NFE2 Induces miR-423-5p to Promote Gluconeogenesis and Hyperglycemia by Repressing the Hepatic FAM3A-ATP-Akt Pathway

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Hepatic FAM3A expression is repressed under obese conditions, but the underlying mechanism remains unknown. This study determined the role and mechanism of miR-423-5p in hepatic glucose and lipid metabolism by repressing FAM3A expression. miR-423-5p expression was increased in the livers of obese diabetic mice and in patients with nonalcoholic fatty liver disease (NAFLD) with decreased FAM3A expression. miR-423-5p directly targeted FAM3A mRNA to repress its expression and the FAM3A-ATP-Akt pathway in cultured hepatocytes. Hepatic miR-423-5p inhibition suppressed gluconeogenesis and improved insulin resistance, hyperglycemia, and fatty liver in obese diabetic mice. In contrast, hepatic miR-423-5p overexpression promoted gluconeogenesis and hyperglycemia and increased lipid deposition in normal mice. miR-423-5p inhibition activated the FAM3A-ATP-Akt pathway and repressed gluconeogenic and lipogenic gene expression in diabetic mouse livers. The miR-423 precursor gene was further shown to be a target gene of NFE2, which induced miR-423-5p expression to repress the FAM3A-ATP-Akt pathway in cultured hepatocytes. Hepatic NFE2 overexpression upregulated miR-423-5p to repress the FAM3A-ATP-Akt pathway, promoting gluconeogenesis and lipid deposition and causing hyperglycemia in normal mice. In conclusion, under the obese condition, activation of the hepatic NFE2/miR-423-5p axis plays important roles in the progression of type 2 diabetes and NAFLD by repressing the FAM3A-ATP-Akt signaling pathway.

In the past decades, type 2 diabetes has become epidemic worldwide (1). Increased hepatic glucose production resulting from insulin resistance is the central event in the pathogenesis of hyperglycemia and type 2 diabetes (2). FAM3A is a member of the family with sequence similarity 3 (FAM3) gene family (3). We recently demonstrated FAM3A is a novel mitochondrial protein and enhances ATP synthesis and secretion. FAM3A plays important roles in repressing hepatic gluconeogenesis and lipogenesis via insulin-independent activation of the ATP–P2 receptor–Akt pathway (4). FAM3A represents a novel target for the treatment of nonalcoholic fatty liver diseases (NAFLDs) and type 2 diabetes. FAM3A is a target gene of peroxisome proliferator–activated receptor-γ (PPARγ), and its expression is induced by PPARγ activation in liver cells (5). However, PPARγ expression is always increased in the livers of obese rodents, obese patients, and NAFLD patients (6–9) with reduced FAM3A expression (4).

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Clearly, hepatic FAM3A repression under the obese condition is not caused by PPARγ inactivation. Exploring the mechanism of hepatic FAM3A repression under the obese condition will shed light on the pathogenesis and treatment of NAFLD and type 2 diabetes. In the past decade, microRNAs (miRNAs) were shown to play important roles in the regulation of glucose and lipid metabolism. To determine whether hepatic FAM3A is repressed by miRNA(s) under obese conditions is of great interest and importance.

miRNA (miR)-214 targets activating transcriptional factor 4 (ATF4) to suppress gluconeogenesis in mouse livers (10). miR-135a targets insulin receptor substrate 2 (IRS-2) to induce insulin resistance in muscle cells (11). miR-181a targets SIRT-1 to induce insulin resistance and cause dysregulated glucose/lipid metabolism (12). miR-122 also plays an important role in regulation of glucose and lipid homeostasis in the liver (13). Recently, there is increasing evidence that circulating miRNAs are dysregulated in populations with type 2 diabetes and diabetic complications (14,15). However, the links between dysregulated circulating miRNAs and their roles in glucose homeostasis within tissues are missing, and the circulating miRNA profiles in patients with diabetes vary greatly in different clinical studies (14,15). Mature miR-423-5p and miR-423-3p are both processed from the miR-423 precursor with identical sequences in mouse and human. Several recent clinical studies revealed that the circulating miR-423-5p level is decreased in patients with impaired glucose tolerance, type 2 diabetes, or morbid obesity (16–18). In particular, the circulating miR-423-5p level is associated with fasting blood glucose variance in patients with type 2 diabetes (18). In contrast, the circulating miR-423-5p level is increased in obese children (19). So far, the role and mechanism of miR-423-5p in regulating glucose and lipid metabolism remain unknown. Bioinformatic prediction revealed that human, mouse, and rat FAM3A mRNAs are the potential targets of miR-423-5p (Supplementary Fig. 1*A*), suggesting that miR-423-5p may regulate glucose and lipid metabolism by repressing FAM3A expression and its downstream signaling transduction.

The current study revealed that in the livers of diabetic mice and NAFLD patients, nuclear factor erythroid-derived 2 (NFE2) expression was increased. NFE2 induced miR-423-5p expression to repress the FAM3A-ATP-Akt pathway, promoting hepatic gluconeogenesis and lipid deposition.

RESEARCH DESIGN AND METHODS

Experimental Mice

The study used 8–10-week-old male C57BL/6J mice (weight 22–25 g). Hepatic inhibition of miR-423-5p was performed in 8–10–week-old-male C57BL/6J mice fed a 45% high-fat diet (HFD) for 12 weeks. All animal protocols were approved by the Peking University Health Science Center Animal Research Committee.

Cell Culture

Murine NCTC 1469 cells were cultured in high-glucose DMEM medium (Invitrogen) supplemented with 10% horse serum (HyClone). Human HepG2 cells and HEK293 cells were cultured at 37°C in DMEM (high-glucose) medium with 10% FBS (Gibco, Carlsbad, CA). Cells were infected with 25 multiplicity of infection of adenovirus (Ad) green fluorescent protein (GFP) or Ad-miR-423 (or Ad-miR-423-5p sponge) for 36 h, followed by treatment with suramin (P2 receptor antagonist) or chlorpromazine (CPZ), a calmodulin (CaM) inhibitor, for 1 h before protein analysis. The primary mouse hepatocytes were cultured in 1640 medium (Invitrogen) and infected with Ads for 24 h before analysis.

Real-Time PCR Assay

Total RNA was extracted from tissues or cultured cells using the High-purity Total RNA Rapid Extraction Kit (BioTeke Corp., Beijing, China), and the reverse transcription was performed with using RevertAid First Strand cDNA Synthesis Kit (Fermentas K1622) according to the manufacturer's instruction. Real-time PCR was performed on a Stratagene Mx3000P real-time quantitative PCR system (Agilent Technologies) using β-actin as the housekeeping gene. The primer sequences for the quantitative PCR assays are listed in Supplementary Table 1.

Bulge-Loop Real-time RT-PCR

Complementary DNA was reverse transcribed from 2 μg total RNA mixing with 2 μL (500 nmol/L) miRNA-specific Bulge-Loop RT primers, then added to 11 μL using RNase-free water, well mixed, kept at 70°C for 10 min, and placed in ice for 2 min. Then, 5 μL 5× RT Buffer, 0.5 μL deoxynucleotide mix (10 mmol/L), 1 μL RiboLock RNase Inhibitor (20 units/μL), 0.5 μL RevertAid RT (200 units/μL), and nuclease-free water was added to 14 μL, followed by incubating for 60 min at 42°C and deactivating for 10 min at 70°C. The PCR reaction mixture was performed in a volume of 20 μL using TansStart Top Green qPCRSuperMix. The PCR reaction was performed by using hot start of 95°C for 20 s, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 70°C for 1–10 s. The relative miRNA levels were firstly normalized to small nuclear RNA U6 and then normalized to control group data. All the Bulge-Loop RT primers for both miRNAs and U6 were purchased from RiboBio Co., Ltd. (Guangzhou, China) (20).

Construction of miR-423-5p Sponge Plasmid

Oligonucleotide sequence was designed with six repeated miRNA binding sites that are separated by a spacer containing four nucleotide residues (CCGG), and four nucleotides mismatch were introduced between positions 9 and 11 from the 5′ end of the mature miRNA to create a bulge to prevent AGO2-mediated endonucleolytic cleavage (21,22). The sequence was as the following: 5′-aagctt***TAG***-AAAGTCTCGCTAGAGCCCCTCA*CCGG*AAAGTCTCGCTAGA-GCCCCTCA*CCGG*AAAGTCTCGCTAGAGCCCCTCA*CCGG*AA-AGTCTCGCTAGAGCCCCTCA*CCGG*AAAGTCTCGCTAGAGC-CCCTCA*CCGG*AAAGTCTCGCTAGAGCCCCTCAggatcc-3′. The sponge sequence was cloned into p-EGFP-C3–control vector using *Hin*dIII/*Bam*HI restriction enzymes.

Ad Vector Construction

The expression plasmid for mouse miR-423 precursor was purchased from Beijing OriGene Technology Co., Ltd.,which contains premicroRNA (60–70 nucleotides) with 250–300 nucleotides up- and downstream flanking sequence. Ads expressing miR-423 precursor/miR-423-5p sponge were constructed at the Chinese National Human Genome Center of Beijing (SinoGenoMax Co., Ltd.).

