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Research paper

Down-regulation of MicroRNA-592 in obesity contributes to hyperglycemia and insulin resistance

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ABSTRACT

Background: Many studies have demonstrated that microRNAs, a class of small and non-coding RNA molecules, play an important role in the regulation of glucose and lipid homeostasis. In the present study, we sought to investigate the function of miR-592 in the development of obesity-associated metabolic disorders, including hyperglycemia andinsulin resistance.

Methods: The expression levels of miR-592 were measured in the liver of obese mice and humans by quantitative reverse transcription PCR. Loss- and gain-of function experiments were employed to explore the metabolic function of miR-592 using locked nucleic acids and adenovirus in lean and obese mice, respectively. The molecular target of miR-592 was determined by western blotting and luciferase reporter assays.

Findings: We found a significant decreased expression of miR-592 in the liver of obese mice and humans. Inhibition of miR-592 led to elevated blood glucose levels, enhanced gluconeogenesis and reduced insulin sensitivity in lean mice. In contrast, adenovirus-mediated overexpression of hepatic miR-592 improved metabolic disorders in obese mice. Mechanistically, we found that the transcription factor forkhead box O1 (FOXO1) is a direct target gene of miR-592 to mediate its metabolic functions. miR-592 was able to inhibit the mRNA and protein expression of FOXO1 by binding to its 3′-untranslated region.

Interpretations: Our findings demonstrate that obesity-associated down-regulation of miR-592 plays an important role in the progression of metabolic diseases. Restoration of hepatic miR-592 could improve glucose and lipid metabolism in obese mice.

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1. Introduction

Glucose and lipid homeostasis, which is controlled by many organs, is crucial for maintaining mammalian health. Of note, the liver that senses and integrates nutrients, hormones and other stimuli, plays an essential role in regulating whole-body metabolism [1]. In the fasted state, glucagon from pancreatic α cells and glucocorticoids from adrenal glands can activate hepatic gluconeogenesis and fatty acid oxidation to provide energy supply [2]. Upon feeding, insulin will be released from pancreatic β cells and reach hepatocytes to inhibit gluconeogenesis, promote glycogen synthesis and *de novo* lipogenesis [2]. On the other hand, due to selective insulin resistance, hyperactivation of

Abbreviations: 3′-UTR, 3′-untranslated region; FOXO1, forkhead box O1; G6Pase, glucose-6-phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFD, high fat diet; miRNA, microRNA; MPH, mouse primary hepatocyte; PEPCK, phosphoenolpyruvate carboxykinase 1; TG, triglyceride; WAT, white adipose tissue.

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Research context

Evidence before this study

Metabolic diseases, including type 2 diabetes (T2DM) and non-alcoholic fatty liver disease (NAFLD), have become a worldwide epidemic. FOXO1, a member of forkhead transcription factors, plays a critical role in the regulation of glucose and lipid metabolism. It has been established that mRNA and protein levels of FOXO1 were increased in livers of diabetic rodents and patients. However, the molecular basis for its dysregulation in the development of metabolic diseases remains poorly understood. MicroRNAs (miRNAs), a class of small and non-coding RNAs, have been implicated in the glucose and lipid homeostasis. Previous studies demonstrated that miR-592 can regulate cell proliferation, migration and tumorigenesis. However, whether miR-592 modulates glucose and lipid metabolism remains unknown since no study was found on PubMed database with the searching term 'miR-592' and 'obesity' or 'metabolic disease'.

Added value of this study

In the present study, the expression levels of miR-592 were measured in the livers of obese mice and humans. Loss- and gain-of function experiments were further employed to explore the metabolic functions of miR-592. It was found that miR-592 was significantly downregulated in the livers of obese mice and humans. Inhibition of miR-592 led to elevated blood glucose levels, enhanced gluconeogenesis and reduced insulin sensitivity in lean mice. In contrast, adenovirus-mediated overexpression of hepatic miR-592 improved metabolic disorders in obese mice. Mechanistically, the transcription factor FOXO1 was identified as a direct target gene of miR-592 to mediate its metabolic functions.

Implication of all the available evidence

Our findings of miR-592 downregulation in obese livers and its functions in glucose and lipid metabolism suggest that miR-592 might be implicated in the pathologenesis of metabolic diseases, indicating the clinical potential of miR-592 as a potential target for treating obesity-induced metabolic disorders.

gluconeogenesis and lipogenesis in obesity contributes to hyperglycemia, liver steatosis and hyperlipidemia, which are critical components of type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD) [3]. Therefore, understanding the regulatory network in the development of abnormal hepatic glucose and lipid metabolism would help to identify new therapeutic targets for metabolic diseases.

FOXO1, a member of forkhead transcription factors, plays a critical role in cell proliferation, apoptosis, differentiation and metabolism [4,5]. It has been shown that FOXO1 could bind to the promoter regions of hepatic gluconeogenic genes (PEPCK and G6Pase) to stimulate gluconeogenesis through acting in concert with PGC-1α and cAMP/CREB pathway [6,7]. As a result, mice overexpressing a constitutively active FOXO1 in the liver exhibited hyperglycemia, hyperinsulinemia, hypertriglyceridemia and hepatosteatosis [8]. On the other hand, liver-specific FOXO1 knockout mice showed a decrease of blood glucose concentrations, reduced hepatic glucose production and triglyceride contents [9]. In agreement, suppression of FOXO1 by antisense oligonucleotides improved insulin resistance and liver steatosis in high-fat-diet-induced obese mice [10]. In addition, FOXO1 was shown to mediate the roles of other transcription factor, transcription cofactor or cytokines in the regulation of hepatic glucose and lipid homeostasis [11–14]. More importantly, mRNA and protein levels of FOXO1 were increased in livers of diabetic rodents and patients with steatohepatitis [10,15,16], suggesting that its abnormal expression is involved in the pathogenesis of hyperglycemia, insulin resistance and NAFLD. However, the molecular basis for its dysregulation in the development of metabolic disorders remains poorly understood.

A growing body of evidence has shown that MicroRNAs (miRNAs), a class of small and non-coding RNAs, play a substantial role in the regulation of cell metabolism, which adds an additional level of complexity to the maintenance of glucose and lipid homeostasis [17]. For examples, several miRNAs have been implicated in the insulin resistance, glucose tolerance, inflammatory process, and/or hepatosteatosis, such as miR-122, miR-802, miR-26 and miR-676 [18–21]. Therefore, further evaluating the roles of miRNAs might provide new therapeutic targets for treating metabolic diseases. Besides, it has been shown that expression of many hepatic metabolic genes, such as PGC-1α, PPARα and p110α, are regulated by miRNAs, leading to altered glucose and lipid metabolism [22–24]. Therefore, we explored whether miRNAs regulates expression levels of FOXO1 in obese livers and by which contributes to metabolic disorders. As a result, we show that miR-592 prevents obesity-induced hyperglycemia, insulin resistance and hepatosteatosis, at least in part, through targeting FOXO1 expression.

