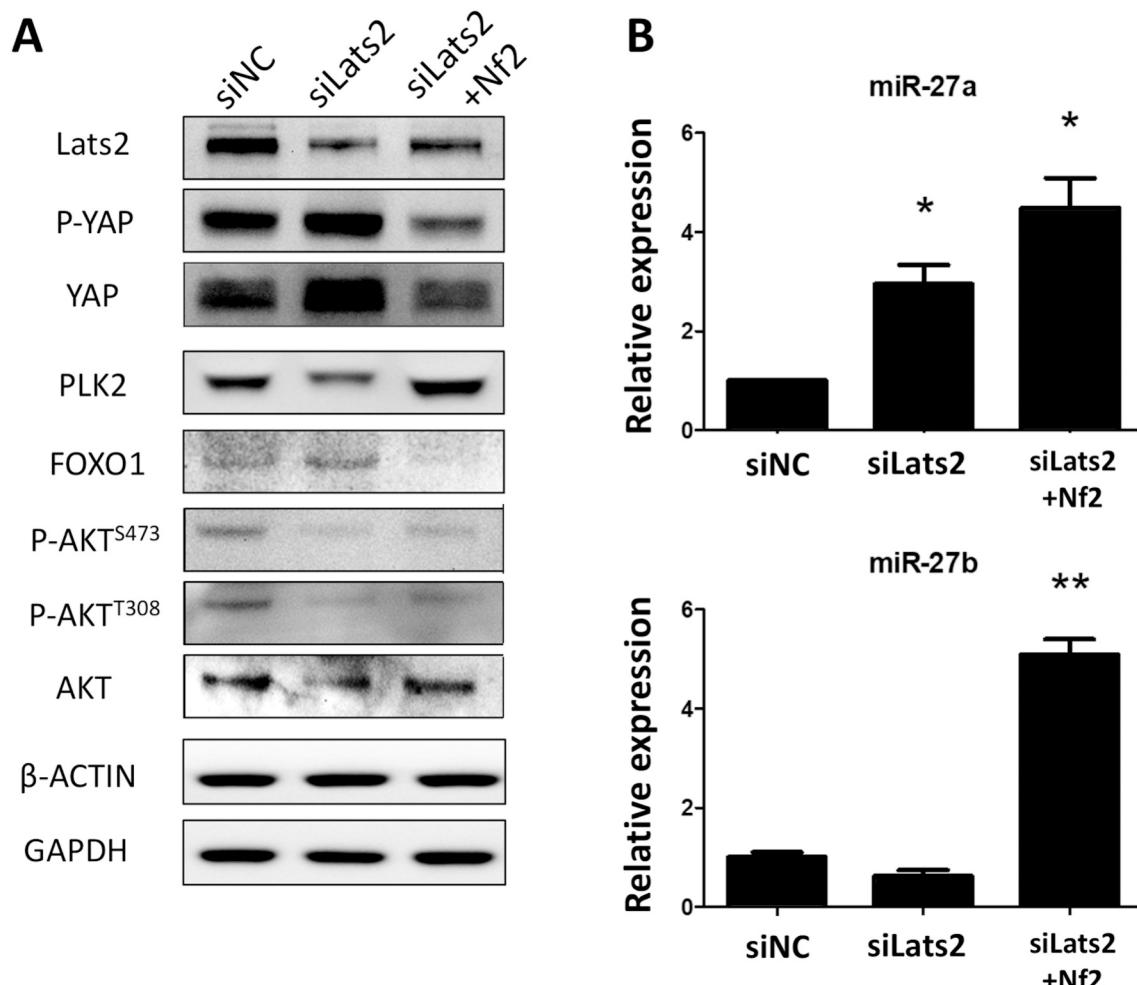


Fig. 5. SiRNA-mediated deactivation of Hippo signaling upregulated miR-27. AML12 cells were transfected with siRNA against Lats2 and/or Nf2 (50 nM). Negative control siRNA (siNC) was used as control. Protein and RNA were extracted after 72 h. (A) Western blot analysis was performed to profile gene expression. (B) QPCR was performed to measure miR-27 levels. GAPDH or β -actin protein expression was used as a loading control for western blot. Expression values of miRNAs were normalized to those of *Rnu6b*. Data are presented as fold changes compared to the control group. * $P < 0.05$, ** $P < 0.01$. Graphs show mean \pm SEM.