Luciferase Reporter Assay

The 3′-untranslated region (UTR) region of mouse FAM3A mRNA was cloned in the pMIR-REPORT vector (Ambion, Inc.). Either pFAM3A 3′-UTR-luciferase or pFAM3A mutant-luciferase was cotransfected with miR-423 expression plasmid (or miR-423-5p sponge plasmid) or pEGFP-C3 in HepG2 cells or HEK293 cells using VigoFect transfection reagent (Vigorous Biotechnology Beijing Co., Ltd.) according to the instructions. pRL-TK vector containing *Renilla* luciferase (kindly provided by Professor Yuansheng Gao) was also cotransfected with each condition as a transfection control. At 12 h after transfection, the transfection medium was replaced with fresh medium. The activities of firefly and *Renilla* luciferases were measured using the Dual-Luciferase Reporter Assay (Promega) after 24 h.

Ad Overexpression or Knockdown of miR-423-5p in Mouse Livers

To overexpress or knockdown miR-423-5p in the liver of normal mice or HFD mice, 1.0×10^9 plaque-forming units Ad-miR-423 or Ad-miR-423-5p sponge (Ad-GFP as control) were injected via tail vein as previously described (4,23). Oral glucose tolerance tests (OGTT) were performed at 4 and 7 days after Ad injection. Insulin tolerance tests (ITT) or pyruvate tolerance tests (PTT) were performed at 7 days after Ad treatment. The fed animals were sacrificed for experimental analysis on day 8.

Serum was collected for measurement of triglyceride (TG), cholesterol (CHO), insulin, and free fatty acid (FFA) levels. Liver tissue (20–40 mg) was homogenized in 1 mL chloroform/methanol (2:1 vol/vol) solvent (Beijing Chemical Technology) on ice. The homogenized samples were incubated for 16 h at 4°C, and then 300 μL distilled water was added to each sample, followed by vortexing and centrifuging at 12,000 rpm for 10 min at 4°C. The organic phase was collected and dried. The dried pellets were solubilized in PBS buffer containing 5% Triton X-100. The total TG and total CHO were quantitated by using TG and CHO assay kits (BIOSINO Bio-Technology and Science Inc.). The data were normalized by protein content in each sample (4,23).

Plasmid Overexpression or Small Interfering RNA Knockdown of NFE2 in Hepatocytes

HepG2 cells or primary mouse hepatocytes were plated in six-well plates and transfected with 2 μg NFE2 plasmid using the VigoFect transfection kit (Vigorous Biotechnology, Cat No T001) for 12 h, and then the medium was replaced with normal medium. The cells were cultured for another 24 h before analysis. Human NFE2 p45 subunit expression plasmid was purchased from Vigenebio Ltd. (Jinan, China; Cat #CH854191, PubMed ID: NM_006163). To knockdown NFE2 p45 expression in HepG2 cells, the cells were transfected with 50 nmol/L small interfering (si)RNAs against homo NFE2 mRNA (siRNA sequences are provided in Supplementary Table 2). At 36 h after transfection, the cells were lysed for analysis.

Hydrodynamics-Based Plasmid Overexpression of NFE2 in C57BL/6J Mouse Livers

Hydrodynamics-based transfection in animals was detailed previously (24–26). Briefly, 50 μg endotoxin-free pEGFP-C3 or pNFE2 was dissolved in sterile saline, with the volume 10% of the bodyweight at room temperature, and injected into tail vein in 7 s. OGTT/PTT were performed at 72 h after plasmid injection. Mice were sacrificed 24 h later for assays, as above.

Metabolic Phenotyping

OGTT was performed in fasted mice (8 A.M. to 2 P.M.) using a dose of 3 g/kg glucose. Blood glucose levels were monitored at various times after the glucose load (27), with blood glucose levels at 0 min used as the fasting blood glucose. Serum insulin levels were measured using the Rat/Mouse Insulin ELISA kit (Millipore) (28) at fed states before sacrifice. ITT (insulin at the dose of 1 unit/kg body wt) and PTT (pyruvate at the dose of 0.75 mg/kg body wt) were performed in mice fasted for 4 h and 16 h, respectively. Blood glucose levels were monitored at various times.

ATP Content Determination

For determination of relative ATP level in the cells, the ATP content values (nmol) were first normalized to the protein mount (nmol/mg protein) in the same sample and then normalized to the control values. For determination of relative ATP level in the medium, the absolute concentration was determined and normalized to the control value (4,23).

Glucose Production Assay in HepG2 Cells

Cells were infected with Ad-miR-423-5p sponge for 24 h and then washed three times by PBS buffer. Cells were cultured in glucose and phenol red-free DMEM (fDMEM) medium, supplemented with 20 mmol/L sodium lactate and 2 mmol/L sodium pyruvate for 13 h. Insulin (100 nmol/L) was added to the cells for an additional 3 h. Glucose content in the medium was determined using Glucose Assay Kit (Sigma-Aldrich, GAGO-20) and normalized to total cellular protein content.

Hyperinsulinemic-Euglycemic Clamp Experiments

At 7 days after miR-423-5p repression, HFD mice underwent a hyperinsulinemic-euglycemic clamp. Mice were fasted for 12 h, and insulin was infused after a 30-min basal period with a primed continuous infusion rate of 4 $mU \cdot kg^{-1} \cdot min^{-1}$, glucose concentration was determined at 10-min intervals to adjust the glucose infusion rate for maintaining blood glucose level between 100 and 150 mg/dL (4).

Lipolysis of Adipose

The protocol for lipolysis of adipose tissue was detailed previously (29). In brief, at 7 days after Ad-miR-423 or

Ad-miR-423-5p sponge injection, 50 mg epididymal adipose tissue was quickly dissected from the mice and suspended in 500 μL fDMEM containing 5 mmol/L glucose, and horseradish peroxidase–conjugated IgG on ice and cut into 1-mm^3 pieces. The samples were incubated at 37°C in 5% CO_2 and 95% air in 500 μL fDMEM for 30 min, and then were washed three times quickly, followed by incubating in 500 μL fDMEM. The culture medium was collected at 30 and 60 min for glycerol release measurement as an index of lipolysis using an Applygen Technologies (Beijing) Detection Kit. The glycerol level was normalized by protein content.

Human Liver Sample

The human liver biopsies and clinical procedures were performed within the diagnostic workup of NAFLD in our previous study, in which 11 patients with steatosis and 11 healthy control subjects were included (30). In the current study, 6 steatosis and healthy liver samples were selected from these 11 pairs of liver samples due to the run out of the other liver samples. The physiological and biochemical characteristics of six steatosis patients and healthy subjects are provided in Supplementary Table 3. (These basic data were previously published among 11 pairs of patients [30]). The application for patient-derived materials was approved by the Beijing You-An Hospital Research Ethics Committee (Number 2015-35), and written consent was obtained from all of the patients (30).

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance of differences between groups was analyzed by the unpaired Student *t* test or by one-way ANOVA when more than two groups were compared.

RESULTS

FAM3A mRNA Is a Direct Target of miR-423-5p

Bioinformatic prediction revealed one potential site specific for miR-423-5p in 3′-UTRs of human, rat, and mouse FAM3A mRNAs (Supplementary Fig. 1*A*). Both miR-423-5p and miR-423–3p are processed from miR-423 precursor gene (Supplementary Fig. 1*A*), and ubiquitously expressed among mouse tissues (Supplementary Fig. 1*B* and *C*). To determine whether miR-423-5p was involved in type 2 diabetes, its expression in main metabolic tissues of obese diabetic mice was analyzed. The miR-423-5p level was increased in the liver and epididymal adipose tissue but was reduced in the skeletal muscle of HFD-fed mice. Its expression was increased in the liver but was decreased in skeletal muscle and remained unchanged in adipose tissue of *db*/*db* mice (Fig. 1*A* and *B*). miR-423-3p expression was increased in HFD mouse liver but remained unchanged in *db*/*db* mouse liver (Fig. 1*A* and *B*). Levels of both miR-423-5p and miR-423-3p remained unchanged in the heart, hypothalamus, and pancreas of HFD and *db*/*db* mice (Fig. 1*A* and *B*). FAM3A mRNA and protein levels were decreased in liver and adipose tissue but remained unchanged in muscle of HFD and *db*/*db* mice (Supplementary Fig. 2*A*–*C*). FAM3A protein level was also reduced in *db*/*db* mouse livers (Supplementary Fig. 2*D*). Notably, levels of both miR-423-5p and miR-423-3p were increased in the steatotic livers of humans with reduced FAM3A mRNA level compared with healthy livers (Fig. 1*C*). Hematoxylin and eosin staining revealed significant lipid droplets in the livers of patients with fatty liver, but no significant lipid droplets were detected in the livers of healthy subjects (Supplementary Fig. 3*A*). Patients with fatty liver have higher serum TG and CHO levels than healthy subjects (Supplementary Table 3). Moreover, although patients with fatty liver did not have diabetes, they had higher fasting blood glucose level than healthy subjects (Supplementary Fig. 3*B*). There were no differences in other clinical and biochemical characteristics, including BMI, waist circumstance, metabolic syndrome, and liver function, between healthy subjects and patients with fatty liver (Supplementary Table 3). Overall, the miR-423-5p level is negatively correlated with FAM3A level in the liver.