2. Materials and methods

2.1. Animal experiments and human samples

All of the animal experiments were conducted in accordance with the guideline of the Animal Care Committee of Shanghai Jiao Tong University School of Medicine. Male C57BL/6 mice and *db*/*db* mice were purchased from the Shanghai Laboratory Animal Company (Shanghai, China) and Nanjing Biomedical Research Institute (Nanjing, China), respectively. Mice were housed with a humidity of 55 ± 10% and a 12-h light/12-h dark cycle at 22 ± 1 °C with free access to food and water. Before the experiment started, C57BL/6 mice were allowed to accommodate to the new environment for 2 weeks and then fed with a high-fat-diet or normal chow diet for 12 weeks. The high-fat diet (HFD) (D12492, Research Diets, New Brunswick, New Jersey, USA) contained 60% kcal from fat, 20% kcal from carbohydrate, and 20% kcal from protein. The normal chow diet (NCD) contained 10% kcal from fat, 70% kcal from carbohydrate, and 20% kcal from protein. For analysis of hepatic miRNA and FOXO1 expression, liver biopsy was performed in those subjects who donated their partial livers for liver transplantation, as described previously [25,26]. The human study was approved by the Human Research Ethics Committee of Zhongshan Hospital, Fudan University. Written informed consent was obtained by each human participant.

2.2. LNA and adenovirus

Locked nucleic acids (LNA) targeting miR-592 were designed and synthesized as unconjugated and fully phosphorothiolated oligonucleotides by Qiagen (Shanghai, China). LNA were intravenously delivered to C57BL/6 mice at a concentration of 20 mg/kg. Mice were injected on two consecutive days and euthanized 15 days after LNA administration. To overexpress miR-592 and knockdown FOXO1, DNA fragments encoding miR-592 and shRNA directed against the FOXO1 gene, respectively, were constructed into pENTR/U6 vector under the control of the human U6 promoter. Then, recombinant adenoviruses were generated using the BLOCKiT Adenoviral RNAi Expression System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Sequence of the shRNA against FOXO1 is 5-GGATAAGGGCGACAGC AAC-3′. Viruses were diluted in 0.25 ml PBS and administered to mice *via* tail vein injection using $1*10^9$ plaque-forming units. Mice were euthanized 15 days after the adenovirus injection.

2.3. Primary hepatocyte isolation

Mouse primary hepatocytes (MPH) were isolated from male C57BL/6 mice aged 10 weeks. Shortly, the liver was perfused with 50 ml Hanks buffer containing 1 mM EDTA and digested with 20 ml 0.05% collagenase type IV solution at 37 °C for 15 min and then purified by centrifugation. Fresh prepared hepatocytes were seeded in 6-well plates in attachment media (Science Cell, USA).

2.4. Triglyceride, glucose and insulin analysis

Liver triglyceride contents were measured using commercial kits from Biovision company (Milpitas, California, USA). Glucose tolerance tests were performed by intraperitoneal injection of D-glucose (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 2.0 mg/g body weight after a 16-h fast. Pyruvate tolerance tests were performed by intraperitoneal injection of sodium pyruvate (Sigma-Aldrich) at a dose of 1.5 mg/g body weight after a 16-h fast. For insulin tolerance tests, mice were injected with regular human insulin (Eli Lily, Indianapolis, Indiana, USA) at a dose of 1.0 U/kg body weight after a 6-h fast. Blood glucose levels were determined using a portable blood glucose meter (Lifescan, Johnson & Johnson, New Jersey, USA). Plasma insulin levels were measured using commercial ELISA kits from Millipore company (Bedford, MA, USA).

2.5. Cell culture, transfection and luciferase assays

All cell lines were purchased from the Cell Bank of Type Culture Collection, Chinese Academy of Sciences (CAS, Shanghai, China) and cultured in DMEM (Gibco, Shanghai) containing 10% FBS (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). microRNA mimics, antisense and lock nucleic acids (LNA) were purchased from Qiagen company (Shanghai). The 3′-untranslated regions of human FOXO1 gene were amplified and cloned into a CMV-driven Firefly luciferase cassette. For luciferase assays, HepG2 cells were transfected in 24-well plates in duplicate wells together with firefly luciferase using Lipofectamine 3000 (Invitrogen, Shanghai). Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA).

2.6. Glucose production assays

Mouse primary hepatocytes were isolated and transfected with miR-592 adenovirus or antisense for 24 h. Then, cells were washed three times with PBS and treated with forskolin (20 μM)/dexamethasone (100 nM) for another 16 h in glucose-free DMEM containing sodium pyruvate (2 mM). Glucose concentrations in the medium were measured using glucose assay kits from Sigma-Aldrich company.