Table 1

Insulin signaling pathway was ranked third among the top predicted biological functions of miR-27 by FAME.

Rank	KEGG pathway	FAME p-value	FAME q-value	FAME z-score	FAME enrichment factor	Targets with function	HG p-value
1	Fc epsilon RI signaling pathway	0.0100	0.5500	3.78	2.32	11	0.00910
2	GnRH signaling pathway	0.0100	0.2750	3.48	1.90	14	0.00352
3	Insulin signaling pathway	0.0100	0.1833	2.71	1.57	19	0.00270
4	T cell receptor signaling pathway	0.0100	0.1375	4.05	2.09	18	0.00041
5	Glycerophospholipid metabolism	0.0100	0.1100	2.93	2.23	8	0.03714
6	Neuroactive ligand-receptor interaction	0.0100	0.0917	4.05	2.14	16	0.08851
7	MAPK signaling pathway	0.0100	0.0786	2.42	1.40	30	0.01004

Biological functions of miR-27 in human were predicted by the FAME (functional assignment of miRNAs via enrichment) algorithm based on the enrichment of miRNA targets in a set of genes involved in a specific process or pathway in the KEGG database. The top seven predicted KEGG pathways for miR-27a/b family with FDR < 0.1 are shown. All KEGG pathways were ranked in an increasing order of p-value. In case of ties, all pathways were ranked in decreasing order of q-value. The detailed computation of FAME p-value and FAME enrichment factors are described in the previous study [16]. As a comparison to the FAME-based prediction, p-value of hypergeometric (HG) test was additionally generated by the FAME algorithm using hypergeometric test.

To test this *in vitro*, we treated human HepaRG and mouse Hepa1-6 cells with different concentrations of metformin (0, 5, and 10 mM) for 48 h. Metformin was found to significantly suppress CREB protein expression (Fig. 7A and C). Furthermore, 10 mM metformin significantly reduced miR-27a levels (Fig. 7B and D). These data indicate that metformin downregulates miR-27 via the CREB axis *in vitro* and it could be applied as a chemical miR-27 regulator in the context of NAFLD.

4. Discussion

In this study, we investigated the mechanism by which miR-27 is regulated in livers with insulin resistance. Our data revealed a coordinated regulation of miR-27 by insulin/CREB/Hippo signaling pathways. First, in the context of insulin resistance, in which insulin and Hippo signaling pathways are downregulated and CREB signaling is upregulated, miR-27 was significantly upregulated in both the PA-treated cell