miR-423 overexpression reduced FAM3A mRNA and protein levels in HepG2 cells (Supplementary Fig. 4). Ad-miR-423-5p sponge treatment reduced the miR-423-5p level without affecting the miR-423-3p level in human HepG2 cells and murine NCTC cells (Supplementary Fig. 5). miR-423 overexpression repressed, whereas miR-423-5p silencing activated mouse FAM3A 3′-UTR luciferase reporter activity in HepG2 and HEK293 cells (Fig. 1*D* and *E*). Mutation of the potential miR-423-5p binding site abolished the regulatory effects of miR-423-5p overexpression or inhibition on the reporter activity in HepG2 and HEK293 cells (Fig. 1*D* and *E*). Clearly, FAM3A mRNA is a target gene of miR-423-5p, but not of miR-423-3p.

miR-423-5p Targeted FAM3A to Repress the ATP-P2 Receptor–CaM–Akt Pathway in Hepatocytes

Because FAM3A enhances ATP production to activate Akt via ATP–P2 receptor–CaM signaling (4), the effect of miR-423-5p overexpression or inhibition on the ATP-Akt pathway was determined in cultured hepatocytes. miR-423-5p overexpression or inhibition reduced or increased intracellular and extracellular ATP content in HepG2 cells (Fig. 2*A*) and primary mouse hepatocytes (Fig. 2*B*). miR-423-5p overexpression reduced whereas miR-423-5p inhibition increased FAM3A and phosphorylated (p)Akt protein levels in HepG2 cells (Fig. 2*C*) and mouse hepatocytes (Fig. 2*D*). miR-423-5p overexpression increased, whereas miR-423-5p inhibition reduced PEPCK expression in HepG2 cells and mouse hepatocytes (Fig. 2*C* and *D*). Akt activation induced by miR-423-5p inhibition was blocked by the P2 receptor antagonist suramin and CaM inhibitor CPZ in HepG2 cells and mouse hepatocytes (Fig. 2*E* and *F*).

miR-423-5p Overexpression Induced Glucose Intolerance and Hepatic Lipid Deposition in C57BL/6J Mice

miR-423 was overexpressed in C57BL/6J mouse livers via tail vein injection of Ad-miR-423 to evaluate its

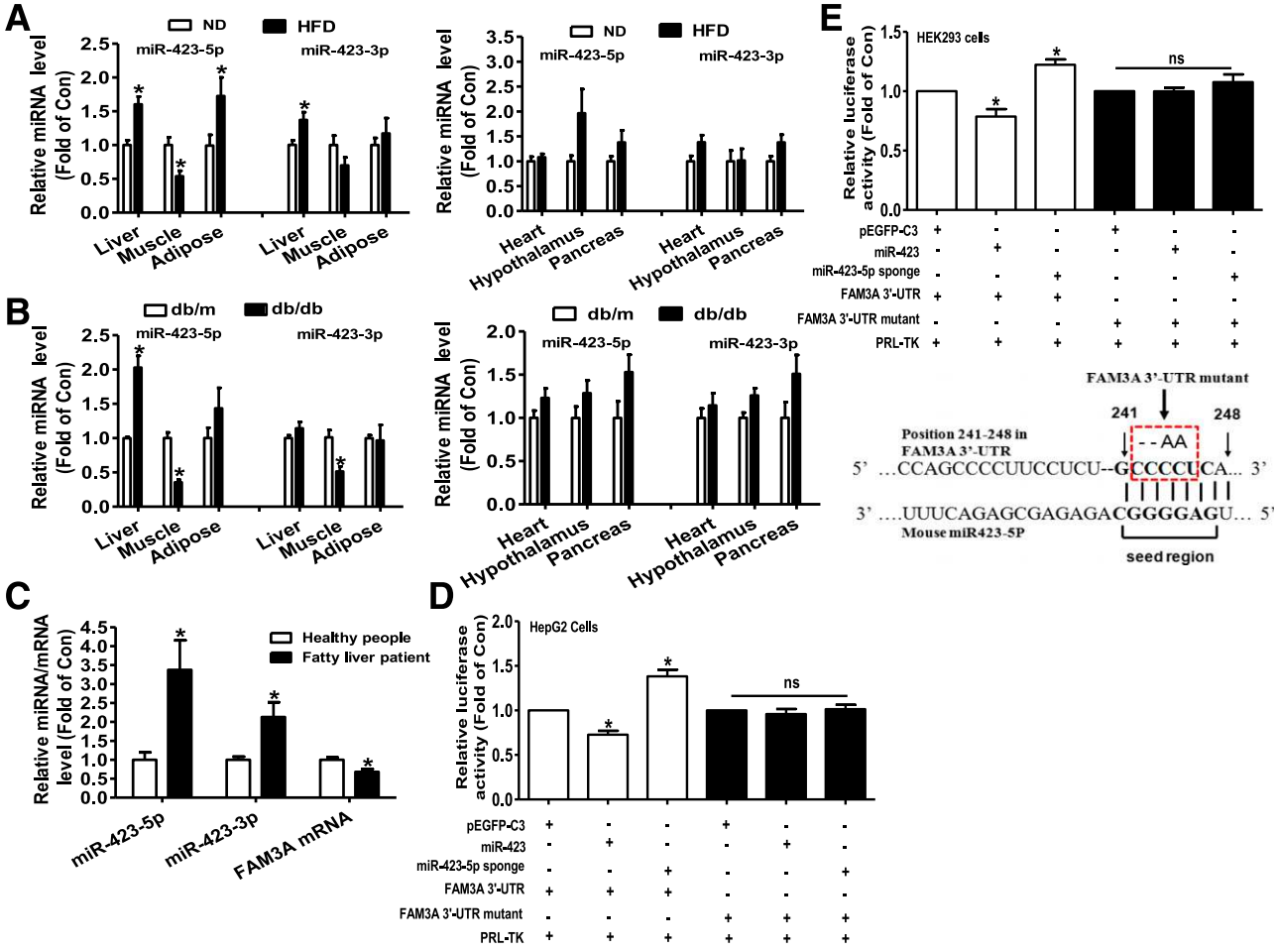

Figure 1—miR-423-5p level was increased in livers of obese diabetic mice and NAFLD patients. *A*: Expression of miR-423-5p and miR-423-3p in main metabolic tissues of HFD mice and normal diet (ND) mice (n = 6–8). *P < 0.05 vs. ND mice. *B*: Expression of miR-423-5p and miR-423-3p in main metabolic tissues of *db/db* mice (n = 6–8). Adipose, epididymal adipose tissue. *P < 0.05 vs. *db/m* mice. *C*: miR-423-5p expression was increased with a reduction in FAM3A mRNA expression in the livers of NAFLD patients (n = 6). *P < 0.05 vs. healthy subjects. miR-423 overexpression inhibited, whereas miR-423-5p inhibition activated, the luciferase activity of mouse FAM3A 3′-UTR reporter in HepG2 cells (*D*) and HEK293 cells (*E*). Mutation of the potential site for miR-423-5p abolished the inhibition or activation effects of miR-423 or miR-423-5p sponge on the luciferase activity of the mouse FAM3A 3′-UTR reporter in both HepG2 and HEK293 cells. n = 5–6. *P < 0.05 vs. control cells or between two indicated groups; ns, not significant.

effect on glucose and lipid metabolism. Ad-miR-423 injection significantly increased miR-423-5p and miR-423-3p levels in liver and slightly increased miR-423-5p level in muscle without significant effect on the miR-423-3p level. In contrast, Ad-miR-423 injection had little effect on miR-423-5p and miR-423-3p levels in adipose tissue and the pancreas (Supplementary Fig. 6). These findings revealed the specific overexpression of miR-423 in the liver. On days 4 and 7 after Ad injection, Ad-miR-423–treated mice exhibited glucose intolerance and fasting hyperglycemia compared with Ad-GFP–treated mice (Fig. 3*A*–*C*). Oil Red O staining assay indicated that miR-423 overexpression increased lipid deposition in mouse livers (Fig. 3*D*). Quantitative assays confirmed that miR-423 overexpression increased TG content but not CHO content in mouse livers (Fig. 3*E*). Hepatic miR-423 overexpression had little effect on serum TG and CHO levels (Fig. 3*F*).

miR-423-5p Inhibition Attenuated Glucose Intolerance and Fatty Liver in HFD Mice

To further confirm the role of miR-423-5p in hepatic glucose and lipid metabolism, its expression in the livers of HFD mice was inhibited by injection of Ad-miR-423-5p sponge. On days 4 and 7 after Ad-miR-423-5p sponge injection, glucose intolerance and hyperglycemia of HFD mice were significantly improved (Fig. 4*A*–*C*). Oil Red O staining assays indicated that hepatic lipid deposition was reduced after Ad-miR-423-5p sponge treatment (Fig. 4*D*). Quantitative assays confirmed Ad-miR-423-5p sponge treatment reduced TG content but not CHO content in mouse livers (Fig. 4*E*). In contrast,