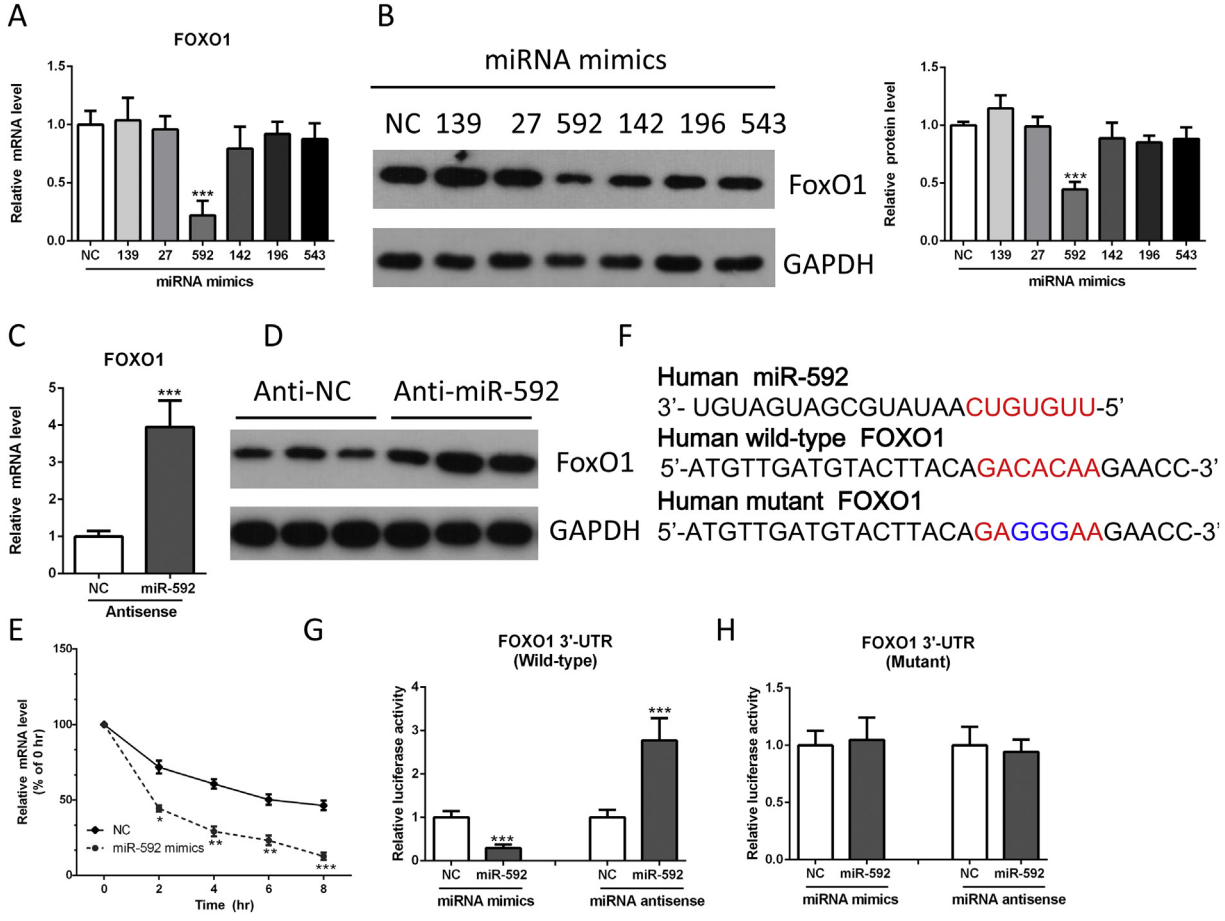

Fig. 1. Effects of miR-592 on FOXO1 expression in HepG2 cells. (A) Relative mRNA levels of FOXO1 in HepG2 cells transfected with various miRNA mimics or negative control (NC) for 36 h. $n = 4$. (B) Protein levels of FOXO1 in HepG2 cells were determined by wester blots in HepG2 cells harvested 48 h after transfection. The quantification plot was analyzed by scanning densitometry using the Image J software (v 1.8.0). (C) Relative mRNA levels of FOXO1 in HepG2 cells transfected with miR-592 antisense or negative control (NC) for 36 h. n = 4. (D) Protein levels of FOXO1 in HepG2 cells transfected with miR-592 antisense or NC for 48 h. (E) Time course of FOXO1 mRNA degradation in HepG2 cells transfected with the miR-592 mimics or NC and incubated with actinomycin D. (F) Sequence alignment of the 3'-UTR of the FOXO1 and miR-592 reveal an miR-592 response element. Seed sequences are highlighted in red and the mutant sequences were highlighted in blue. (G-H) The activity of the reporter containing the wild-type (G) or mutant (H) 3'-UTR of FOXO1 was determined in HepG2 cells in the presence of miR-592 mimics or antisense. *$P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

2.7. RNA isolation and real-time PCR

Total RNA of tissues or cell lysates were extracted using TRIzol reagent (Invitrogen, Shanghai). After reverse transcription by using PrimeScript RT reagent Kit (TaKaRa, Dalian, China), quantitative real-time PCR was performed using a SYBR Green Premix Ex Taq (TaKaRa) on Light Cycler480 (Roche, Switzerland). U6 was used for normalization of microRNAs expression and β-actin was used for normalization of mRNAs expression. PCR condition is composed of an initial holding period at 95 °C for 5 min, followed by a two-step PCR program consisting of 95 °C for 5 s and 60 °C for 45 cycles. Relative quantitation analysis of gene expression results was performed according to the $2^{-\Delta\Delta Ct}$ method. The primer sequence for determining expression of mir-592 is: 5-ACAT CATCGCATATTGACACAA-3′ (Human) and 5-ACATCATCGCATATTGACA CAAT-3′ (Mouse).

2.8. Western blotting

Tissues or cells were lysed in radioimmunoprecipitation (RIPA) supplemented with protease and phosphatase inhibitors. 25 μg lysates were loaded on 10% SDS-PAGE and transferred onto polyvinylidenedifluoride (PVDF) membranes (Millipore) and probed with the indicated antibodies. The membranes were visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to the manufacturer's instructions. Antibodies for FOXO1 (#2880, 1:1000, Cell Signaling Technology) and GAPDH (#KC5G5, 1:8000, KangChen Bio-tech, Shanghai) were used.

2.9. Statistical analysis

All data were expressed as mean ± standard error of mean (SEM) of at least three independent experiments. Statistical differences were determined by 2-way ANOVA with Bonferroni-adjusted post-test or by Student's t-test. Statistical significance is displayed as $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

3. Results

3.1. Repression of FOXO1 expression by miR-592

Using the TargetScan software, which predicts the relationship of target messenger RNAs and miRNAs based on seed recognition [27], we found that many miRNAs could interact with the 3′-untranslated region of FOXO1 (Data not shown). Then, two criteria were established to narrow down the candidate miRNAs. First, we would like to identify novel miRNAs that could regulate FOXO1 expression. Therefore, several miRNAs that have been shown to downregulate FOXO1 expression in human cancer cells, such as miR-9, miR-96, miR-183 and miR-215 [28–30], were excluded. Second, those miRNAs that have been studied in other biological process were selected. Here, 6 candidate miRNAs were selected for further experiments in the current study. To test it, specific miRNA mimics were transfected into human hepatoma HepG2 cells. Whereas expression of miR-139, miR-27, miR-142, miR-196 or miR-543 had little or minimal effect, expression of miR-592 dramatically decreased mRNA levels of FOXO1 as measured by quantitative PCR (qPCR) (Fig. 1A). Our qPCR analysis showed that miR-592