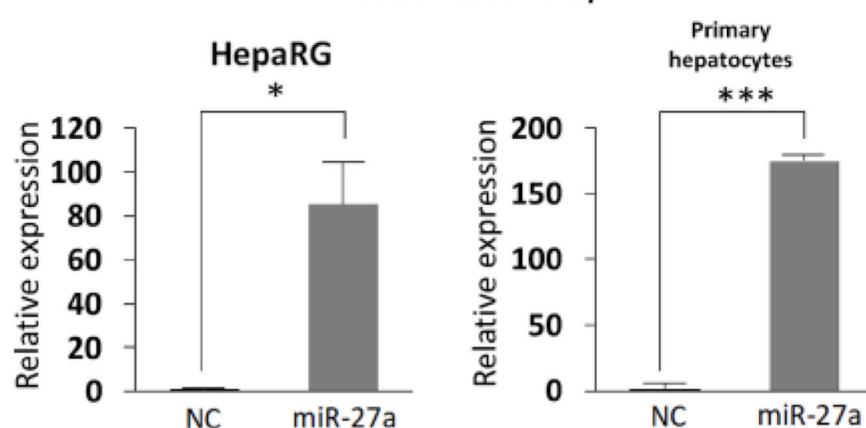
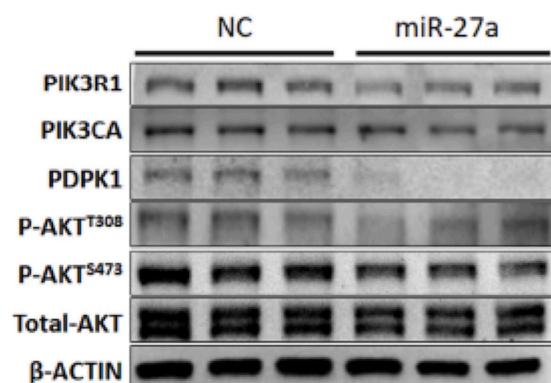
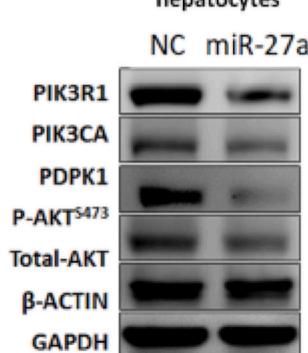
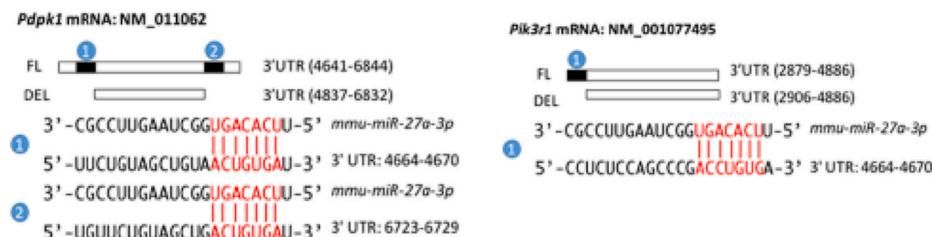
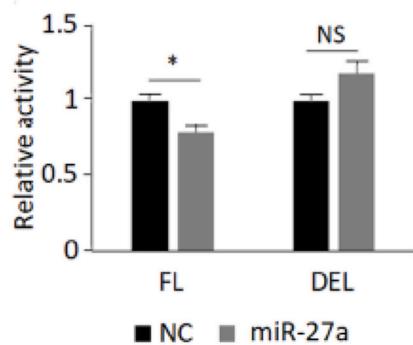
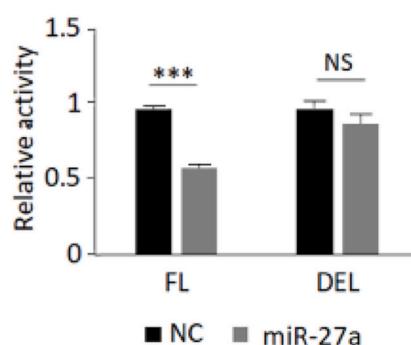
A*mmu-miR-27a-3p***B****HepaRG****C****Primary hepatocytes****D****E***Pdpk1* 3'UTR*Pik3r1* 3'UTR

Fig. 6. Overexpression of miR-27 repressed AKT signaling in human HepaRG cells and primary mouse hepatocytes via *Pdpk1* and *Pi3kr1*. HepaRG cells and primary hepatocytes were transfected with miR-27a mimics at 50 nM for 48 and 72 h before RNA and protein extraction, respectively. (A) QPCR was performed to determine miR-27a levels. (B-C) Western blot analysis was performed to profile genes involved in AKT signaling in HepaRG cells (B) and primary mouse hepatocytes (C). (D) Schematics of mouse *Pdpk1* and *Pi3kr1* 3'UTR that contained predicted miR-27a 3'UTR seed sequences (full length, FL) or with the seed sequences deleted (deletion length, DEL). Both sequences were cloned into the luciferase reporter psiCHECK2 and were cotransfected at 200 ng per well into NIH3T3 cells with control (NC) or miR-27a mimic (miR-27a) at 50 nM. (E) Luciferase activity was measured 48 h after transfection. Graphs show Renilla luciferase activity normalized to firefly luciferase activity expressed by the same plasmid. GAPDH or β -actin protein expression was used as a loading control for western blot. Expression values of miRNAs were normalized to those of *Rnub6b*. The data were analyzed in triplicate. *** $P < 0.001$. * $P < 0.05$. NS, not significant. Graphs show mean \pm SEM.