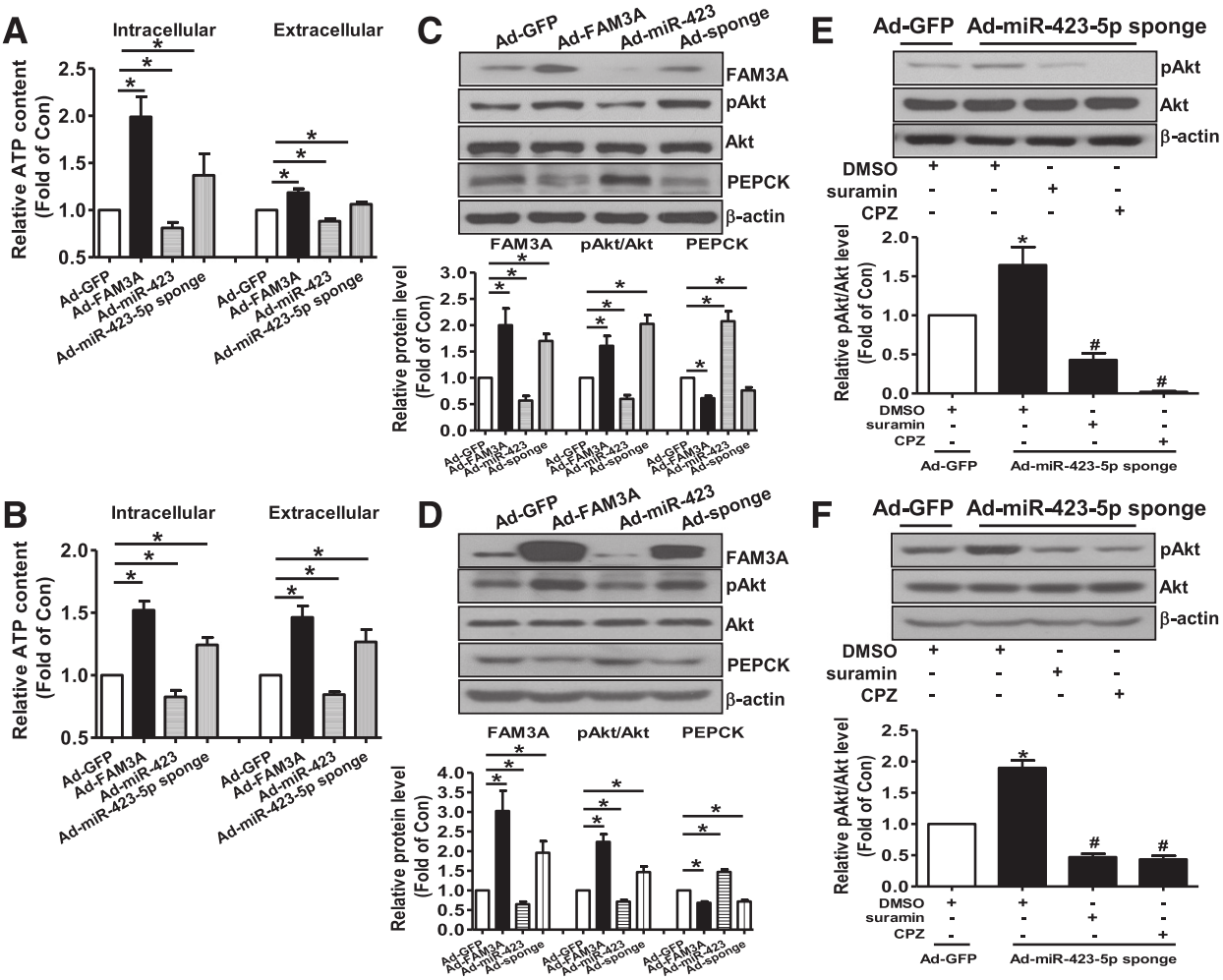

Figure 2—miR-423-5p targets FAM3A to inhibit the ATP–P2 receptor–CaM–Akt pathway in cultured hepatocytes. miR-423-5p overexpression or knockdown on intracellular and extracellular ATP content in HepG2 cells (*A*) and primary mouse hepatocytes (*B*). miR-423-5p overexpression or knockdown on FAM3A expression and Akt activation in HepG2 cells (*C*) and mouse hepatocytes (*D*). Ad-sponge, Ad-miR-423-5p sponge. Inhibition of P2 receptors or CaM blocked Akt activation induced by miR-423-5p knockdown in HepG2 cells (*E*) and mouse hepatocytes (*F*). The infected cells were treated with suramin (50 μmol/L) and CPZ (100 μmol/L) for 1 h before pAkt analysis. $n = 3–5$. *$P < 0.05$ vs. control cells or between two indicated groups; #$P < 0.05$ vs. Ad-miR-423-5p sponge–infected cells without inhibitor treatment.

Ad-miR-423-5p sponge treatment had little effect on serum TG and CHO levels (Fig. 4*F*).

miR-423-5p Overexpression or Knockdown Induced or Improved Global Insulin Resistance

ITT revealed that hepatic miR-423 overexpression induced global insulin resistance with an elevation in serum insulin level in normal mice (Fig. 5*A–C*). Moreover, miR-423 overexpression increased hepatic glucose production as evaluated by PTT in normal mice (Fig. 5*D* and *E*). In contrast, insulin resistance was improved by Ad-miR-423-5p sponge treatment with reduced serum insulin level in HFD mice (Fig. 5*F–H*). Hyperinsulinemic-euglycemic clamp further confirmed that hepatic miR-423-5p inhibition enhanced global insulin sensitivity in HFD mice (Supplementary Fig. 7*A–C*). Ad-miR-423-5p sponge injection suppressed hepatic glucose production in HFD mice (Fig. 5*I* and *J*). In support, miR-423-5p inhibition repressed glucose production in HepG2 cells (Supplementary Fig. 7*D*). Hepatic miR-423 overexpression slightly increased the lipolysis of epididymal adipose tissue with a significant elevation in serum FFA levels in normal mice (Supplementary Fig. 8*A* and *B*), whereas hepatic miR-423-5p inhibition repressed the lipolysis of epididymal adipose tissue with a reduction in circulating FFA levels in HFD mice (Supplementary Fig. 8*C* and *D*).

miR-423-5p Overexpression or Inhibition on Metabolic Gene Expression in Mouse Livers

In normal mouse livers, miR-423 overexpression reduced the FAM3A protein level (Fig. 6*A*) and ATP content (Fig. 6*B*). miR-423 overexpression reduced pAkt level with increased protein levels of PEPCK, G6pase, and FAS in mouse livers (Fig. 6*A*). miR-423 overexpression reduced the mRNA

