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METABOLIC LIVER DISEASE

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MicroRNA-30b regulates insulin sensitivity by targeting SERCA2b in non-alcoholic fatty liver disease

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Abstract

Background & Aims: Insulin resistance is strongly associated with non-alcoholic fatty liver disease, a chronic, obesity–related liver disease. Increased endoplasmic reticulum (ER) stress plays an important role in the development of insulin resistance. In this study, we investigated the roles of miRNAs in regulating ER stress in the liver of rats with obesity.

Methods: We used miRNA microarray to determine the miRNA expression profiles in the liver of rats fed with a high fat diet (HFD). We used prediction algorithms and luciferase reporter assay to identify the target gene of miRNAs. To overexpress the miRNA miR-30b or inhibit miR-30b rats were injected with lentivirus particles containing PGLV3-miR-30b or PGLV3-miR-30b antimiR through tail vein. Hepatic steatosis was measured using transient elastography in human subjects.

Results: Our data showed that miR-30b was markedly up-regulated in the liver of HFD–treated rats. Bioinformatic and in vitro and in vivo studies led us to identify sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b), as a novel target of miR-30b. Overexpression of miR-30b induced ER stress and insulin resistance in rats fed with normal diet, whereas inhibition of miR-30b by miR-30b antimiR suppressed ER stress and insulin resistance in HFD–treated rats. Finally, our data demonstrated that there was a positive correlation between serum miR-30b levels and hepatic steatosis or homoeostasis model assessment of insulin resistance (HOMA-IR) in human subjects.

Conclusions: Our findings suggest that miR-30b represents not only a potential target for the treatment of insulin resistance, but also a non-invasive disease biomarker of NAFLD.

KEYWORDS
endoplasmic reticulum stress, insulin resistance, microRNA, NAFLD

Abbreviations: 3′-UTR, 3′-untranslated region; CHOP, CCAAT/enhancer–binding protein homologous protein; ER, endoplasmic reticulum; HFD, high fat diet; miRNA, microRNA; NAFLD, non-alcoholic fatty liver disease; qRT-PCR, quantitative real-time PCR; SERCA2b, sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b.

Li-Li Dai, Shu-De Li and Yi-Cheng Ma contributed equally to this work.

1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is considered as the hepatic manifestation of obesity and encompasses simple steatosis, non-alcoholic steatohepatitis and cirrhosis.[1,2] The liver is one of the major organs of insulin action. Insulin resistance is pivotal for the development of NAFLD.[2-4] Insulin resistance leads to an increase in hepatic de novo lipogenesis and subsequent accumulation of fatty acids in the liver.[4] On the other hand, excessive fat accumulation (steatosis) in liver may promote the pathogenesis of hepatic insulin resistance by inducing inflammation and endoplasmic reticulum (ER) stress.[5,6] Thus, NAFLD and insulin resistance are intertwined in obesity.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that are approximately 22 nucleotides long. In animals, miRNAs mainly target specific mRNAs through imperfect complementarity with the 3′-untranslated region (3′-UTR) of these mRNAs resulting in either translational repression or mRNA cleavage.[7] An increasing number of studies have identified misregulated miRNAs in the liver from dietary–induced and genetic (ob/ob) models of murine obesity and patients with severe NAFLD,[8-13] suggesting a potential role for miRNAs in the pathogenesis of NAFLD and insulin resistance. For example, the expression of miR-103/107 is up-regulated in ob/ob mice and diet–induced obese mice.[9] Silencing of miR-103/107 restores the protein levels of caveolin-1, a regulator of the insulin receptor, thereby improving glucose homeostasis. Furthermore, upregulation of miR-24 promotes hepatic lipid accumulation by repressing insulin–induced protein 1 in the liver of mice fed with high–fat diet (HFD).[11] miR-21, which is up-regulated after HFD treatment, suppressing its targets Hbp1 and p53, leading to hepatic lipid accumulation in mice.[12] A recent study has revealed that the expression of miR-206 is significantly reduced in the liver of mice fed with HFD.[13] Delivery of miR-206 into the liver of dietary obese mice promotes insulin signalling and inhibits hepatic lipogenesis by suppressing the expression of protein tyrosine phosphatase, non-receptor type 1.

Endoplasmic reticulum is the main site of protein folding, lipid and cholesterol biosynthesis and cellular calcium storage. Increased load of misfolded proteins that enter the ER leads to ER stress and the activation of the unfolded protein response.[14,15] ER stress induces dissociation of the ER chaperone glucose regulated protein 78 from three ER stress signalling mediators: the ribonuclease inositol–requiring protein-1 (IRE-1), the PKR-like ER kinase (PERK) and the activating transcription factor 6 (ATF6).[15] PERK phosphorylates and activates eIF2α leading to an increase in translation of transcription factor ATF4 and its downstream target CCAAT/enhancer–binding protein homologous protein (CHOP). IRE-1 splices an intron from transcription factor XBP1 mRNA, producing the activated 'spliced form' of XBP1 (XBP1s).[14,15] Accumulating evidence indicates that ER stress plays a critical role in the development of insulin resistance in obesity.[16-19] Sarco/ER calcium ATPase (SERCA) is a Ca^{2+}-transport ATPase that is involved in the reuptake of Ca^{2+} from the cytosol into the ER lumen.[20] In the liver, the main isoform of SERCA is sarco(endo) plasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b). Previous studies have shown that SERCA2b expression or activity is dramatically reduced, leading to ER stress and insulin resistance in the liver of leptin–deficient obese (ob/ob) mice.[20,21] Overexpression of SERCA2b in the livers of obese mice reduces ER stress and increases glucose tolerance.[20,21] A recent study has indicated that activation of SERCA2b by its allosteric activator, CDN1163, markedly ameliorates ER stress and improves glucose homeostasis in ob/ob mice.[22] These results highlight a pathological role for SERCA2b dysfunction in the development of metabolic abnormalities in insulin resistance and diabetes.