lines and HFD-fed mouse livers. Second, miR-27 expression was negatively regulated by insulin and Hippo signaling pathways, but positively regulated by CREB in both human and mouse cell lines. Third, miR-27 overexpression led to AKT dephosphorylation and downregulation of several reported miR-27 targets, such as FOXO1 and PLK2. Furthermore, miR-27 repressed AKT phosphorylation by directly targeting *Pdpk1* and *Pik3r1*. Fourth, metformin treatment, a well-known diabetic medicine, induced CREB and miR-27 expression in human and mouse cell lines. Altogether, as shown in Fig. 8, our data suggested that insulin may downregulate miR-27 and eventually promote AKT phosphorylation. However, this mechanism is abolished by aberrant CREB overexpression and Hippo downregulation in the context of insulin resistance. To the best of our knowledge, our findings are the first to discuss the biological role of insulin/CREB/Hippo-regulated miR-27 expression in the development of insulin resistance. In the context of obesity, the aberrant overexpression of CREB and miR-27 may be the cause of insulin insensitivity. It is speculated that insulin fails to activate AKT phosphorylation and results in high levels of CREB, which leads to miR-27 overexpression and subsequent AKT dephosphorylation; in other words, there is a positive feedback loop between insulin insensitivity (i.e., AKT dephosphorylation) and miR-27 expression. Previous studies have reported that CREB depletion helps to restore insulin resistance [8]. Further studies are required to determine whether miR-27 depletion may also exert a beneficial effect similar to that of obesity-induced diabetes.

Many miRNAs regulate the insulin signaling pathway and influence liver metabolism. For instance, miR-26a [19], miR-33 [20], and miR-802 [21] affect hepatic insulin sensitivity to regulate glucose metabolism. In this study, the role of miR-27 in insulin/AKT signaling in the liver was elucidated. In fact, insulin may serve as a negative regulator of miR-27a. High levels of insulin in the liver result in rapid alternation of hepatic homeostasis, such as glycogenesis, lipogenesis, and suppression of gluconeogenesis [22–24]. Our findings also provide a microRNA-mediated link between insulin and rhythmic AKT signaling. Furthermore, our data demonstrated a rapid CREB-mediated expression of miR-27 in response to insulin. Interestingly, recent studies have highlighted the potential of microRNAs as fast-responsive genes in fast-paced biological events. For example, Salzman et al. discovered that miR-34 is pre-processed and stored in an inactive form, and only functions when phosphorylated in response to DNA damage [25]. Similarly, Bartel's

group demonstrated that miR-16 is dynamically expressed during cell cycle progression [26]. These findings suggest that microRNAs can be temporally regulated. Our results further characterize that miR-27 was regulated by insulin in response to rapid metabolic changes after food intake, indicating that miR-27 is another example of a fast-responsive microRNA.

MiR-27 has been shown to be a therapeutic target in NAFLD mouse models, where antagomir-mediated miR-27 silencing ameliorates steatosis in HFD-induced mouse liver [1]; therefore, the discovery of a miR-27 negative-regulator could be valuable in the treatment of NAFLD. In the last part of our result, we tested the hypothesis that metformin could be used to manipulate miR-27 expression. The most profound effect of metformin is to reduce hepatic glucose production and plasma glucose and, therefore, improve the progression of diabetes [27,28]. Our *in vitro* findings demonstrate that metformin may effectively downregulate miR-27 via downregulation of CREB, which plays an important role in activating gluconeogenesis during fasting. Therefore, to control miR-27 expression via CREB, metformin could be an ideal option, and it would be necessary to design a chemical compound that directly blocks the transcriptional activity of CREB without disturbing AKT signaling.