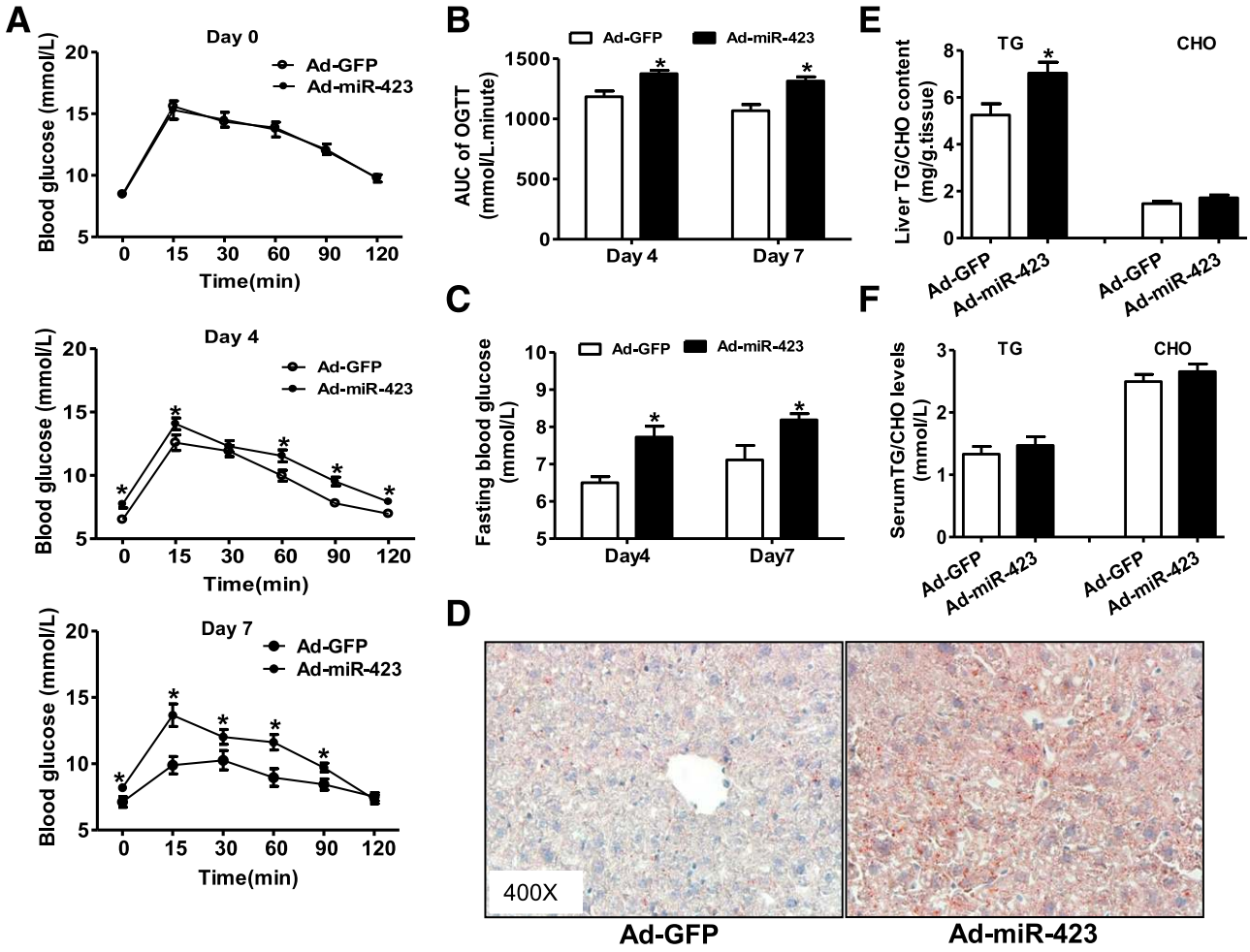

Figure 3—Hepatic miR-423 overexpression induced glucose intolerance and fatty liver in C57BL/6J mice. Male C57BL/6J mice (8–10 weeks old) were treated with 1.0×10^9 plaque-forming units of Ad-GFP or Ad-miR-423 via tail vein injection. *A*: Hepatic miR-423 overexpression induced glucose intolerance in C57BL/6J mice. OGTTs were performed on day 0 (upper), day 4 (middle), and day 7 (lower) after Ad injection. *B*: Area under curve (AUC) of OGTT data presented in panel *A*. *C*: Hepatic miR-423 overexpression elevated fasting blood glucose levels in C57BL/6J mice. The blood glucose levels at 0 min in panel *A* are presented as fasting blood glucose. n = 8–10. $^*P < 0.05$ vs. Ad-GFP mice. *D*: Representative images of liver samples stained with Oil Red O. *E*: Quantitative assays of TG and CHO content in mouse livers. *F*: Serum TG and CHO levels of mice. n = 14–18. $^*P < 0.05$ vs. Ad-GFP mice.

level of FAM3A and increased that of PEPCK, G6Pase, and FAS (Fig. 6*C*). miR-423-5p inhibition increased FAM3A and pAkt protein levels and ATP content, and reduced PEPCK, G6pase, and FAS protein levels in HFD mouse livers (Fig. 6*D* and *E*). miR-423-5p inhibition upregulated the mRNA level of FAM3A and reduced that of PEPCK, G6pase, and FAS in HFD mouse livers (Fig. 6*F*). The mRNA levels of SREBP1, ACC1, CPT1α, and AOX were not significantly affected by miR-423-5p overexpression or inhibition in normal or HFD mouse livers (Supplementary Fig. 9*A* and *B*).

NFE2 Upregulated miR-423-5p to Repress FAM3A Expression in Liver Cells

To probe the mechanism for hepatic miR-423-5p activation under the diabetic condition, the potential transcriptor binding sites in the promoter region of miR-423 precursor gene were analyzed. The analyses revealed that the potential binding sites for transcriptors OCT1, BACH1, and NFE2 are present in the human miR-423 precursor gene promoter. One potential NFE2 binding site is also predicted to be present in mouse miR-423-precuror gene promoter (Supplementary Fig. 10). In the livers of NAFLD patients and HFD mice, the mRNA level of NFE2 was increased, whereas the levels of OCT1 and BACH1 remained unchanged (Fig. 7*A* and Supplementary Fig. 11*A*). The NFE2 protein level was increased in the livers of HFD and *db*/*db* mice (Supplementary Fig. 11*B* and *C*). Plasmid overexpression of NFE2 increased miR-423-5p and miR-423-3p levels with reduced FAM3A mRNA level in HepG2 cells (Fig. 7*B*). NFE2 overexpression reduced the FAM3A protein level, intracellular and extracellular ATP content and pAkt level, and increased PEPCK mRNA and protein levels (Fig. 7*B–D*). In contrast, NFE2 silencing reduced miR-423-5p expression with increased FAM3A mRNA and

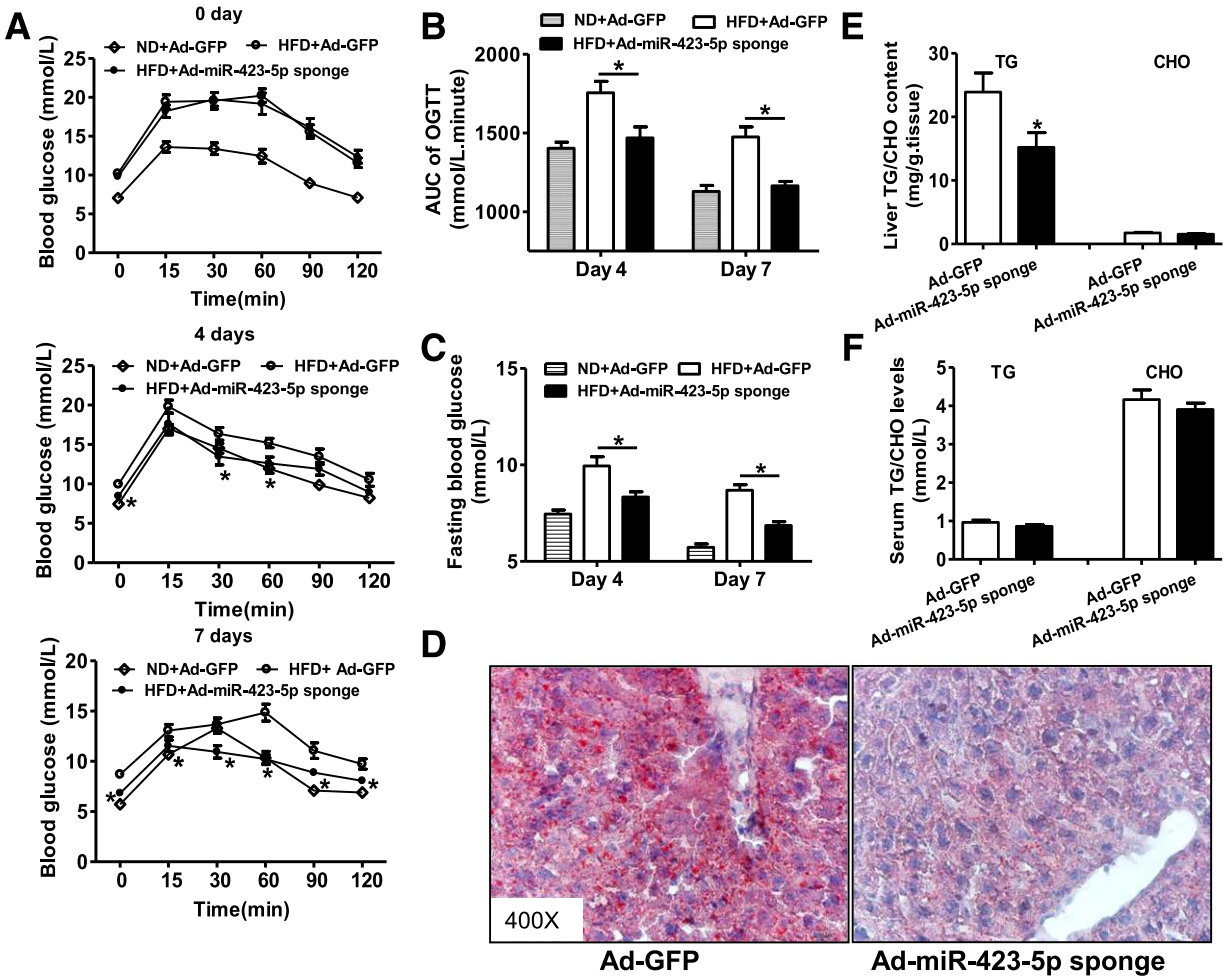

Figure 4—Inhibition of hepatic miR-423-5p improved glucose intolerance and fatty liver in HFD mice. ND, normal diet. *A*: Inhibition of miR-423-5p attenuated glucose intolerance in HFD mice. OGTTs were performed on day 0 (upper), day 4 (middle), and day 7 (lower) after Ad injection. *B*: Area under the curve (AUC) of OGTT data presented in panel *A*. *C*: Inhibition of miR-423-5p ameliorated fasting hyperglycemia in HFD mice. The blood glucose levels at 0 min in panel *A* are presented as fasting blood glucose. n = 8–10. $^*P < 0.05$ vs. HFD+Ad-GFP mice. *D*: Representative images of liver samples stained with Oil Red O. *E*: Quantitative assays of TG and CHO content in HFD mouse livers. *F*: Serum TG and CHO levels of HFD mice. n = 14–18. $^*P < 0.05$ vs. HFD+Ad-GFP–treated mice.