To date, how miRNAs modulate ER stress during NAFLD has not been addressed. In this report, we performed miRNA microarray analysis on the livers of rats fed HFD. We demonstrated that miR-30b was markedly up-regulated in the livers of HFD–treated rats and promoted ER stress by targeting SERCA2b. Overexpression of miR-30b induced insulin resistance. Finally, our data showed that there was a positive correlation between the serum miR-30b levels and hepatic steatosis in human subjects.

Key points

- Expression of miR-30b is up-regulated in the livers of HFD–treated rats.
- miR-30b activates endoplasmic reticulum (ER) stress by repressing SERCA2b expression in the livers of rats.
- Activation of ER stress in liver impairs insulin sensitivity in rats.
- Serum miR-30b is positively correlated with hepatic steatosis or HOMA-IR in human subjects.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Sprague-Dawley (SD) rats (12 weeks old, 220-240 g) rats were obtained from the Animal Center, Kunming Medical University (Kunming City, Yunnan, China). These animals were housed at a constant temperature of 20-22°C, with a 12-hour light/dark cycle. All animal procedures conform to the Guide for the Care and Use of Laboratory Animals that was published by the US National Institute of Health (NIH Publication No. 8523, revised 1985). This study was approved by the Animal Care and Use Committee of Kunming Medical University.

3 | RESULTS

3.1 | Induction of ER stress is accompanied by down-regulation of SERCA2b expression in the livers of rats on HFD

Activation of ER stress has been observed in the liver from dietary (HFD–induced) and genetic (ob/ob) models of murine obesity.[20,23]

We measured the expression of several molecular indicators of ER stress in the livers of rats and found that the protein levels of CHOP, ATF4, GRP-78/BIP, ATF-6, p-PERK, p-IRE-1 and p-eIF2α were significantly up-regulated in the liver of rats on HFD (Figure 1A,B; Figure S1A), compared with those in rats fed normal diet. Using quantitative real-time PCR (qPCR), we observed a significant increase in XBP1 splicing in the liver of rats on HFD (Figure 1C). hese results suggest that ER stress is activated in the livers of obese rats.

Inadequate or depleted Ca^{2+} content in ER lead to ER stress.[15] A previous study has demonstrated that the protein levels of SERCA2b are dramatically reduced by 90%, whereas its mRNA levels are down-regulated about a two-fold in the livers of ob/ob mice.[20] Consistent with these observations, we found that the levels of SERCA2b protein and SERCA2b mRNA were reduced approximately fold-fold and 1.8-fold respectively, in the liver of rats fed with HFD (Figure 1D-F). In addition, immunohistochemistry analysis confirmed a significant reduction in the protein levels of SERCA2b in the liver of rats fed with HFD (Figure 1G).

We also determined the protein levels of SERCA2b in the liver of rats fed with methionine- and choline-deficient diet (MCD). After six weeks of MCD feeding, a significant increase in hepatic lipid accumulation was observed (Figure S2A). The protein levels of SERCA2b were decreased (Figure S2B,D), whereas the protein levels of CHOP, ATF4, GRP-78/BIP and the mRNA levels of XBP1 splicing were significantly up-regulated in the liver of mice fed MCD (Figure S2C-E).

3.2 | miR-30b is up-regulated and suppresses SERCA2b translation in the liver of rats on HFD

The disproportional changes in the levels of SERCA2b transcript and SERCA2b protein in obese rats implicate that the expression of SERCA2b is likely regulated by post-transcriptional mechanisms. Since miRNAs exert such mechanisms, we investigated whether miRNAs were involved in the suppression of SERCA2b protein. We first determined the miRNA expression profiles using miRNA microarray in the liver of rats and found that 17 miRNAs were up-regulated at least two-fold (*P*-value ≤ 0.05) in the liver of rats fed with HFD (Figure 2A,B; Table S1). We used prediction algorithms (Miranda and Targetscan) to identify their target gens (Figure 2C). We selected miRNAs that meet the following three criteria: (a) The predicted target gene of miRNAs is SERCA2b; (b) The miRNAs are conserved in human and rodents; (c) Conserved binding sites for miRNAs. We found that only the miR-30b and let-7 family met these criteria (Figure 2C; Table S2). Two previous studies have demonstrated that let-7 overexpression induces insulin resistance by repressing multiple components of the insulin-PI3K-mTOR pathway, including the insulin–like growth factor 1 receptor, the insulin receptor and the insulin receptor substrate 2.[24,25] We thus focused on the