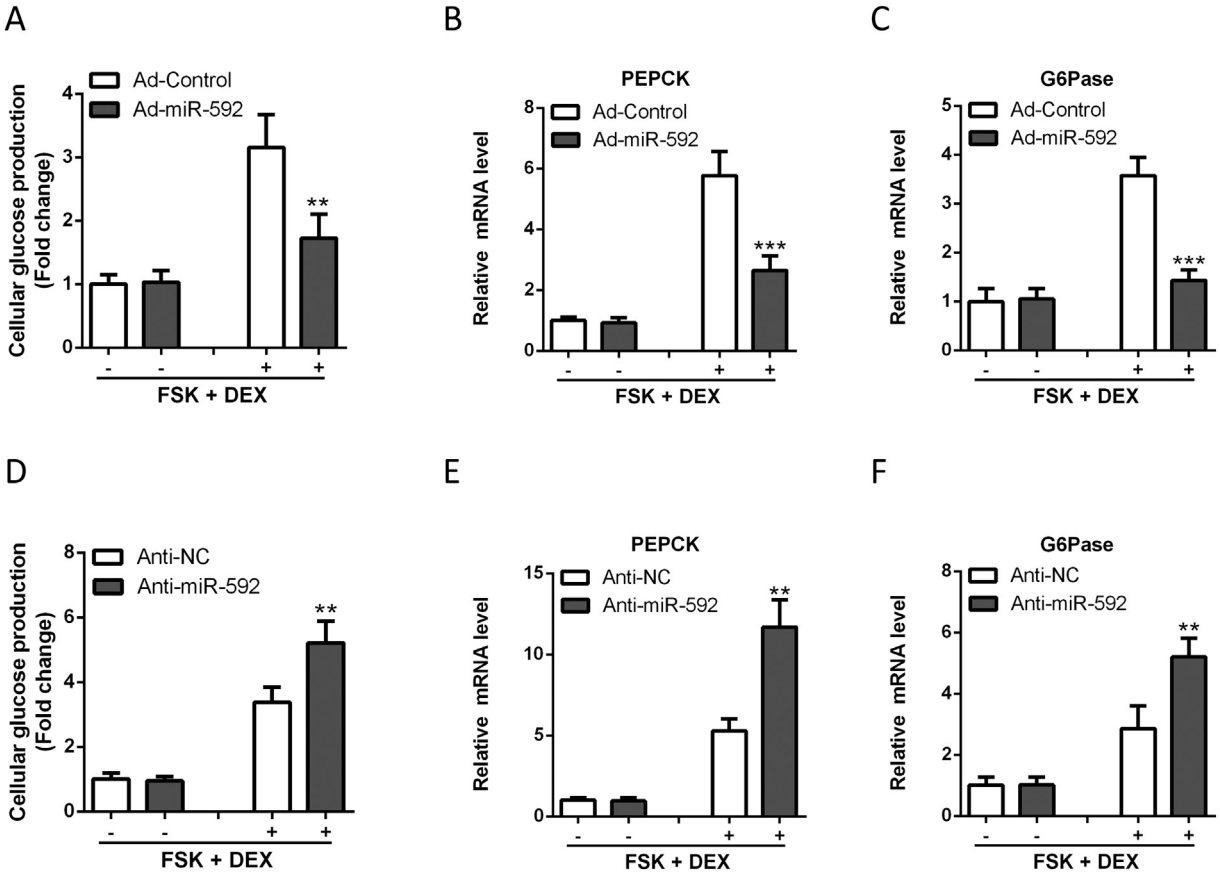

Fig. 2. miR-592 inhibits glucose production in mouse primary hepatocytes. (A) Cellular glucose production was assayed in mouse primary hepatocytes. Cells were transfected with adenovirus containing miR-592 or control for 24 h and then treated with FSK/DEX (forskolin/dexamethasone) or vehicle for another 16 h. n = 4. (B–C) Relative mRNA levels of PEPCK (B) and G6Pase (C) in mouse primary hepatocytes as indicated in (A) n = 4. (D) Cellular glucose production was assayed in mouse primary hepatocytes. Cells were transfected with miR-592 antisense control for 24 h and then treated with FSK/DEX (forskolin/dexamethasone) or vehicle for another 16 h. n = 4 (*E*-F) Relative mRNA levels of PEPCK (E) and G6Pase (F) in mouse primary hepatocytes as indicated in (D). n = 4. $^{**}P < 0.01$ or $^{***}P < 0.001$.

expression was higher in liver and heart, and was also present at a lower level in white adipose tissue (WAT), skeletal muscle (SKM), kidney and spleen (Supplementary Fig. 1A). Besides, we isolated primary non-hepatocytes and primary hepatocytes from C57BL/6 mice. As a result, compared with that in non-hepatocytes, miR-592 expression was 12-fold higher in hepatocytes (Supplementary Fig. 1B), indicating that hepatic parenchymal cells represent the main source of miR-592 expression in the liver.

The downregulation of FOXO1 by miR-592 mimics was further confirmed by western blots (Fig. 1B). Moreover, inhibition of endogenous miR-592 by its antisense oligonucleotides increased mRNA and protein amounts of FOXO1 (Fig. 1C and D). Similar results were also observed in other types of human or mouse hepatoma cells (HuH7, Hep3B, Hep1–6) and mouse primary hepatocytes (MPH) (Supplementary Fig. 1C-D). These results identify FOXO1 as a target gene of miR-592 in hepatocytes.

3.2. miR-592 interacts with 3′-UTR of FOXO1 to inhibit its expression

To understand how miR-592 regulates FOXO1 mRNA levels, HepG2 cells were transfected with miR-592 mimics or negative control and then treated with actinomycin D, a widely used transcription inhibitor. As a result, FOXO1 mRNA decreased more rapidly in cells overexpressing miR-592 than controls, suggesting that this miRNA may accelerate mRNA degradation of FOXO1 (Fig. 1E). To further explain the molecular basis of this regulation, human FOXO1 3′-untranslated region (3-UTR) containing the potential miR-592 response site was cloned into a luciferase reporter (Fig. 1F). Subsequent analysis revealed that miR-592 mimics reduced, while anti-miR-592 increased the luciferase activity of the reporter in HepG2 cells (Fig. 1G). In contrast, mutation of the miR-592 target sites abrogated miR-592-associated regulation in luciferase activity (Fig. 1F and H), suggesting a direct interaction of miR-592 with FOXO1 3-UTR. Besides, 3-UTR of mouse FOXO1 mRNA was also targeted by mouse miR-592 (Supplementary Fig. 1E–G).

3.3. miR-592 regulates gluconeogenesis in mouse primary hepatocytes

As described above, FOXO1 plays a critical role in the hepatic gluconeogenesis [6,7]. Therefore, we aim to determine whether miR-592 could exert a similar function cell-autonomously. To test it, adenoviruses containing miR-592 or negative control were generated and transfected into MPH, which were further incubated with or without forskolin/dexamethasone (FSK/DEX) to mimic the action of glucagon and glucocorticoids. As a result, overexpression of miR-592 did not alter glucose production in the absence of FSK/DEX. However, it significantly reduced glucose production in MPH treated with FSK/DEX (Fig. 2A). Quantitative real-time PCR analysis further confirmed that forced expression of miR-592 reduced mRNA levels of two gluconeogenic enzymes (PEPCK and G6Pase) in the presence of cocktails (Fig. 2B-2C). On the other hand, inhibition of miR-592 by its antisense led to increased cellular glucose production and up-regulation of gluconeogenic genes (Fig. 2D-2F). Therefore, these data suggest that miR-592 could suppress gluconeogenesis and glucose production in a cell autonomous manner.

3.4. Hepatic miR-592 expression is reduced in obese mice and humans

Considering that FOXO1 is upregulated in obese livers [10,15,16], we speculated that miR-592 expression might be altered in obesity. Therefore, we compared the expression levels of hepatic miR-592 and FOXO1 in different mouse models. Our data showed that the expression levels of miR-592 were reduced during fasting conditions and reduced upon refeeding in C57BL/6 mice (Fig. 3A). Hepatic miR-592 was also reduced while FOXO1 mRNA was increased in the livers of mice with high-fat-diet (HFD)-induced obesity or genetic obesity caused by leptin receptor (*db/db*) deficiency, compared with those in the corresponding control lean mice (Fig. 3B-3C). Moreover, expression of miR-592 negatively correlated with the mRNA levels of FOXO1 in these lean and obese mice (Fig. 3D). However, miR-592 expression in epididymal and