protein levels in HepG2 cells (Fig. 7*E* and *F*). NFE2 silencing increased intracellular and extracellular ATP content and pAkt level, with reduced PEPCK mRNA and protein levels (Fig. 7*E*–*G*). To further confirm that NFE2 directly regulated miR-423 precursor gene expression, a 1.9-kb fragment of the human miR-423 precursor gene promoter containing the potential NFE2 binding site was cloned from HepG2 cells (Supplementary Fig. 12). NFE2 overexpression activated whereas NFE2 silencing repressed human miR-423 precursor promoter activity in HepG2 cells (Fig. 7*H*). Furthermore, mutation of the potential NFE2 binding site abolished the regulatory effects of NFE2 overexpression or silencing on the reporter activity (Fig. 7*H*). NFE2 overexpression similarly upregulated miR-423-5p and reduced FAM3A expression, ATP production, and Akt activity in mouse hepatocytes (Fig. 8*A*–*C*). Plasmid overexpression of NFE2 in normal mouse liver caused glucose intolerance and fasting hyperglycemia (Fig. 8*D* and *E*). NFE2 overexpression upregulated miR-423-5p and reduced FAM3A expression, ATP content, and pAkt level with increased gluconeogenic and lipogenic gene expression in mouse livers (Fig. 8*F*–*H*). Consistently, NFE2 overexpression increased hepatic gluconeogenesis and lipid deposition in C57BL/6J mouse livers (Supplementary Fig. 13), with little effect on serum TG and CHO levels (data not shown). In HepG2 cells, chronic exposure to FFAs upregulated NFE2 and miR-423-5p levels (Supplementary Fig. 14). Overall, NFE2 induced miR-423-5p expression to repress the FAM3A-ATP-Akt pathway in liver cells, causing dysregulated hepatic and global glucose and lipid metabolism.

DISCUSSION

miRNAs inhibit gene expression mainly via translational repression and mRNA decay. The contribution of translational repression to overall miRNA silencing effects on target genes will prevail over that of mRNA decay in some

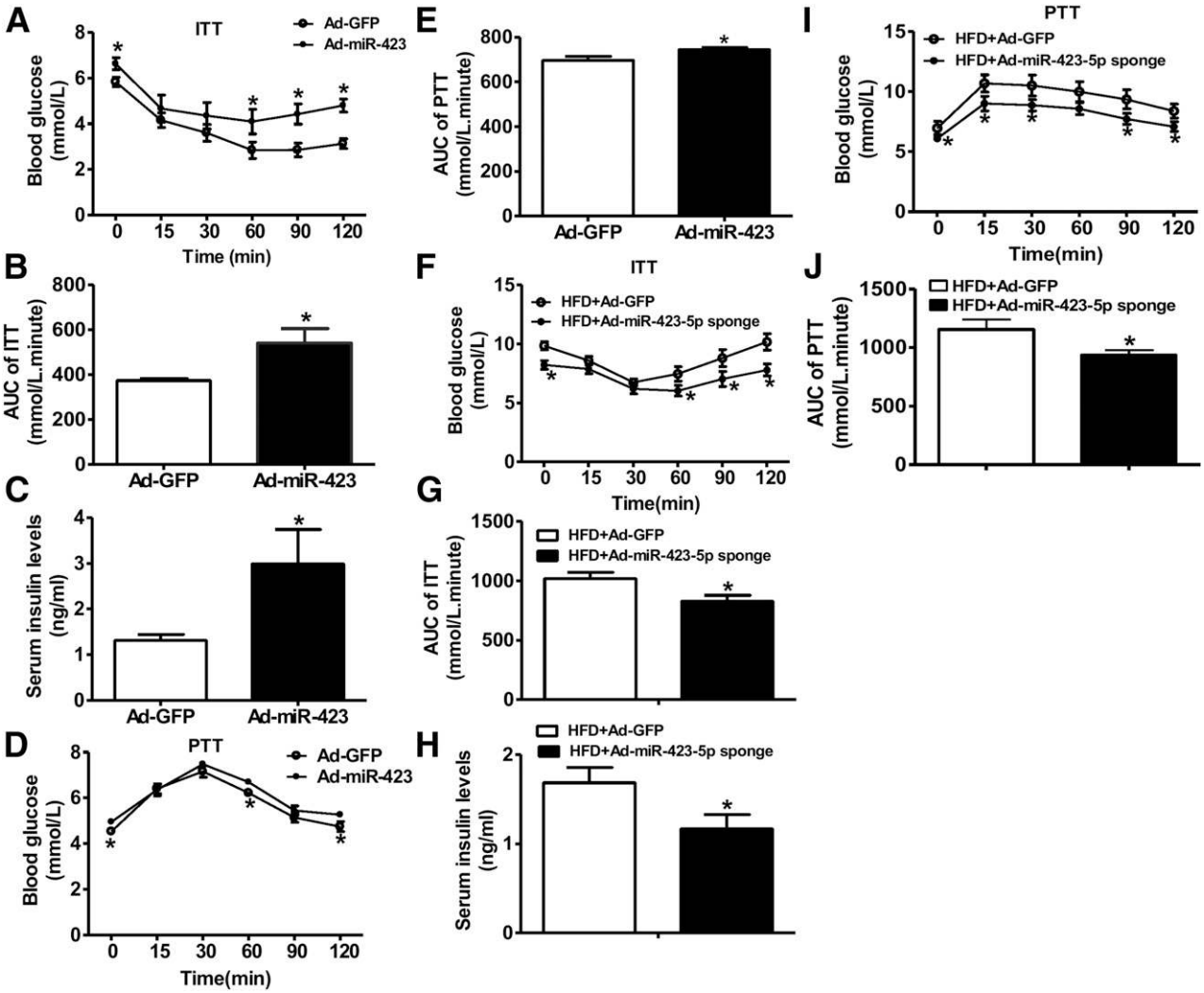

Figure 5—miR-423-5p overexpression or inhibition on global insulin sensitivity and hepatic glucose production. At day 7 after Ad infection, ITTs and PTTs were performed as described in RESEARCH DESIGN AND METHODS. *A*: Hepatic miR-423 overexpression induced insulin resistance in C57BL/6J mice. *B*: Area under the curve (AUC) of ITT data presented in panel *A*. *C*: miR-423 overexpression elevated serum insulin levels in fed states. *D*: miR-423 overexpression increased hepatic glucose production. *E*: AUC of the data presented in *D*. *F*: Inhibition of miR-423-5p increased insulin sensitivity in HFD mice. *G*: AUC of ITT data presented in panel *F*. *H*: miR-423-5p inhibition reduced serum insulin levels in fed states. *I*: miR-423-5p inhibition suppressed hepatic glucose production. *J*: AUC of the data presented in panel *I*. n = 14–18 for insulin measurement and n = 6–8 for others. *$P < 0.05$ vs. HFD+Ad-GFP–treated mice.

conditions (31,32). Although miR-423-5p and miR-423-3p are processed from the miR-423 precursor in humans and mice (33), they exert distinct biological functions by targeting different mRNAs. For example, miR-423-3p but not miR-423-5p targets p21Cip1/Waf1 to promote cell growth and cell cycle progression in hepatocellular carcinoma cells (34). Moreover, miR-423-3p but not miR-423-5p promotes the proliferation of breast cancer cells (33). Our findings revealed that miR-423-5p but not miR-423-3p targets FAM3A to repress ATP production and Akt activity in liver cells. Under obese or diabetic condition, miR-423-5p expression in the liver was increased, with reduced FAM3A expression and cellular ATP level observed in our previous studies (4,23). Gain- and loss-of-function studies revealed that miR-423-5p promotes hepatic gluconeogenesis and lipid deposition and induces glucose intolerance, insulin resistance, and hyperglycemia by inhibiting the FAM3A-ATP-Akt pathway in the liver. In our previous study, we found that FAM3A overexpression repressed FAS expression in obese diabetic mouse livers (4). Because FOXO1 has been reported to directly regulate FAS gene expression beyond its regulatory roles on PEPCK and G6Pase in liver cells (35), it is likely that FAM3A activates Akt to repress FOXO1 activity and FAS expression in obese diabetic mouse livers. Consistent with the change of FAM3A expression, miR-423 overexpression or miR-423-5p knockdown increased or decreased FAS expression in mouse livers.