5. Conclusions

Our data from cell lines and animals clearly indicated that miR-27 expression was negatively regulated by insulin/Hippo signaling but positively regulated by CREB. Moreover, in liver cells, miR-27 overexpression suppressed Akt phosphorylation by targeting *Pdpk1* and *Pik3r1*. Based on these data, we concluded that dysregulation of insulin/CREB/Hippo in PA-treated cells and HFD-fed mice aberrantly increased miR-27, which subsequently inhibited Akt phosphorylation and contributed to insulin resistance. All in all, miR-27 is a critical gene in insulin signaling and dysregulation of its upstream regulators, including insulin/CREB/Hippo, may further promote preexisting insulin resistance and exacerbate associated NAFLD. In the future, miR-27 can be further tested as a potential therapeutic target for patients with NAFLD to restore insulin sensitivity of hepatocytes.

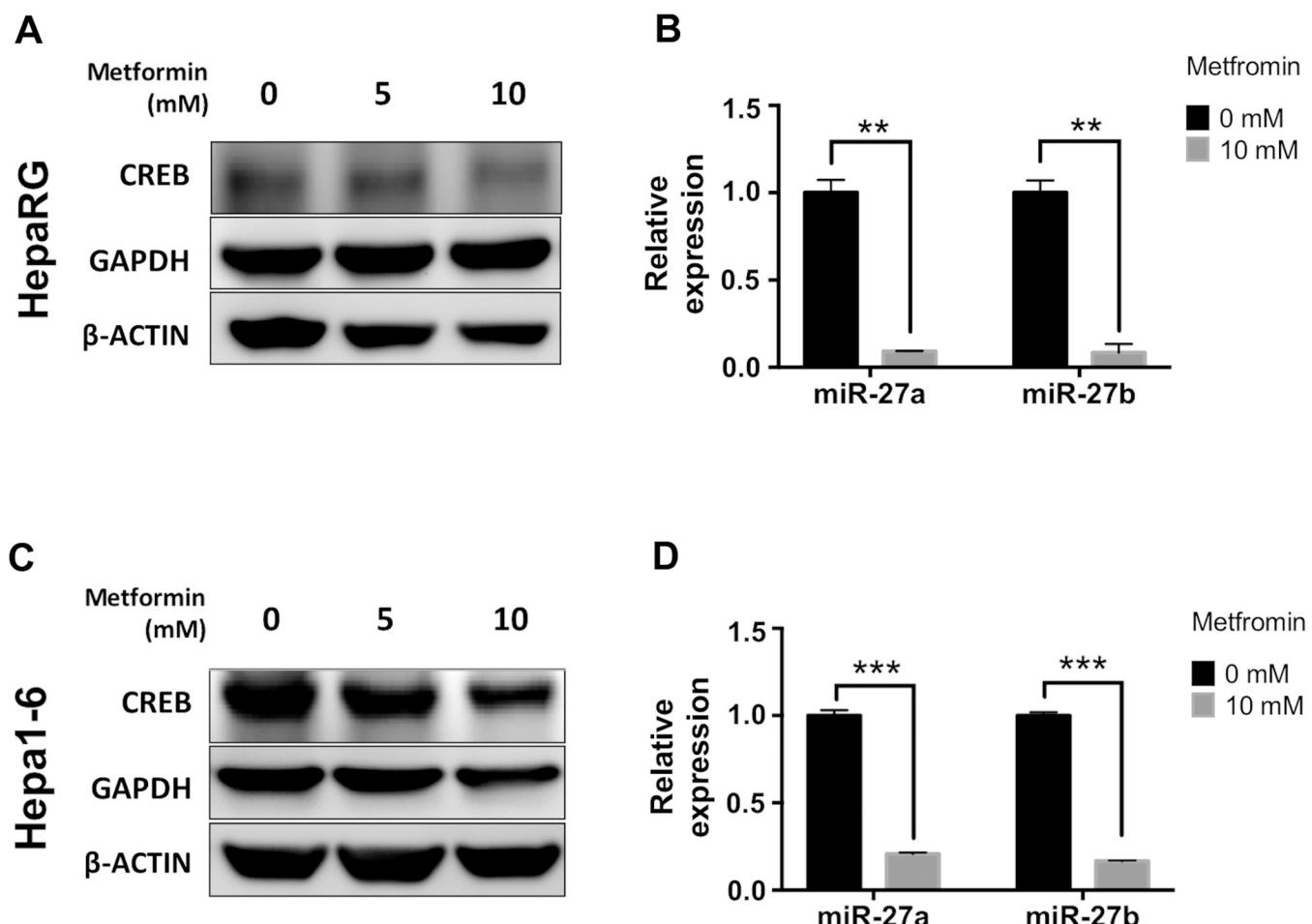


Fig. 7. Metformin downregulated CREB to reduce miR-27 expression in cells. CREB protein expression and miR-27 expression were determined by western blot analysis and qPCR, respectively, in (A-B) HepaRG and (C-D) Hepa1-6 cells treated with metformin (0, 5, and 10 mM) for 48 h. GAPDH or β-actin protein expression was used as a loading control for western blot. Expression values of miRNAs were normalized to those of *Rnub6b*. Data were presented as fold changes compared to the control. *P < 0.05, **P < 0.01, ***P < 0.001. NS, not significant. Graphs show mean ± SEM.

Ethics approval

All animal studies were performed according to the guideline by Council of Agriculture Executive Yuan Guideline for the Care and Use of Laboratory Animals, Taiwan. All of the animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University College of Medicine.

Competing interests

No commercial association or conflict of interest that needs to be declared.

Availability of data and materials

All data and materials are fully available and are shown within the manuscript.

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Author contributions

S. Hsu designed the experiments; Y. Chen, L. Chueh, Shi-Yun Lee, and Peng-Fang Ma conducted the experiments; S. Hsu, Y. Chen, and L. Chueh analyzed the data; P. Chen and S. Hsu edited the manuscript.

CRediT authorship contribution statement

Yen-Ju Chen: Formal analysis, Investigation, Methodology, Resources, Validation, Visualization. **Li-Yun Chueh:** Formal analysis, Investigation, Methodology, Resources, Validation, Visualization. **Shi-Yun Lee:** Investigation, Validation. **Peng-Fang Ma:** Investigation, Validation. **Po-Chun Chen:** Writing - original draft, Writing - review & editing. **Shu-hao Hsu:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing - original draft, Writing - review & editing.