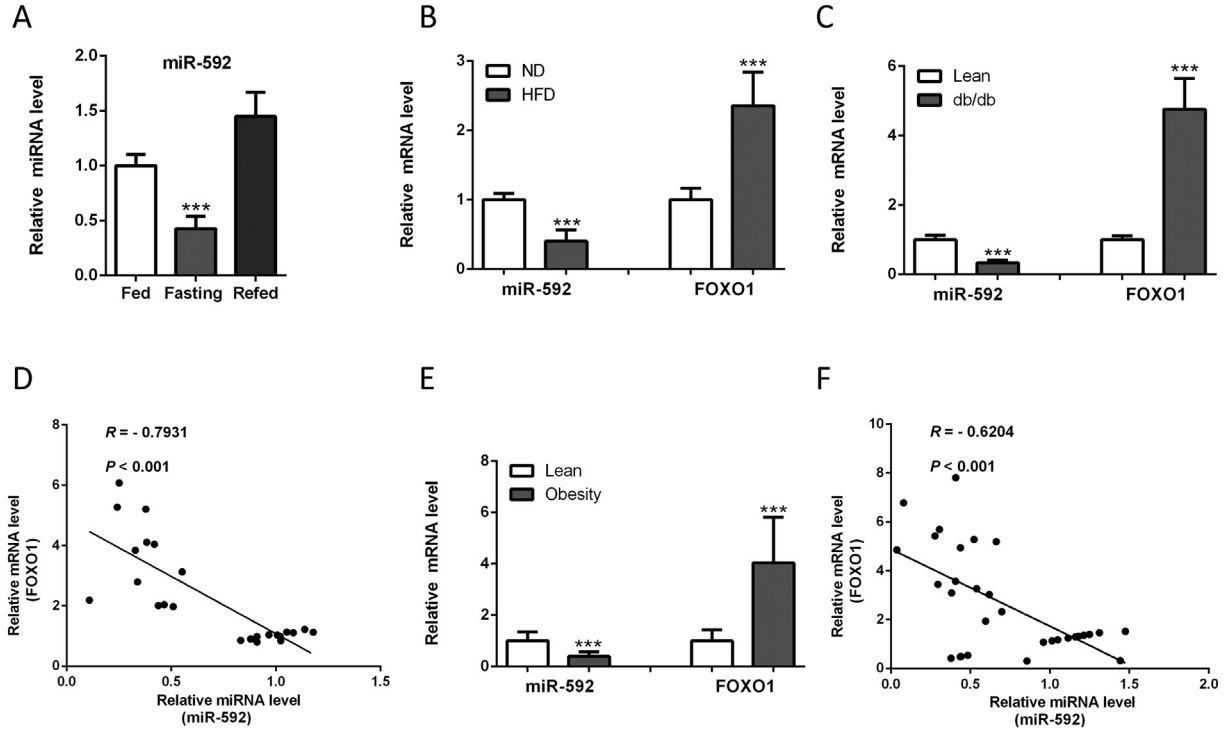

Fig. 3. Abnormal expression of miR-592 and FOXO1 in obesity. (A) Real-time PCR analysis of hepatic miR-592 expression in mice. C57BL/6 mice were placed under fed or 18-h fasted or 18-h fasted/6-h refed conditions. (B) Relative expression levels of miR-592 and FOXO1 mRNA in livers of C57BL/6 mice fed a normal diet (ND) or high-fat-diet (HFD) for 12 weeks. $n = 6$. (C) Relative expression levels of miR-592 and FOXO1 mRNA in livers of lean and *db/db* mice. n = 6. (D) Correlation between hepatic miR-592 levels and FOXO1 mRNA in the lean and obese mice as indicated in (A) and (B). ($n = 24$). (E) Relative analysis of human miR-592 and FOXO1 expression in livers of lean ($n = 18$) and obese ($n = 15$) individuals. (F) Correlation between hepatic miR-592 levels and FOXO1 mRNA in the lean and obese human subjects as indicated in (D). ($n = 33$). *** $P < 0.001$.

subcutaneous WAT and SKM was comparable between lean and obese mice (Supplementary Fig. 2A-B), suggesting its downregulation might be tissue-specific.

To examine whether expression levels of miR-592 and FOXO1 was also altered in obese humans, a small cohort of individuals were employed. We found that hepatic miR-592 was significantly downregulated while FOXO1 mRNA levels were increased in obese subjects (body mass index >28) compared with lean individuals (body mass index <24) (Fig. 3E). In addition, a significant correlation between expression of miR-592 and FOXO1 was also observed in human subjects (Fig. 3F).

To investigate which factors contribute to the downregulation of miR-592, we treated MPH with proinflammatory cytokines (TNFα, IL-1β), palmitate or insulin. As a result, miR-592 expression levels were inhibited by TNFα, IL-1β and palmitate, but not insulin, in a dose-dependent manner (Supplementary Fig. 2C-D), suggesting that downregulation of miR-592 in obesity might be attributed to overnutrition and related hepatic inflammation.

3.5. Inhibition of miR-592 leads to metabolic dysfunctions in healthy mice

To investigate whether reduced miR-592 expression is causally associated to the pathogenesis of obesity-induced metabolic disorders, male C57BL/6 mice aged 10 weeks were injected with lock nucleic acids (LNA), a modified RNA nucleotide [31], to specifically target miR-592. As a result, we observed a significant reduced expression of miR-592 in the liver of mice receiving anti-miR-592 LNA treatment compared to those receiving control LNA (Fig. 4A). However, its expression in SKM and WAT was only slightly reduced (Fig. 4A). Although body weight and food intake were comparable between two groups of mice (Supplementary Fig. 3A-B), anti-miR-592 LNA-treated mice showed an obvious symptom of diabetes, including elevated fasting glucose concentrations, enhanced hepatic gluconeogenesis, impaired glucose tolerance and reduced insulin sensitivity, as shown by pyruvate, glucose and insulin tolerance tests (Fig. 4B-4E). Besides, ratio of liver weight to body weight was increased in miR-592 LNA-infected mice (Fig. 4F), which might be