Increased lipolysis in adipose tissues resulting from insulin resistance also plays important roles in the development of fatty liver by promoting lipid transfer from adipose tissues to

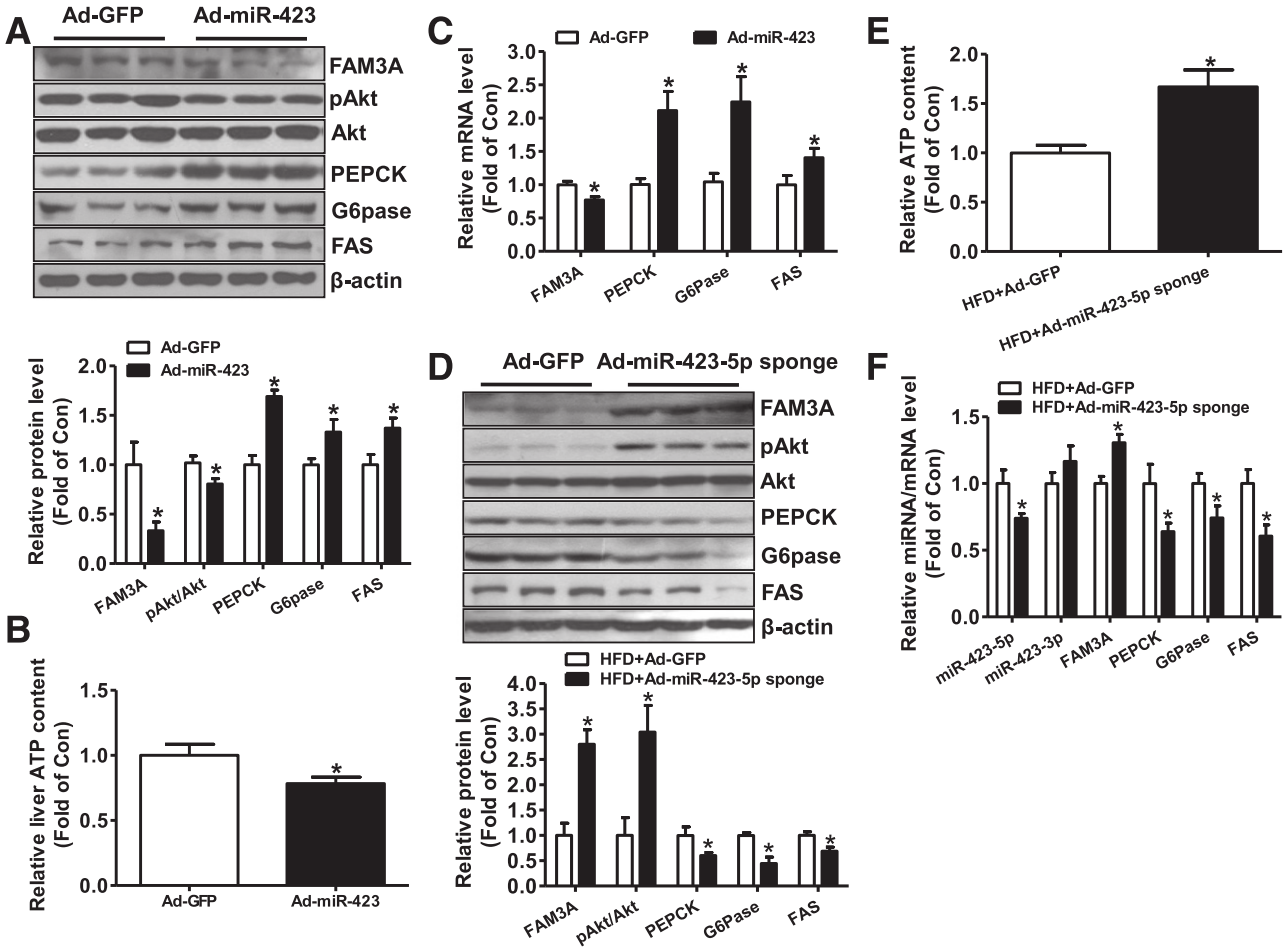

Figure 6—miR-423-5p overexpression or inhibition on the expression of FAM3A and other metabolic genes in mouse livers. *A*: miR-423 overexpression on the protein levels of glucose and lipid metabolizing genes in normal mouse livers. Representative gel images are shown in the upper panel and quantitative data in the lower panel. *B*: miR-423 overexpression reduced cellular ATP content in normal mouse livers. *C*: miR-423 overexpression on the mRNA levels of glucose and lipid metabolizing genes in normal mouse livers. *D*: miR-423-5p inhibition on the protein levels of glucose and lipid metabolizing genes in HFD mouse livers. Representative gel images are shown in the upper panel and quantitative data in the lower panel. *E*: miR-423-5p inhibition increased cellular ATP content in HFD mouse livers. *F*: miR-423-5p inhibition on the mRNA levels of glucose and lipid metabolizing genes in HFD mouse livers. n = 8–10. $^*P < 0.05$ vs. Ad-GFP–treated control mice.

liver (36). Hepatic miR-423-5p activation induces global insulin resistance to enhance the lipolysis of epididymal adipose tissue and increase serum FFAs levels, which should increase FFAs influx into the liver and contribute much to hepatic lipid deposition. Collectively, miR-423-5p promotes hepatic lipid deposition by stimulating de novo FFA synthesis and increasing lipid transfer from adipose tissue to the liver (Fig. 8*I*). One recent study revealed that polymorphism (rs6505162) of miR-423 is associated with abnormal HDL levels in patients with angiographic coronary artery disease (37), further supporting an important role of miR-423-5p in lipid metabolism. Overall, miR-423-5p plays important roles in regulating hepatic glucose and lipid metabolism by repressing the FAM3A-ATP-Akt signaling pathway. Increased miR-423-5p expression explains hepatic FAM3A repression under the obese and diabetic condition.

The transcriptor NFE2 belongs to the Cap'n'Collar basic leucine zipper (*CNC*-bZIP) gene family, including LCR-F1, Nrf1, Nrf2, and other members (38). NFE2 consists of two subunits, designated as p45 and p18, respectively. Mouse and human p45 protein share a homology of 88% with the identical DNA binding domain (38). So far, although Nrf2 has been shown to regulate glucose metabolism (39), the role of NFE2 in glucose and lipid metabolism remains unknown. Our findings revealed that NFE2 directly induces miR-423-5p to repress the FAM3A-ATP-Akt pathway in liver cells. The NFE2/miR-423-5p axis was activated in the livers of obese mice and in NAFLD patients. In human with steatosis, activation of the hepatic NFE2/miR-423-5p axis was correlated with the fasting blood glucose level. Repression of FAM3A via the upregulated NFE2/miR-423-5p axis provides a novel explanation for decreased hepatic ATP content observed in patients with diabetes and NAFLD patients (40). Interestingly, one recent report revealed that miRNA expression change in tissues is negatively correlated with that in plasma in cancer (41). miR-122 level is reduced