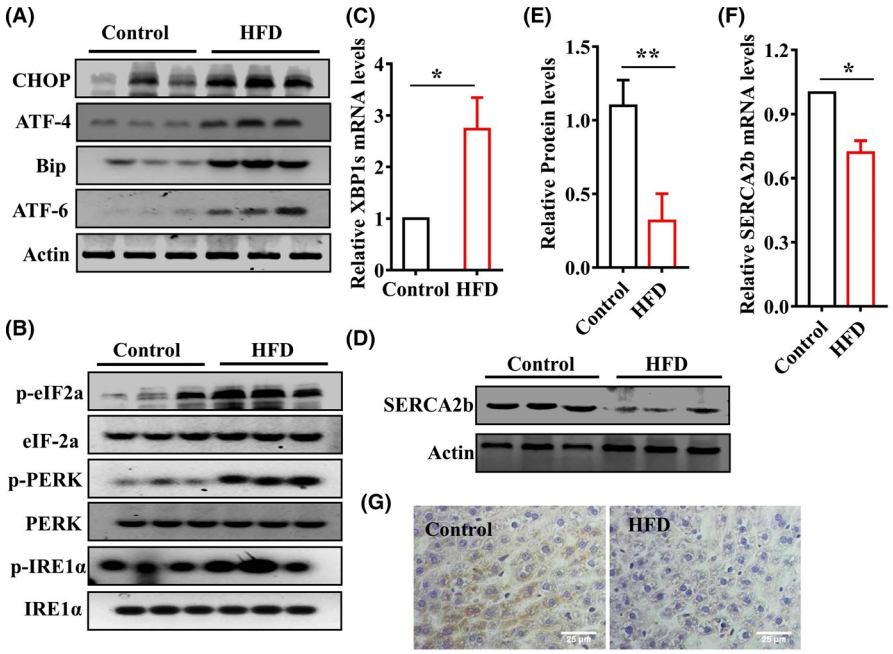

FIGURE 1 High fat diet (HFD) activated endoplasmic reticulum (ER) stress and decreased the expression of sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) in the liver of rats. (A,B) The protein levels of ER stress markers were up-regulated in the liver of rats fed HFD. The protein levels were measured using Western blotting. C, Quantitative real-time PCR (qPCR) analysis of spliced XBP1 mRNA levels. All results are standardized to the levels of β-actin and are the mean ± SD (n = 6 in each group). **P* < 0.05 relative to control. (D,E) The protein levels of SERCA2b were down-regulated in the liver of rats fed HFD. The SERCA2b levels were measured using Western blotting (n = 6 in each group). Representative Western blots are shown (D). Quantification of the ratio of SERCA2b to β-actin (E). ***P* < 0.01 relative to control (normal diet). F, qPCR analysis revealed that the mRNA levels of SERCA2b were down-regulated in the liver of rats fed HFD. All results are standardized to the levels of β-actin and are the mean ± SD (n = 6 in each group). **P* < 0.05 relative to control. G, The protein expression of SERCA2b in the liver was determined using immunohistochemistry

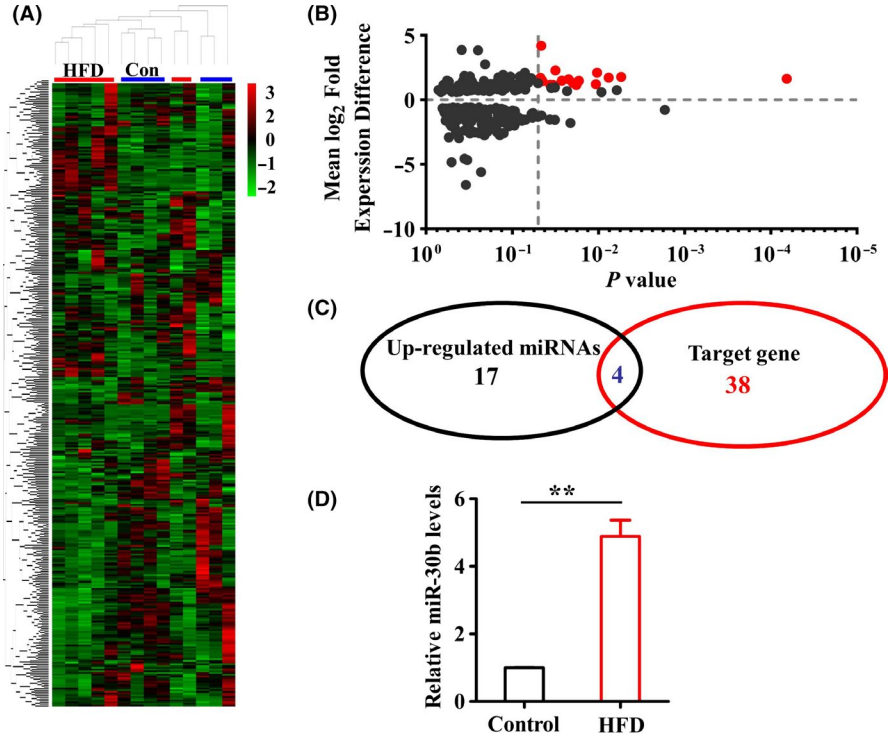

FIGURE 2 High fat diet (HFD) regulates a set of miRNAs in the liver of rats. A, Heat maps show significantly up- and down-regulated miRNAs in the livers of rats on HFD. B, Volcano Plot for differential miRNA expression in the liver of rats. The red dots represent the significantly up-regulated miRNAs. C, A Venn diagram comparing the overlap in up-regulated miRNAs and conserved miRNAs that putatively regulate sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b. D, qPCR analysis confirmed that the miRNA-30b levels were up-regulated in the livers of rats on HFD. $^{**}P < 0.01$ relative to control (normal diet)

role of miR-30b in ER stress and insulin resistance. Quantitative real-time PCR (qRT-PCR) assay confirmed that miR-30b expression was notably increased in the liver of rats fed HFD (Figure 2D). Likewise, miR-30b levels were significantly up-regulated in the liver of rats fed MCD (Figure S2F). Thus, miR-30b represents a promising candidate to regulate SERCA2b.

The increase in miR-30b expression and the decrease in SERCA2b expression led us to hypothesize that SERCA2b is a direct target of miR-30b. We construct a luciferase reporter vector containing the SERCA2b-3′-UTR (Figure 3A). Cotransfection of miR-30b mimic led to a 60% reduction of the luciferase activity from the SERCA2b-3′-UTR in HepG2 cells (Figure 3B). In contrast, the luciferase activity was elevated after miR-30b antagomiR transfection (Figure 4B). Additionally, we examined whether miR-30b regulated SERCA2b endogenously. Transfection of miR-30b mimic markedly suppressed the protein levels of SERCA2b in HepG2 cells (Figure 3C,D). These results indicate that miR-30b targets SERCA2b for translational inhibition.