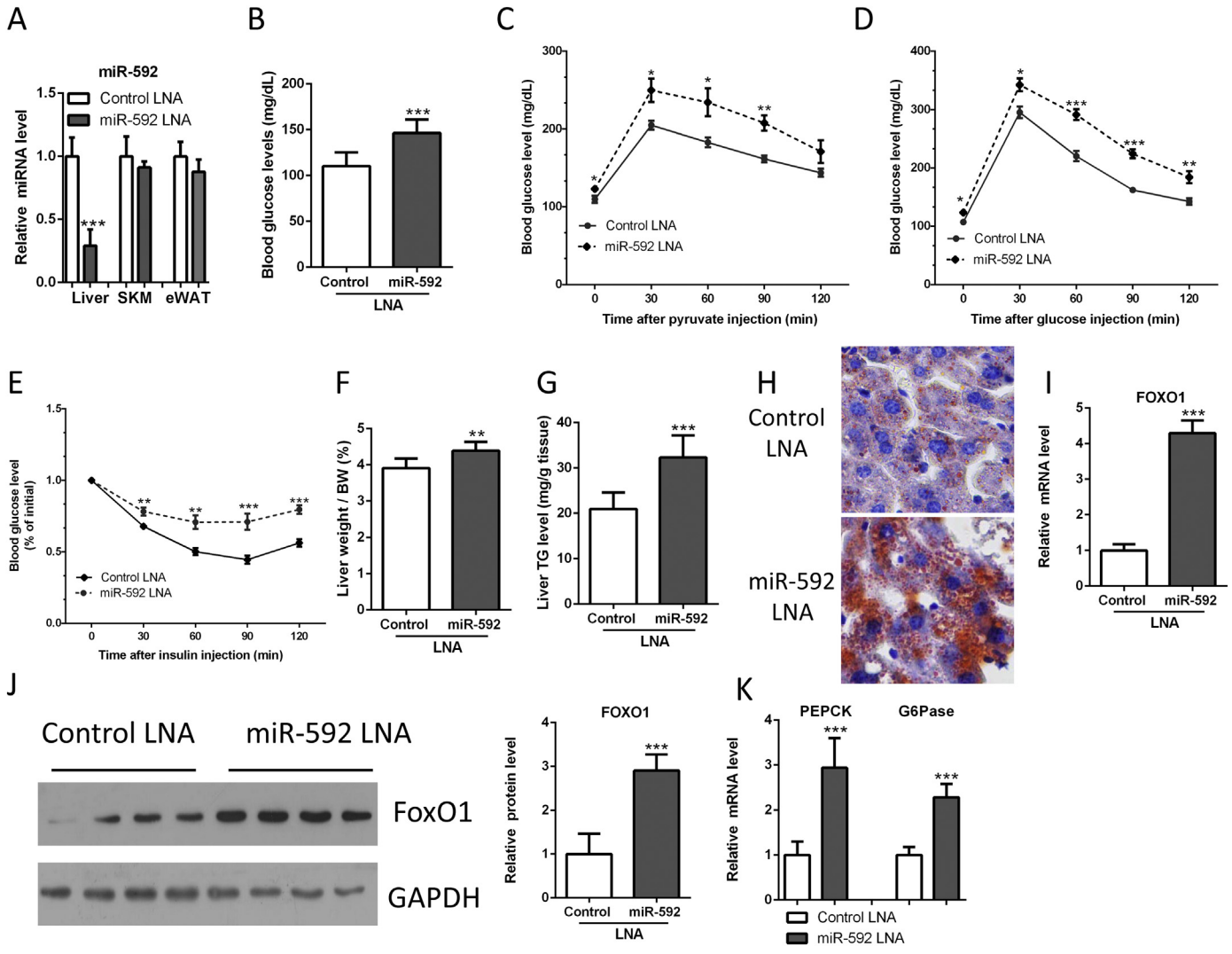

Fig. 4. Suppression of miR-592 expression leads to hyperglycemia and insulin resistance in lean mice. (A) Relative expression levels of miR-592 in C57BL/6 mice after intravenous injection with miR-592 locked nucleic acids (LNA) or control LNA. Mice were sacrificed 15 days after administration of miR-592 LNA or control LNA. (B) Fasting blood glucose concentrations in mice treated with miR-592 LNA or control LNA at day 4. (C-E) Pyruvate (C), glucose (D) and insulin (E) tolerance tests were performed at day 6, 9 and 12 after injection with miR-592 LNA or control LNA. (F-G) Liver weights (F) and triglyceride contents (G) in two groups of mice. (H) Representative oil red O staining showing the triglyceride accumulation in the liver of mice. (I-J) mRNA (I) and protein (J) expression of hepatic FOXO1 in mice treated with miR-592 LNA or control LNA. The quantification plot was analyzed by scanning densitometry using the Image J software (v 1.8.0). (K) Relative mRNA levels of PEPCK and G6Pase in two groups of mice. $n = 8$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

attributed to accumulation of hepatic triglyceride (Fig. 4G and H). Accordingly, expression of FOXO1 and its target gluconeogenic genes (PEPCK and G6Pase) were significantly induced in miR-592-LNA treated mice (Fig. 4I-4K). It has been shown that increased FOXO1 function leads to lipid accumulation partly through activating triglyceride synthesis [8,14]. Consistently, FOXO1 target genes responsible for lipogenesis, including SREBP-1c and FASN (8), were significantly up-regulated in mice after miR-592 LNA treatment (Supplementary Fig. 3C). Besides, it has been shown that FOXO1 could promote insulin signaling through a positive feedback mechanism [8]. Consistently, we found that phosphorylation status of AKT was enhanced in the liver of mice treated with miR-592 LNA (Supplementary Fig. 3D).

To determine whether the roles of anti-miR-592-LNA is mediated by up-regulation of FOXO1, we depleted its expression using adenovirus short hairpin RNA (shRNA), which suppressed the protein levels of FOXO1 in the mouse liver (Fig. 5A). At the same time, knockdown of FOXO1 significantly counteracted the increased fasting glucose levels and hepatic gluconeogenesis induced by miR-592 LNA (Fig. 5B-5C). In agreement, reduced insulin sensitivity, elevated hepatic triglyceride contents, increased expression of gluconeogenic and lipogenic genes were also reversed by FOXO1 shRNA treatment (Fig. 5D-5G). These data suggest that hepatic FOXO1 mediates the function of miR-592 in the regulation of glucose and lipid metabolism.

3.6. Hepatic overexpression of miR-592 alleviates hyperglycemia and hepatosteatosis in obese mice

Finally, we studied whether forced expression of miR-592 could alleviate diabetic phenotypes in obese mice. To test it, adenoviruses expressing miR-592 or negative control were employed. we firstly confirmed that our adenoviruses did not induce liver damage or inflammation as shown by circulating ALT, AST and TNFα mRNA expression (Supplementary Fig. 4A-C). Then, male *db*/*db* mice aged 12 weeks were administrated with miR-592 adenovirus or negative control through tail vein injection (Supplementary Fig. 5A). As a result, treatment of *db*/*db* mice with Ad-miR-592 improved hyperglycemia, hyperinsulinemia, hepatic glucose production and insulin sensitivity compared with control mice (Fig. 6A-6D). Moreover, liver weights, hepatic and plasma triglyceride contents were also decreased by Ad-miR-592 treatment (Fig. 6E-6H). Consistently, expression levels of FOXO1 and gluconeogenic genes were reduced (Fig. 6I-6K). Lipogenic genes, SREBP-1c and FASN, were also down-regulated by Ad-592 (Supplementary Fig. 5B). Insulin signaling, as measured by phosphorylation status of AKT, was enhanced (Supplementary Fig. 5C). However, overexpression of miR-592 in *db*/*db* mice did not alter body weight or food intake (Supplementary Fig. 5D-E). Similar effects on the improvement of glucose metabolism and hepatic TG contents were also observed in HFD-induced obese mice transduced with miR-592 adenovirus (Supplementary Fig. 6A-G). Moreover, overexpression of miR-592 also led