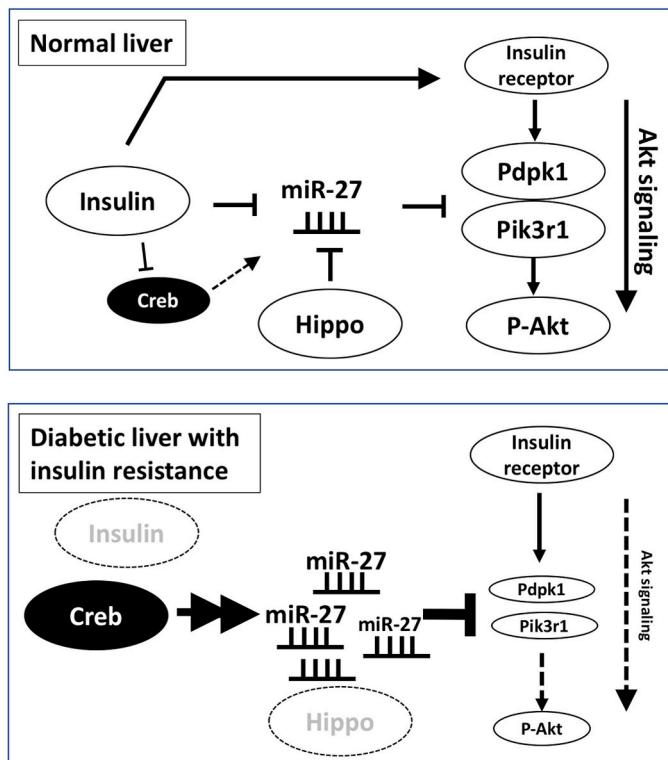


Fig. 8. Schematics of insulin/CREB/Hippo-regulated miR-27 expression and AKT phosphorylation in normal and diabetic livers. The faded-out shapes represent the decreased expression or activity of the designated genes or signalling pathways.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellsig.2021.109930>.