3.3 | miR-30b regulate ER stress and insulin resistance

To test the effect of miR-30b on ER stress and insulin resistance in vivo, we injected normal or HFD–fed rats once weekly for four consecutive weeks with lentivirus particles containing PGLV3-miR-30b, PGLV3-miR-30b antimiR or PGLV3-NC through tail vein. After the last injection, we harvested the liver tissues. We first measured the mRNA levels of SERCA2b using qPCR and found that miR-30b overexpression and miR-30b antimiR injection did not significantly alter the amount of the SERCA2b transcript in the livers of normal and HFD–fed rats respectively (Figures 4C & 5C). Overexpression of miR-30b resulted in a significant decrease in the protein levels of SERCA2b in the liver of rats fed normal diet as shown by Western blotting and immunohistochemistry analysis (Figure 4A, 4B; Figure S3). In contrast, miR-30b antimiR injection dramatically up-regulated the protein levels of SERCA2b in the liver of HFD–fed rats (Figure 5A,B). The protein levels of ER stress markers, such as CHOP, ATF4, GRP-78/BIP, ATF-6, p-PERK, p-IRE-1 and p-eIF2α were markedly increased in the liver of normal mice with miR-30b overexpression (Figure 4D; Figure S1B) and were reduced in the liver of HFD–fed rats injected with miR-30b antimiR (Figure 5D; Figure S1D). qRT-PCR analysis showed that overexpression of miR-30b up-regulated the spliced XBP1 mRNA levels in the liver of rats (Figure 4E). Injection of miR-30b antimiR significantly down-regulated the mRNA levels of XBP1 splicing in the liver of HFD-fed rats (Figure 5E).

To examine the effect of miR-30b on insulin sensitivity in rats, we calculated HOMA-IR (Homeostatic model assessment for insulin resistance). We found that overexpression of miR-30b induced an increase in HOMA-IR in control rats (Figure S4A), whereas injection of miR-30b antimiR reduced HOMA-IR in rats fed HFD (Figure S4B). Furthermore, glucose tolerance testing showed that overexpression of miR-30b induced glucose intolerance in control rats after an intraperitoneal glucose injection (Figure 4F). In contrast, we found that glucose intolerance was improved in rats fed HFD that were injected with miR-30b antimiR (Figure 5F). Meanwhile, insulin–tolerance testing revealed that miR-30b overexpression induced an increase in insulin sensitivity in control rats (Figure S5A), whereas injection of miR-30b antimiR reduced insulin sensitivity in rats fed HFD (Figure S5B). Finally, we found that the phosphorylation levels of IRS-1 and AKT, the major intermediates of insulin signaling pathway, were

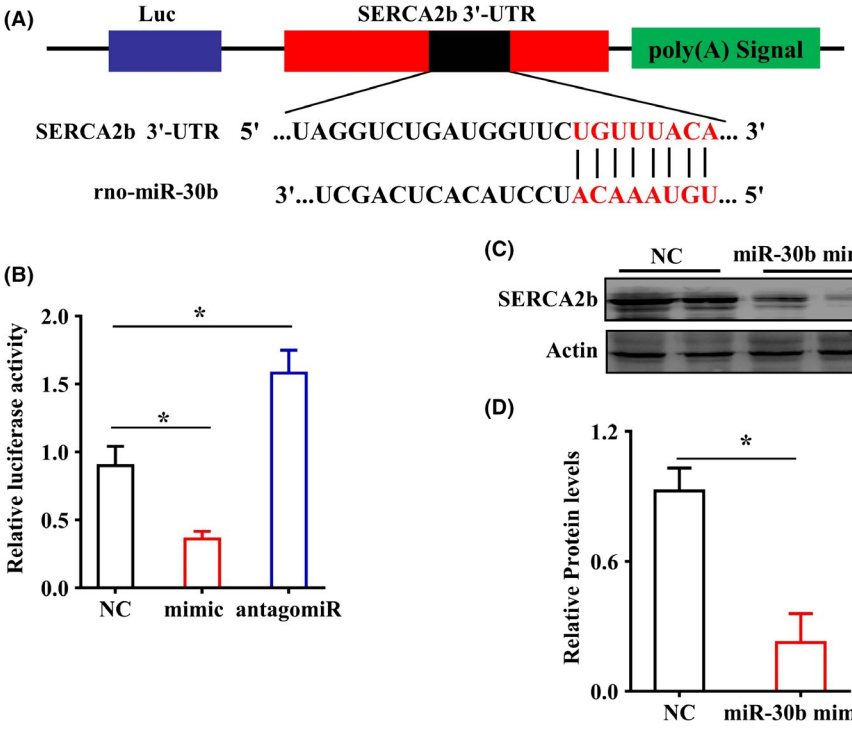

FIGURE 3 Sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) is a target gene of miR-30b. A, Complementarity between the 3′-UTR of SERCA2b gene and miR-30b. Seeds are marked in red, Watson-Crick base pairing with a straight line. B, Luciferase analysis of a reporter vector harbouring the 3′-UTR of SERCA2b in HepG2 cells transfected with negative control (NC) or miR-30b mimics or miR-30b antagomiR for 36 h. *P < 0.05 relative to NC. (C,D) Western blot analysis for SERCA2b protein levels in HepG2 cells transfected with NC or miR-30b for 36 h (n = 4). Representative Western blots are shown (C). Quantification of the ratio of proteins to β-actin (D). *P < 0.05 relative to NC