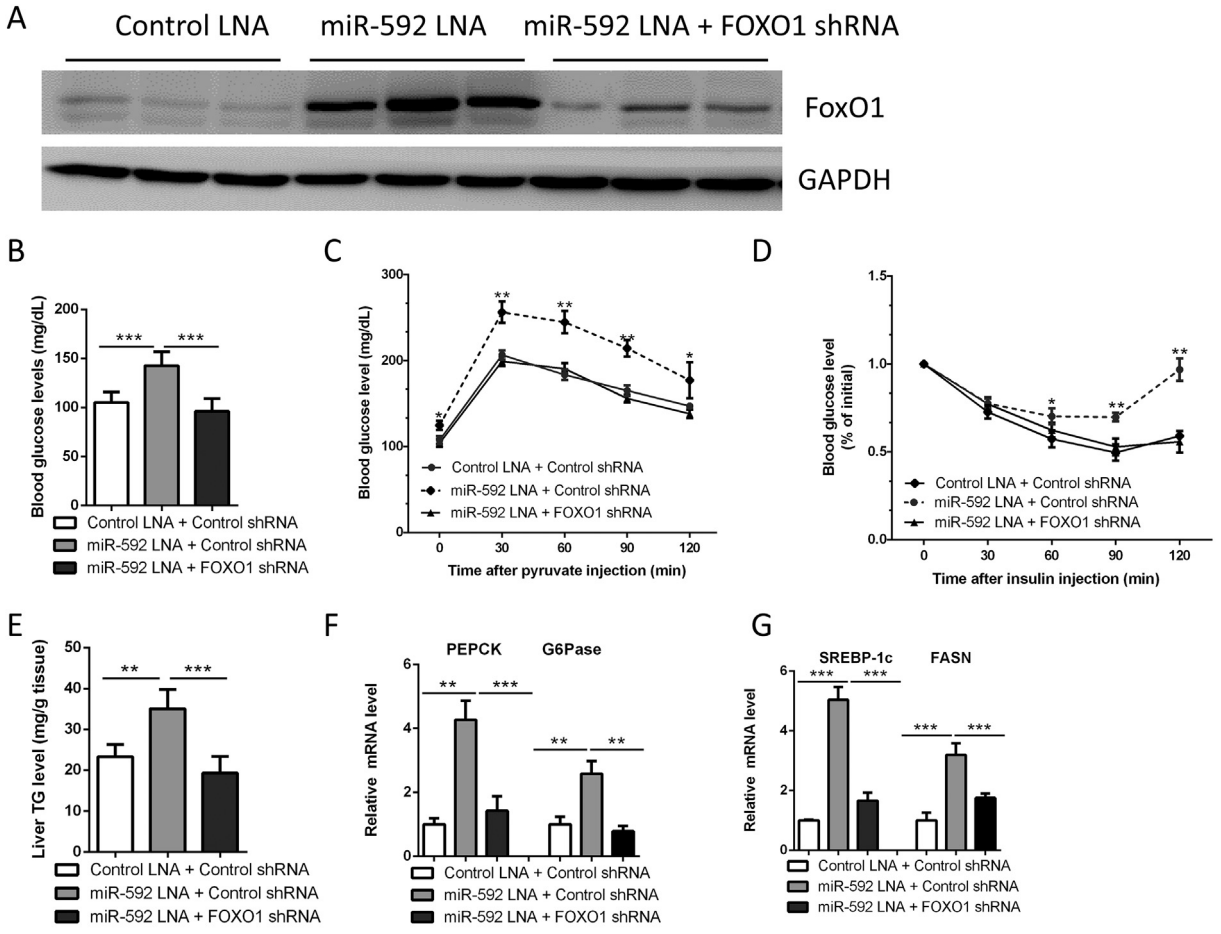

Fig. 5. FOXO1 mediates the roles of miR-592 in the glucose and lipid metabolism. (A) Protein expression of FOXO1 in mice treated with control LNA, miR-592 LNA, miR-592 LNA plus FOXO1 shRNA. (B) Fasting blood glucose concentrations in three groups of mice. (C-D) Pyruvate (C) and insulin (D) tolerance tests in three groups of mice. (E) Liver triglyceride contents in three groups of mice. (F) Relative mRNA levels of PEPCK and G6Pase in three groups of mice. (G) Relative mRNA levels of SREBP-1c and FASN in three groups of mice. n = 8 per group. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

to a down-regulation of FOXO1 and gluconeogenic genes in HFD mice (Supplementary Fig. 7A-C). Therefore, our data indicate that restoration of miR-592 in the livers of obese mice could improve metabolic disorders, including hyperglycemia, insulin resistance and hepatosteatosis.

4. Discussion

Previous studies have demonstrated that miR-592 plays important roles in many biological events. For instance, Irmady K et al. identified miR-592 as a key regulator of neurotrophin receptor p75 (NTR) expression and a potential therapeutic candidate to limit neuronal apoptosis after ischemic injury [32]. Besides, recent studies demonstrated that miR-592 is abnormal expressed in many types of human cancers. It has been shown that miR-592 could promote the proliferation of prostate, colorectal and gastric cancer cells [33–35]. On the other hand, miR-592 was shown to act as a tumor suppressor in breast cancer, non-small cell lung cancer and glioma [36–38]. These inconsistent observations suggest that the role of miR-592 might be tissue- or cell-specific. Notably, miR-592 was shown to target two oncogenic proteins: DEK and WSB1, to inhibit hepatocellular carcinoma (HCC) growth [39,40]. As a result, miR-592 levels were frequently decreased in HCC tissues and cell lines, and its reduction was associated with aggressive clinicopathological features and poor prognosis of HCC patients [39,40].

In the present study, we found that hepatic miR-592 expression levels were reduced in several mouse models of obesity. Given that obesity and hepatosteatosis is a critical risk factor contributing to the development of HCC [41,42], we speculate that down-regulation of miR-592 might be a causal factor linking obesity to the initiation and/or progression of HCC. We further showed that inhibition of endogenous miR-592 results in hyperglycemia, insulin resistance and hepatic TG retention in lean mice. In addition, overexpression of miR-592 could alleviate the diabetic phenotypes in *db/db* and HFD-induced obese mice. Overall, our results suggest that miR-592 may play an important role in the regulation of hepatic lipid metabolism. Interestingly, a recent study showed that miR-592 was significantly reduced in the liver of mice fed a high-fat-diet for 7 weeks [43], which is consistent our results. However, they further found that miR-592 was not changed in the liver of mice fed a high-fat-diet for 17 weeks. Although the reason behind these conflicting data remains unclear, we speculate that the expression of miR-592 might be altered by different nutrition status. Therefore, further studies using liver-specific knockout or transgenic mice are still needed to fully investigate the role of miR-592 in glucose and lipid metabolism.