References

- [1] M. Zhang, W. Sun, M. Zhou, Y. Tang, MicroRNA-27a regulates hepatic lipid metabolism and alleviates NAFLD via repressing FAS and SCD1, *Sci. Rep.* 7 (2017) 14493.
- [2] M. Karbienier, C. Fischer, S. Nowitsch, P. Opiressnig, C. Papak, G. Ailhaud, et al., microRNA miR-27b impairs human adipocyte differentiation and targets PPARGamma, *Biochem. Biophys. Res. Commun.* 390 (2) (2009) 247–251.
- [3] K.C. Vickers, B.M. Shoucri, M.G. Levin, H. Wu, D.S. Pearson, D. Osei-Hwedieh, et al., MicroRNA-27b is a regulatory hub in lipid metabolism and is altered in dyslipidemia, *Hepatology* 57 (2) (2013) 533–542.
- [4] S.H. Hsu, E.R. Delgado, P.A. Otero, K.Y. Teng, H. Kutay, K.M. Meehan, et al., MicroRNA-122 regulates polypliodization in the murine liver, *Hepatology* 64 (2) (2016) 599–615.
- [5] W. Zhang, P. Wang, S. Chen, Z. Zhang, T. Liang, C. Liu, Rhythmic expression of miR-27b-3p targets the clock gene Bmal1 at the posttranscriptional level in the mouse liver, *FASEB J.* 30 (6) (2016) 2151–2160.
- [6] Z.M. Younossi, M. Otgonsuren, L. Henry, C. Venkatesan, A. Mishra, M. Erario, et al., Association of nonalcoholic fatty liver disease (NAFLD) with hepatocellular carcinoma (HCC) in the United States from 2004 to 2009, *Hepatology* 62 (6) (2015) 1723–1730.
- [7] V.T. Samuel, G.I. Shulman, The pathogenesis of insulin resistance: integrating signalling pathways and substrate flux, *J. Clin. Invest.* 126 (1) (2016) 12–22.
- [8] D.M. Erion, I.D. Ignatova, S. Yonemitsu, Y. Nagai, P. Chatterjee, D. Weismann, et al., Prevention of hepatic steatosis and hepatic insulin resistance by knockdown of cAMP response element-binding protein, *Cell Metab.* 10 (6) (2009) 499–506.
- [9] Y. Aylon, A. Gershoni, R. Rotkopf, I.E. Biton, Z. Porat, A.P. Koh, et al., The LATS2 tumor suppressor inhibits SREBP and suppresses hepatic cholesterol accumulation, *Genes Dev.* 30 (7) (2016) 786–797.
- [10] S.H. Jeong, H.B. Kim, M.C. Kim, J.M. Lee, J.H. Lee, J.H. Kim, et al., Hippo-mediated suppression of IRS2/AKT signalling prevents hepatic steatosis and liver cancer, *J. Clin. Invest.* 128 (3) (2018) 1010–1025.
- [11] M.F. Kramer, Stem-loop RT-qPCR for miRNAs, *Curr. Protoc. Mol. Biol.* 95 (1) (2011) 15.10.1–15.10.15 (Chapter 15:Unit 15.10).
- [12] M. Shiwa, M. Yoneda, H. Okubo, H. Ohno, K. Kobuke, Y. Monzen, et al., Distinct time course of the decrease in hepatic AMP-activated protein kinase and Akt phosphorylation in mice fed a high fat diet, *PLoS One* 10 (8) (2015), e013554.
- [13] I.K. Guttilla, B.A. White, Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells, *J. Biol. Chem.* 284 (35) (2009) 23204–23216.