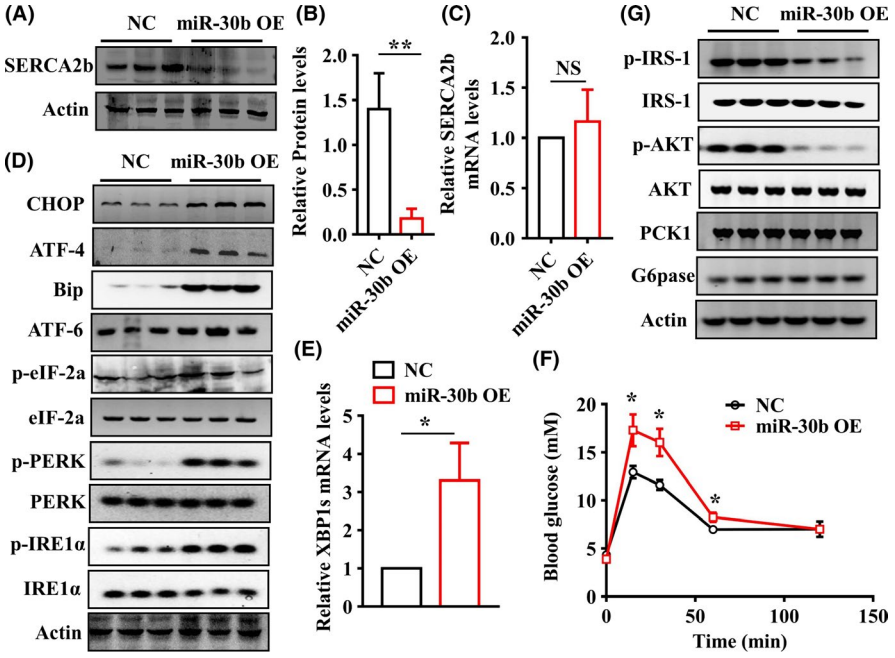

FIGURE 4 Overexpression of miR-30b promotes endoplasmic reticulum (ER) stress and insulin resistance. The rats fed with normal diet were infected with lentivirus particles containing PGLV3-miR-30b (miR-30b OE) or PGLV3-NC (NC). After 4 wk of injection, animals were euthanized for further analysis. (A,B) The sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) protein levels were down-regulated in the liver of rats injected with PGLV3-miR-30b (n = 6 in each group). Representative Western blots are shown (A). Quantification of the ratio of proteins to β-actin (B). **P < 0.01 relative to negative control (NC). C, Quantitative real-time PCR (qPCR) analysis revealed that the mRNA levels of SERCA2b were not altered in the liver of rats injected with PGLV3-miR-30b. D, Overexpression of miR-30b up-regulated the protein levels of ER stress makers in the liver of rats. Representative Western blots are shown. E, qPCR analysis of spliced XBP1 mRNA levels. All results are standardized to the levels of β-actin and are the mean ± SD (n = 6 in each group). *P < 0.05 relative to control (NC). F, Glucose tolerance testing in rats injected with PGLV3-NC and PGLV3-miR-30b. *P < 0.05 relative to NC. G, Western blot analysis of liver extracts from rats fed normal diet injected with PGLV3-miR-30b or PGLV3-NC

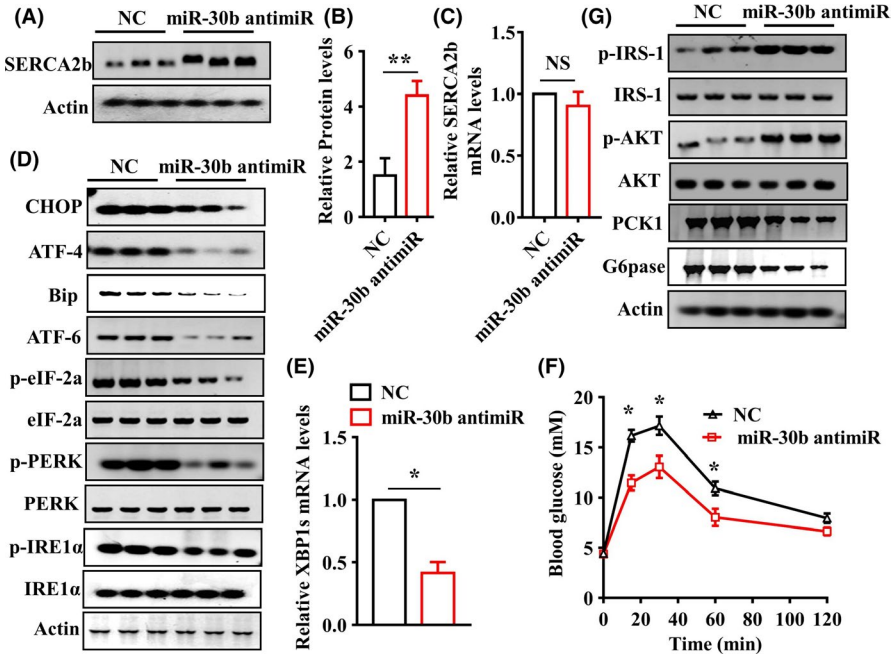

FIGURE 5 Inhibition of miR-30b improves ER stress and insulin resistance. The rats fed high fat diet were infected with lentivirus particles containing PGLV3-miR-30b antimiR (miR-30b antimiR) or PGLV3-NC (NC). After 4 wk of injection, animals were euthanized for further analysis. (A,B) The sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) protein levels were up-regulated in the liver of rats injected with PGLV3-miR-30b antimiR (n = 6 in each group). Representative Western blots are shown (A). Quantification of the ratio of proteins to β-actin (B). $^{**}P < 0.01$ relative to NC. C, Quantitative real-time PCR (qPCR) analysis revealed that the mRNA levels of SERCA2b were not alter in the liver of rats injected with PGLV3-miR-30b antimiR. D, Inhibition of miR-30b down-regulated the protein levels of ER stress makers in the liver of rats. Representative Western blots are shown. E, qPCR analysis of spliced XBP1 mRNA levels. All results are standardized to the levels of β-actin and are the mean ± SD (n = 6 in each group). $^{*}P < 0.05$ relative to control (NC). F, Glucose tolerance testing in rats injected with PGLV3-NC and PGLV3-miR-30b antimiR. $^{*}P < 0.05$ relative to NC. G, Western blot analysis of liver extracts from rats fed high fat diet injected with PGLV3-miR-30b antimiR or PGLV3-NC

markedly reduced in the liver of control rats with miR-30b overexpression (Figure 4G; Figure S1C) and were increased in the liver of rats fed with HFD with injection of miR-30b antimiR (Figure 5G; Figure S1E). Together, these data demonstrate that miR-30b is involved in the development of insulin resistance in obese rats.