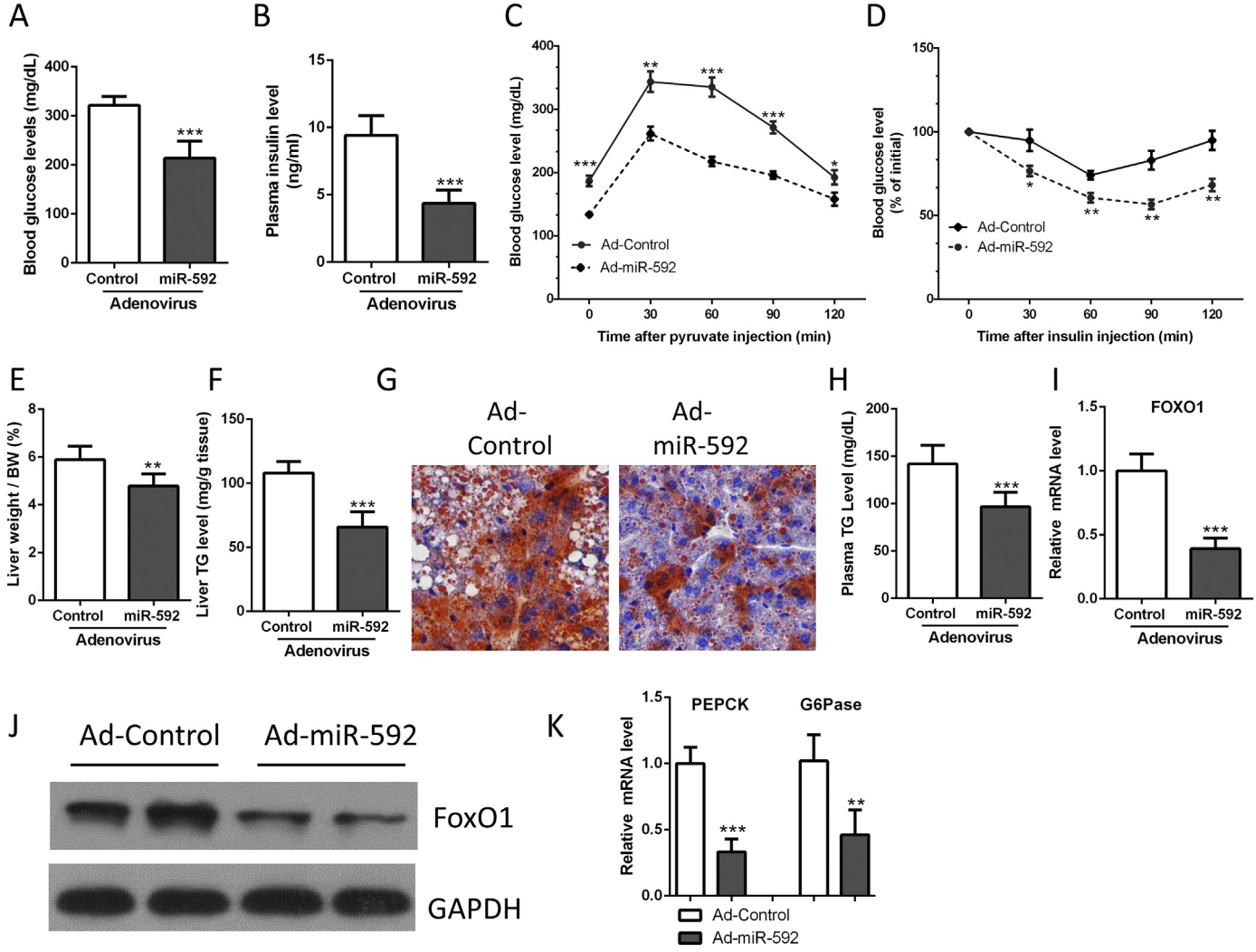

Fig. 6. Hepatic overexpression of miR-592 alleviates hyperglycemia in *db/db* mice. (A-B) Fasting blood glucose concentrations (A) and plasma insulin levels (B) in *db/db* mice treated with miR-592 adenovirus or control adenovirus at day 5. Mice were fasted for 6 h, followed by measurement of blood glucose levels. Besides, aliquots of blood (30μl) were collected from each mouse for the analysis of plasma insulin levels. (C-D) Pyruvate (C) and insulin (D) tolerance tests in two groups of mice were performed at day 7 and 10, respectively. (E-H) Liver weights (E), hepatic (F, G) and plasma (H) triglyceride contents in two groups of db/db mice. (I-J) mRNA (I) and protein (J) expression of hepatic FOXO1 in mice treated with miR-592 adenovirus or control adenovirus. (K) Relative mRNA levels of PEPCK and G6Pase in two groups of mice n = 6 per group. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

At the molecular level, we identified FOXO1 as a direct target of miR-592 in the liver and mediate its role to regulate hepatic metabolism. Recent data from mouse models have demonstrated the metabolic functions of FOXO1 in the liver, using gain- or loss-of-function studies (8, 9). However, dual role of FOXO1 in controlling hepatic glucose and lipid metabolism remains to be defined. Besides, increased expression and transcriptional activity of FOXO1 have been observed in the livers of obese rodents and humans [10,15,16]. Moreover, FOXO1 expression were independently associated with the degree of steatosis and necroinflammatory activity [16], suggesting that obesity-associated persistent steatosis and inflammation are involved in the regulation of FOXO1 expression. Interestingly, our results found that miR-592 is inhibited by proinflammatory cytokines and palmitate (Supplementary Fig. 2C and D), which represent inflammation status and steatosis, respectively. Previous studies have demonstrated that proinflammatory cytokines and palmitate could activate several signaling pathways in the hepatocytes, including NF-kB, JNKs and ER stress [44]. Therefore, further studies are still need to clarify which signaling pathway is responsible for the reduced expression of miR-592 in obesity. Overall, our results propose that miR-592 may mediate the roles of proinflammatory cytokines and over-nutrition in the regulation of FOXO1 expression.

However, considering that one microRNA can affect translation or degradation of many messenger RNAs, we cannot rule out the possibility that miR-592 may exert its function through suppressing other target genes. Especially, some reports showed that miR-592 could regulate FOXO3 expression through directly targeting the 3'-UTR of the FOXO3 transcript [45,46]. Due to sharing similar target consensus sequence, some functions of FOXO1 and FOXO3 appear to be overlapped [47]. For instance, it has been shown that FOXO1 and FOXO3 work in concert to regulate gluconeogenesis and hepatic glucose production [48]. Therefore, whether FOXO3 could mediate the roles of miR-592 in the control of lipid homeostasis needs to be clarified in the future studies.

In addition, recent studies demonstrated that expression levels or protein modifications of FOXO1 could be modulated by several miRNAs, such as miR-146b and microRNA-205-5p, in adipocytes and livers [49,50]. Therefore, these studies together with our results proposed that hepatic miRNAs serve as an important checkpoint in the regulation of FOXO1. Further studies could be conducted to identify novel miRNAs that target FOXO1 expression, which may help to better understand the complex role of FOXO1 in the metabolic diseases.

In summary, our study demonstrates that miR-592 has a protective role in maintaining hepatic glucose homeostasis through targeting FOXO1. Despite the importance of FOXO1 in regulation of hepatic glucose and lipid homeostasis, it remains a poor druggable target due to the lack of a ligand-binding domain. Interestingly, recent studies have shown that miRNAs could be considered as therapeutic targets for human liver diseases [51]. Therefore, our findings may provide a promising novel target for treating obesity-induced metabolic disorders.

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Author contributions

Y. S., L. W., J. Y. and Y. L. contributed to research data, data analysis and wrote the manuscript. M. L., X.X., Z. F., J. Z. and G. Y. contributed to the mouse and cellular experiments. X. C. contributed to discussion and revision of the manuscript; J. Y. and Y. L. conceived and designed the study, reviewed and edited the manuscript.

Conflict of interests

The authors declare that they have no competing interests.

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