- [14] Y. Tian, S. Fu, G.B. Qiu, Z.M. Xu, N. Liu, X.W. Zhang, et al., MicroRNA-27a promotes proliferation and suppresses apoptosis by targeting PLK2 in laryngeal carcinoma, *BMC Cancer* 14 (2014) 678.
- [15] Z. Meng, T. Moroishi, K.L. Guan, Mechanisms of hippo pathway regulation, *Genes Dev.* 30 (1) (2016) 1–17.
- [16] I. Ulitsky, L.C. Laurent, R. Shamir, Towards computational prediction of microRNA function and activity, *Nucleic Acids Res.* 38 (15) (2010), e160.
- [17] Y. Chen, B. Zhang, Y. Jin, Q. Wu, L. Cao, MiR-27b targets PI3K p110alpha to inhibit proliferation and migration in colorectal cancer stem cell, *Am. J. Transl. Res.* 11 (9) (2019) 5988–5997.
- [18] L. He, A. Sabet, S. Djedjos, R. Miller, X. Sun, M.A. Hussain, et al., Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein, *Cell* 137 (4) (2009) 635–646.
- [19] X. Fu, B. Dong, Y. Tian, P. Lefebvre, Z. Meng, X. Wang, et al., MicroRNA-26a regulates insulin sensitivity and metabolism of glucose and lipids, *J. Clin. Invest.* 125 (6) (2015) 2497–2509.
- [20] C.M. Ramirez, L. Goedeke, N. Rotllan, J.H. Yoon, D. Cirera-Salinas, J.A. Mattison, et al., MicroRNA 33 regulates glucose metabolism, *Mol. Cell. Biol.* 33 (15) (2013) 2891–2902.
- [21] J.W. Kornfeld, C. Baitzel, A.C. Konner, H.T. Nicholls, M.C. Vogt, K. Herrmanns, et al., Obesity-induced overexpression of miR-802 impairs glucose metabolism through silencing of Hnf1b, *Nature* 494 (7435) (2013) 111–115.
- [22] L.L. Madison, Role of insulin in the hepatic handling of glucose, *Arch. Intern. Med.* 123 (3) (1969) 284–292.
- [23] J.H. Exton, L.E. Mallette, L.S. Jefferson, E.H. Wong, N. Friedmann, T.B. Miller Jr., et al., The hormonal control of hepatic gluconeogenesis, *Recent Prog. Horm. Res.* 26 (1970) 411–461.
- [24] G.I. Welsh, C.G. Proud, Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B, *Biochem. J.* 294 (Pt 3) (1993) 625–629.
- [25] D.W. Salzman, K. Nakamura, S. Nallur, M.T. Dookwah, C. Methetrairut, F.J. Slack, et al., miR-34 activity is modulated through 5'-end phosphorylation in response to DNA damage, *Nat. Commun.* 7 (2016) 10954.
- [26] O.S. Rissland, S.J. Hong, D.P. Bartel, MicroRNA destabilization enables dynamic regulation of the miR-16 family in response to cell-cycle changes, *Mol. Cell* 43 (6) (2011) 993–1004.
- [27] N.F. Wiernsperger, C.J. Bailey, The antihyperglycaemic effect of metformin: therapeutic and cellular mechanisms, *Drugs* 58 (Suppl. 1) (1999) 31–39 (discussion 75–82).
- [28] R.S. Hundal, M. Krssak, S. Dufour, D. Laurent, V. Lebon, V. Chandramouli, et al., Mechanism by which metformin reduces glucose production in type 2 diabetes, *Diabetes* 49 (12) (2000) 2063–2069.