3.4 | miR-30b partially influences the progress of gluconeogenesis, lipogenesis and glycolysis

Next, we tested the effect of miR-30b on the expression of metabolic enzymes involved in gluconeogenesis, lipogenesis and glycolysis. Firstly, we found that miR-30b overexpression failed to influence the protein levels of the gluconeogenic enzymes, G6Pase and PEPCK, in the liver of control rats (Figure 4G; Figure S1C). In contrast, injection of miR-30b antimiR substantially reduced the protein levels of G6Pase and PEPCK in the liver of rats fed HFD (Figure 5G; Figure S1E). Secondly, miR-30b overexpression reduced the expression of the glycolytic enzyme HK-1 in the liver of control rats (Figure S6A), whereas injection of miR-30b antimiR dramatically increased the expression of HK-1 in the liver of rats fed HFD (Figure S6B).

After 16 weeks of HFD feeding, a significant increase in hepatic lipid accumulation and serum triglyceride (TG) levels was observed (Figure 6A,B). qRT-PCR analysis revealed that the expression of numerous genes-related to hepatic lipogenesis, such as sterol regulatory element binding protein 1c (SREBP1c), carbohydrate response–element binding protein (chREBP) and steroyl-CoA desaturase (SCD-1), was significantly up-regulated in the liver of rats fed HFD (Figure 6C). miR-30b overexpression up-regulated the mRNA levels of SCD-1 in the liver of control rats, while miR-30b antimiR injection down-regulated the mRNA levels of SCD-1 in the liver of control rats fed HFD (Figure 6C). By contrast, both miR-30b overexpression and miR-30b antimiR injection did not significantly alter the mRNA levels of SREBP-1c and chREBP in the livers of control and HFD–fed rats respectively (Figure 6C). It should be noted that miR-30b overexpression did not significantly affect hepatic lipid accumulation (judged by Oil Red-O staining) and TG content in the serum of control rats (Figure 6A,B). In contrast, miR-30b antimiR injection reduced lipid accumulation and TG content in the serum of rats fed HFD (Figure 6A,B).

3.5 | The circulating miR-30b is correlated with hepatic steatosis

MicroRNAs are usually secreted into the bloodstream by packing into vesicles (eg. microvesicles and exosome).[26] In the current study, we found that the miR-30b levels were also significantly elevated in the serum of rats fed HFD (Figure S7). To determine the clinical relevance of the circulating miR-30b in hepatic steatosis in human

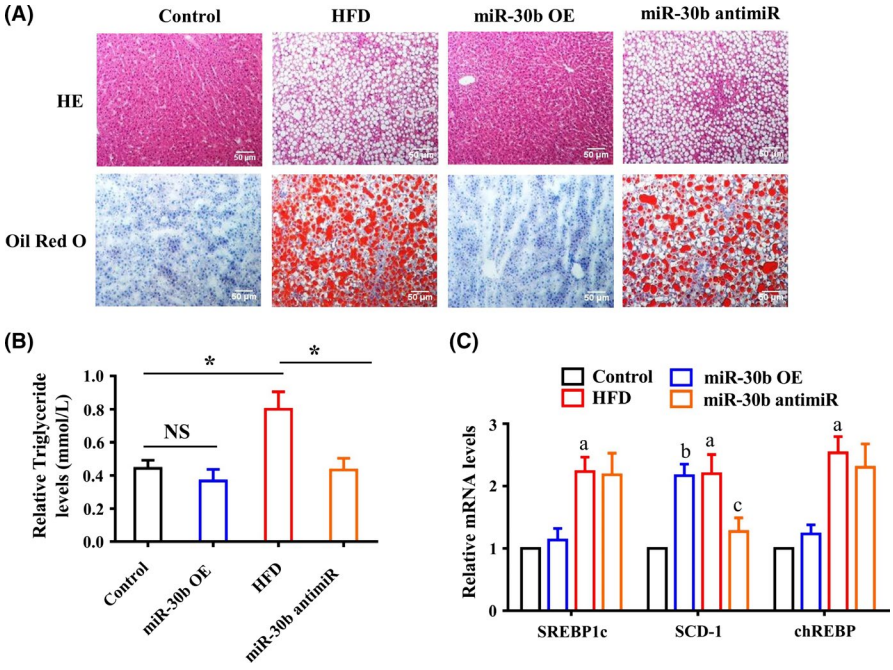

FIGURE 6 Steatosis in liver of rats. A, Hepatic steatosis assessed by hematoxylin and eosin and Oil Red-O staining. B, Measurement of triglyceride (TG) in the serum of rats. $^{*}P < 0.05$. C, The mRNA levels of hepatic lipogenesis genes. $^{a}P < 0.05$ relative to Control, $^{b}P < 0.05$ relative to Control, $^{c}P < 0.05$ relative to high fat diet (HFD)

subjects, we performed a non-invasive diagnosis for the assessment of hepatic steatosis by measuring fat attenuation index (FAI) using transient elastography (FibroTouch).[27] We recruited 165 Chinese individuals with an average age of 54.94 at the First Affiliated Hospital, Kunming Medical University (Table 1). All individuals volunteered to accept FibroTouch examination. We measured the circulating miR-30b levels in these individuals. Partial correlation analysis showed a positive correlation between the plasma miR-30b levels and the FAI or the levels of insulin or glucose or HOMA-IR (Figure 7A-D). These results indicate that higher grades of steatosis and HOMA-IR are associated with highly elevated miR-30b levels.

TABLE 1 Study polulation characteristics

	No.	Mean	SD or 95% CIs
Male/female	165	56/109	
Age (y)	165	54.94	12.56
BMI (kg/m^2)	165	23.96	2.971
Height	165	162.3	7.571
Waist circ (cm)	165	87.25	8.782
Glucose (mmol/L)	165	5.218	1.582
Total cholesterol (mmol/L)	165	4.951	1.058
HDL cholesterol (mmol/L)	165	1.413	0.9659
LDL cholesterol (mmol/L)	165	3.357	0.9859
Triglycerides (mmol/L)	165	1.783	1.282
ALT (IU/L)	165	25.98	23.35
AST (IU/L)	165	31.8	15.89
GGT	165	40.26	79.25

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase; HDL, high density lipoprotein; LDL, low density lipoprotein.

4 | DISCUSSION

Our present study identifies a potentially important role for miR-30b in the development of insulin sensitivity in NAFLD. miR-30b negatively regulates the protein expression of SERCA2b through direct base pairing to the 3′-UTR of its mRNA. Down-regulation of SERCA2b results in the activation of ER stress, which in turn impairs insulin sensitivity (Figure 7E). Finally, our study shows that the circulating miR-30b is positively correlated with hepatic steatosis and HOMA-IR in human subjects. Thus, miR-30b may represent not only a potential target for the treatment of insulin resistance, but also a non-invasive disease biomarker for NAFLD.

It has been well-established that increased ER stress in obesity is involved in glucose intolerance, insulin resistance and ultimately type 2 diabetes.[16-19,23] A reduction in ER Ca^{2+} levels caused by reduced expression or activity of SERCA2b, a key player in the maintenance of ER homeostasis, is a potential mechanism underlying obesity–induced activation of ER stress.[20,21] Indeed, overexpression of SERCA2b or activation of SERCA2b by its agonist greatly improves obesity–induced ER stress and insulin resistance.[20,21] It has been shown that tribbles–related protein 3 (TRB3) contributes to insulin resistance by blocking activation of Akt in liver.[28] Previously, we and others had shown that TRB3 expression is induced by ATF4 and CHOP during the activation of ER stress.[29,30] As down-regulation of SERCA2b expression leads to the activation of ER stress[20] and this study, TRB3 is likely involved in insulin resistance in obesity. Clearly, the role of TRB3 in SERCA2b dysregulation–mediated insulin resistance needs to be investigated further in light of our current study.

However, little information is available regarding the dysregulation of SERCA2b expression in obesity. Using miRNA microarray

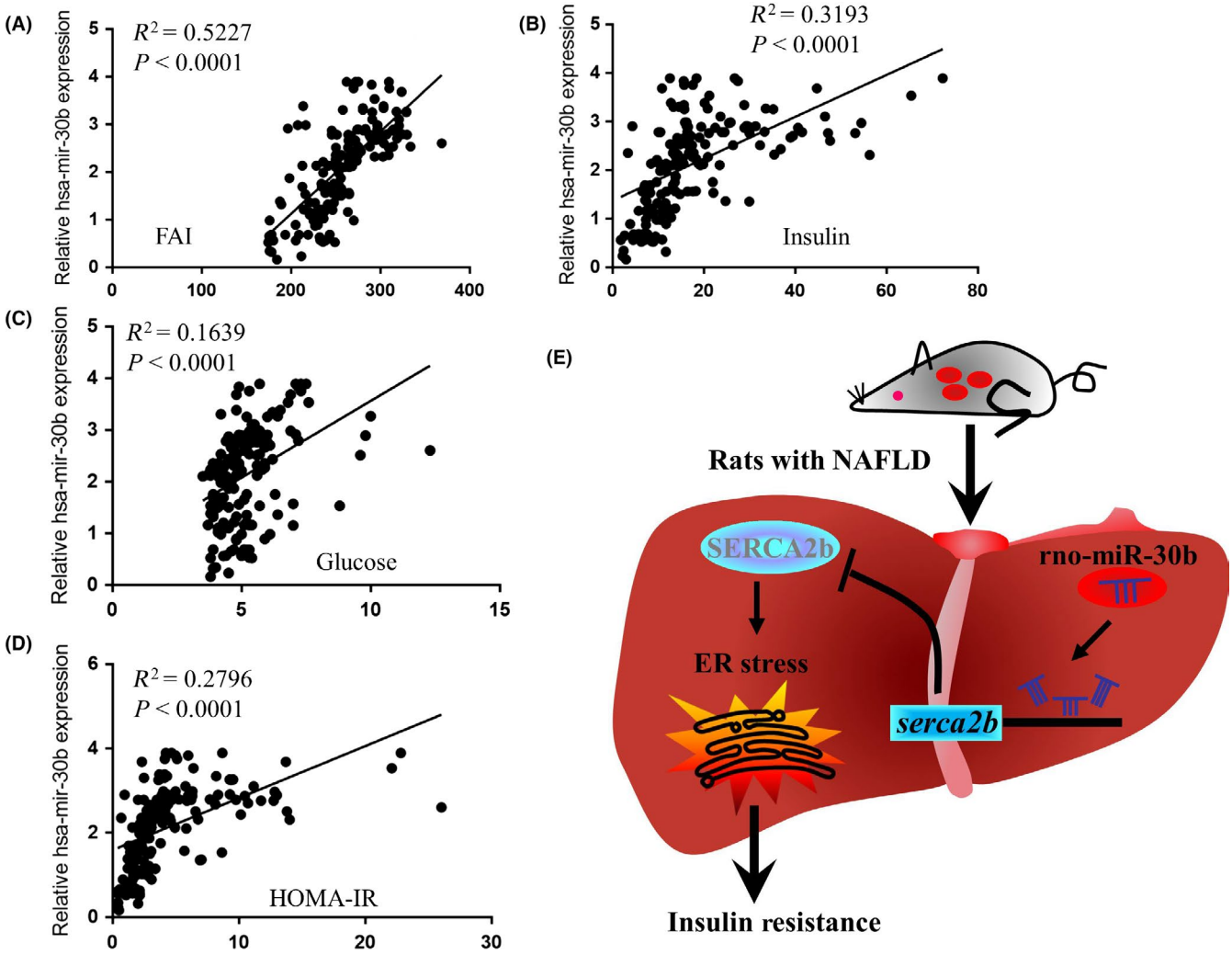

FIGURE 7 Correlation between plasma miR-30b and hepatic steatosis, glucose levels, insulin levels and homeostatic model assessment for insulin resistance (HOMA-IR) (A-D) Correlation analysis between miR-30b levels and FAI (A) or the levels of insulin (B) or glucose (C) or HOMA-IR (D) in human subjects. (E) Proposed mechanism by which miR-30b links insulin resistance and hepatic lipid accumulation

analysis, we show that miR-30b is markedly up-regulated in the liver of rats fed HFD. miR-30b is identified as a potential negative regulator of SERCA2b by two miRNA target prediction algorithms. Our in vitro and in vivo experiments demonstrate that miR-30b suppresses the protein levels of SERCA2b. All these features of miR-30b lead us to focus on its role in ER stress and insulin resistance. Indeed, overexpression of miR-30b significantly upregulates the expression of ER stress markers. In addition, overexpression of miR-30b induces glucose intolerance in rats fed normal diet, whereas antimiR knockdown of miR-30b improves glucose intolerance in HFD–treated rats. Thus, our findings reveal that miR-30b is a negative regulator of insulin sensitivity through the activation of ER stress. Our bioinformatics analysis predicts that let-7 family miRNAs are also potential negative regulators of SERCA2b. The expression of let-7 family miRNAs is markedly up-regulated in the liver from murine models of rats fed HFD.[9] Although let-7 may induce insulin resistance by inhibiting the components of the insulin-PI3K-mTOR pathway,[24,25] it is likely that the miRNA family exerts its effects through induction of ER stress.

In the current study, our data indicate that the expression of SREBP1c, chREBP and SCD-1 is significantly up-regulated in the liver of rats fed HFD. However, only the expression of SCD-1 is significantly increased in the liver of normal rats with miR-30b overexpression and is dramatically reduced in liver of HFD–fed rats with injection of miR-30b antimiR. In contrast, neither miR-30b overexpression nor miR-30b antimiR alters the expression of SREBP1c and chREBP in the livers of normal and HFD-fed rats respectively. Park et al have shown that SERCA2b overexpression reduces lipid accumulation and TG levels and the expression of lipogenesis genes, such as SCD1, DGAT2, FASN and ACC2, in the liver of ob/ob mice.[20] A previous study has revealed that XBP1 directly regulates the expression of lipogenic genes (SCD1, DGAT2, and ACC2) in the liver.[31] However, neither XBP1 nor SERCA2b is involved in regulation of expression of SREBP1c and chREBP.[20,31] These results suggest that SERCA2b regulates lipid accumulation, at least in part, by inhibiting lipogenic genes mediated by XBP1.

The circulating miRNAs have been detected within vesicles (eg microvesicles and exosome) and argonaute proteins (eg. AGO2) and other RNA–binding proteins.[26] When searching the tissue distribution of miR-30b in database,[32] we found that miR-30b is widely expressed in murine and human tissues, including liver. In this study, our data show that miR-30b levels in both the liver and serum are significantly increased in obese rats. Consistent with our observation, some studies have reported that the miR-30b levels are increased in the livers of HFD–induced mice and rats.[9,33] Conversely, a reduction in miR-30b levels is observed in the liver of HFD–induced mice and the serum of HFD–induced rats.[34,35] Some factors, such as different diet compositions and feeding periods, may account for the observed discordances. It is likely that the expression of miR-30b is up-regulated in liver and other donor tissues in obese rats and then secreted into the bloodstream. These observations prompt us to investigate whether circulating miR-30b levels is correlated with hepatic steatosis and HOMA-IR in human subjects. For detection of hepatic steatosis, liver biopsy is the common approach for diagnosing NAFLD and hepatic fibrosis. However, it has several shortcomings, such as lack of accuracy, cost and risk of complications.[36] More importantly, it is poorly accepted by some NAFLD patients. During the last decade, non-invasive approaches have emerged to replace biopsy as various biomarkers and imaging methods, such as transient elastography. Transient Elastography FibroScan® (Echosens, Paris, France) is a non-invasive, and rapid bedside method to assess hepatic fibrosis and steatosis by measuring the liver stiffness and the controlled attenuation parameter (CAP) respectively.[27,37] FibroTouch is a new generation of transient elastography for the detection and quantification of hepatic steatosis using FAI. It has been used for clinical diagnosis in China since 2013.[27] We found that circulating miR-30b levels are positively correlated with FAIs and HOMA-IR in human subjects. Thus, detection of circulating miR-30b may also be useful for monitoring the therapeutic efficacy of hepatocyte steatosis.

In summary, our findings reveal that increased hepatic expression of miR-30b impairs insulin sensitivity in obesity. This miRNA activates ER stress by targeting SERCA2b, thereby inducing insulin resistance. In addition, the association of the circulating miR-30b levels with hepatic steatosis implicates miR-30b as a non-invasive biomarker to detect the extent of hepatic steatosis.

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CONFLICT OF INTEREST

The authors do not have any disclosures to report.

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