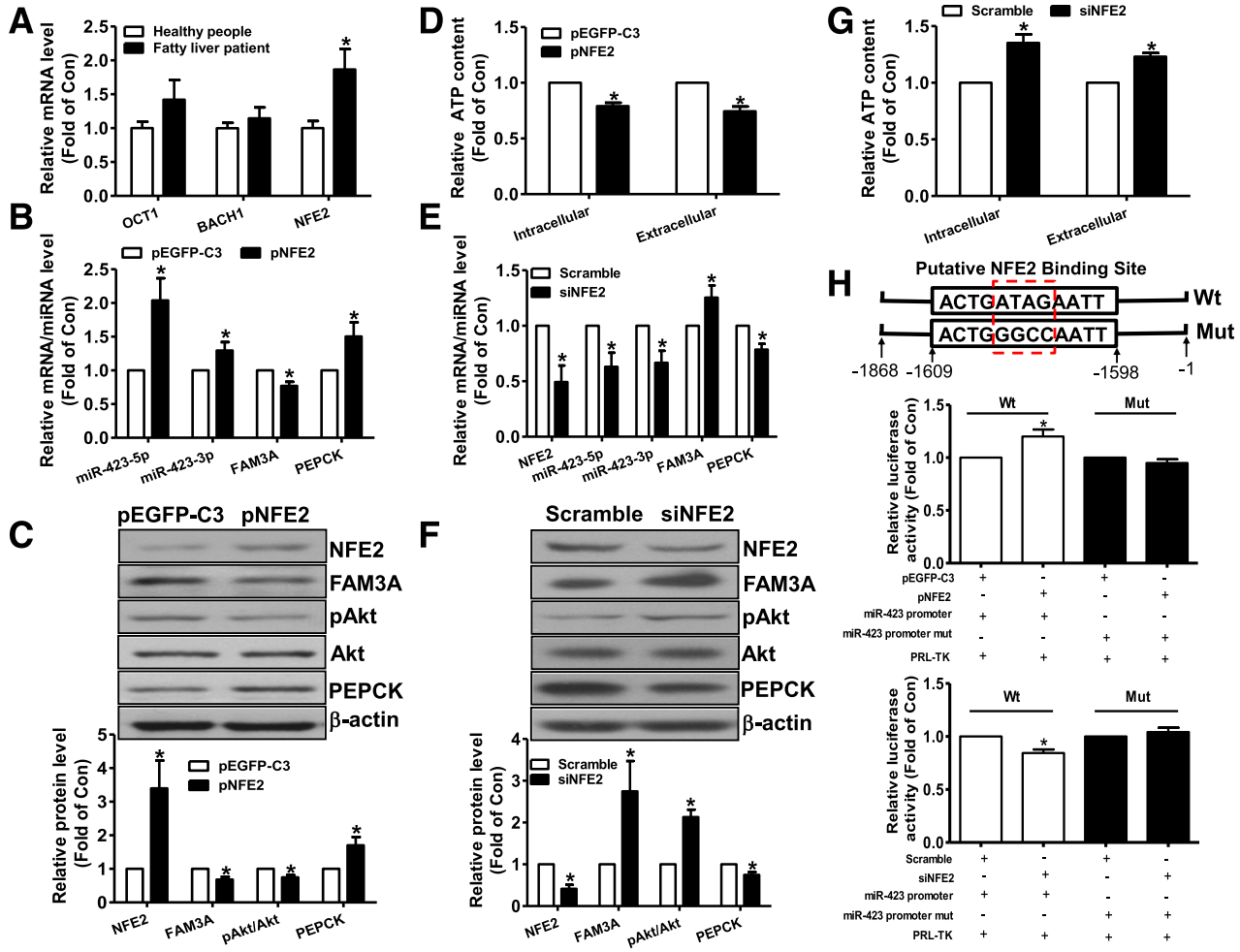

Figure 7—NFE2 induces miR-423 expression to repress the FAM3A-ATP-Akt pathway in liver cells. *A*: NFE2 expression was increased in the livers of NAFLD patients. $n = 6$. $^*P < 0.05$ vs. healthy subjects. *B*: Plasmid overexpression of NFE2 on miR-423-5p and FAM3A mRNA expression in HepG2 cells. *C*: NFE2 overexpression reduced the protein levels of FAM3A and pAkt in HepG2 cells. *D*: NFE2 overexpression reduced intracellular and extracellular ATP levels in HepG2 cells. *E*: siRNA knockdown of NFE2 on miR-423-5p and FAM3A mRNA expression in HepG2 cells. Cells were transfected with 50 nmol/L siNFE2 or scrambled siRNA, and target gene expression was analyzed after 36 h. *F*: NFE2 silencing increased the protein levels of FAM3A and pAkt in HepG2 cells. *G*: NFE2 silencing increased intracellular and extracellular ATP levels in HepG2 cells. *H*: NFE2 overexpression (upper panel) or silencing (lower panel) activated or inhibited the activity of human miR-423 promoter luciferase reporter in HepG2 cells. Mutation of the NFE2 potential binding site completely abolished the regulatory effects of NFE2 on the promoter reporter activity in HepG2 cells. Mut, mutant promoter; Wt, wild-type promoter. $n = 5$. $^*P < 0.05$ vs. control cells.

in liver (42), whereas it is increased in the circulation of patients with nonalcoholic steatohepatitis (43). These findings suggest that the miR-423-5p expression change in the liver is possibly negatively correlated with that in circulation under the diabetic condition. However, the contribution of various tissues to the circulating miR-423-5p level under physiological or diabetic conditions still remains unclear at present. To our knowledge, this is the first report revealing that the NFE2/miR-423-5p axis regulates hepatic glucose and lipid metabolism by repressing FAM3A-ATP-Akt signaling transduction.

The heart has a high rate of ATP production and consumption, and impaired mitochondrial ATP synthesis plays a vital role in the pathogenesis of various cardiac diseases (44,45). ATP content was significantly reduced in the hearts of humans and animals with diabetes (46,47). One recent study revealed that in the hearts of streptozocin-induced type 1 diabetic mice, miR-423 precursor expression was significantly upregulated (48), which was not reversed after normoglycemia with insulin. That repression of FAM3A via miR-423-5p may be involved in diabetic cardiac complications is of great interest. Because chronic exposure to FFAs upregulated NFE2 and miR-423-5p expression in hepatocytes, the NFE2/miR-423-5p axis may also be activated to repress the hepatic FAM3A-ATP-Akt pathway and enhance gluconeogenesis in fasting status, which is beneficial for maintaining blood glucose level. Moreover, given that ATP functions as the main energy

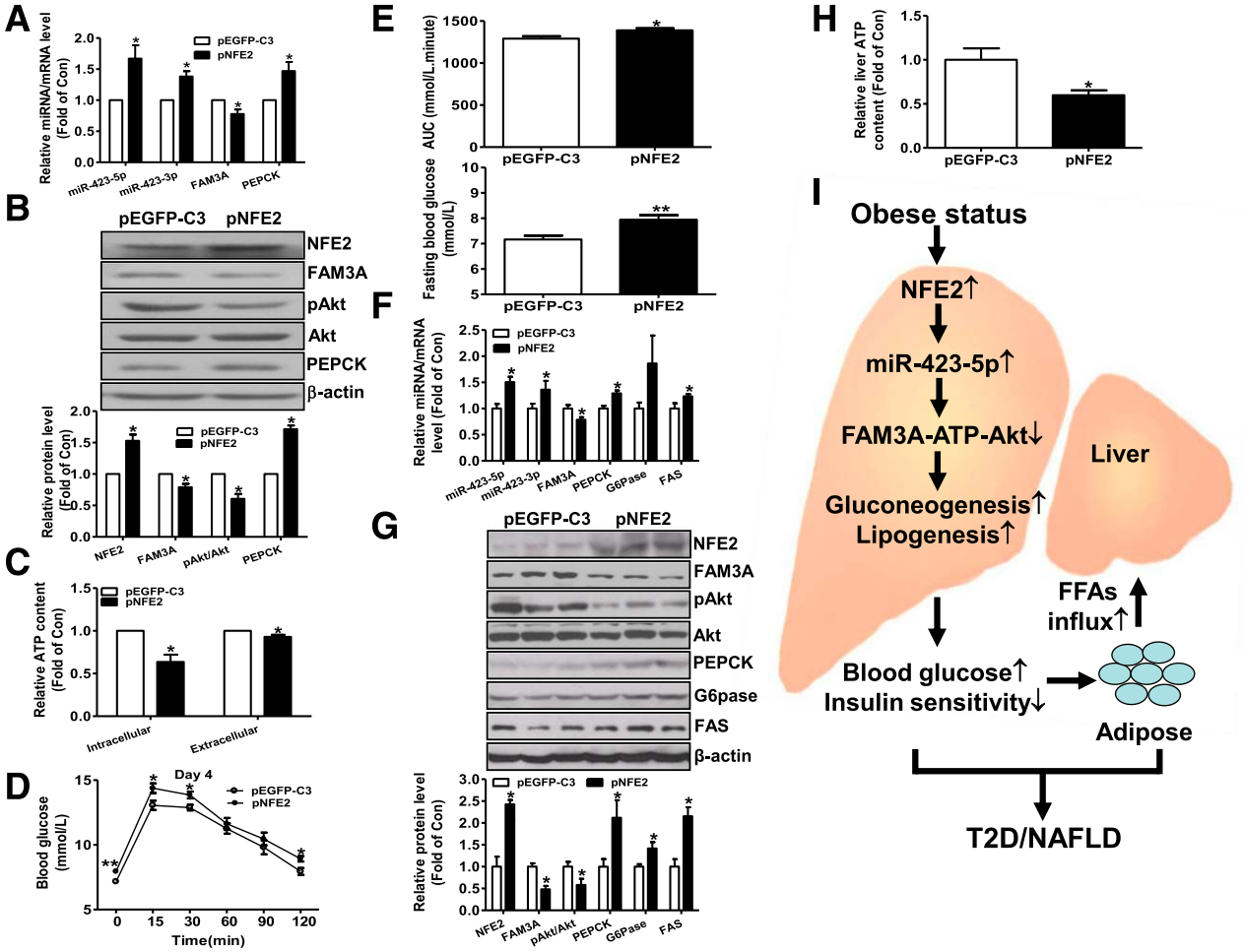

Figure 8—Hepatic activation of NFE2/miR-423-5p axis resulted in dysregulated glucose and lipid metabolism in normal mice. *A*: NFE2 overexpression upregulated miR-423-5p in primary mouse hepatocytes. *B*: NFE2 overexpression reduced FAM3A and pAkt protein levels in primary mouse hepatocytes. *C*: NFE2 overexpression reduced ATP content in primary mouse hepatocytes. $n = 5$. $^*P < 0.05$ vs. control cells. *D*: Hepatic NFE2 overexpression induced glucose intolerance in C57BL/6J mice. Male C57BL/6J mice (8–10 weeks old) were treated with pEGFP-C3 or pNFE2 via tail vein injection of plasmid as detailed in RESEARCH DESIGN AND METHODS. OGTT were performed at 72 h after plasmid injection. *E*: Area under curve (AUC) of OGTT data (upper panel), and fasting blood glucose (lower panel) after NFE2 overexpression. The blood glucose levels at 0 min in panel *D* are presented as fasting blood glucose. $n = 8–10$. $^*P < 0.05$, $^{**}P < 0.01$ vs. pEGFP-C3–treated control mice. *F*: NFE2 overexpression upregulated miR-423-5p expression and repressed FAM3A mRNA expression in mouse livers. *G*: NFE2 overexpression reduced FAM3A and pAkt protein levels in mouse livers. *H*: NFE2 overexpression reduced liver ATP content. $n = 8–10$. $^*P < 0.05$ vs. pEGFP-C3–treated control mice. *I*: Proposed mechanism of hepatic NFE2/miR-423-5p axis in the progression of NAFLD and type 2 diabetes (T2D). Under obese or insulin-resistant conditions, activation of the hepatic NFE2/miR-423-5p axis plays important roles in the progression of NAFLD and T2D by repressing the FAM3A-ATP-Akt pathway to promote gluconeogenesis and lipogenesis. Moreover, hepatic activation of the NFE2/miR-423-5p axis also promotes lipid transfer from adipose tissues to the liver via induction of global insulin resistance.

molecule and as an important signaling molecule, the NFE2/miR-423-5p/FAM3A axis is likely involved in the regulation of other physiological processes, such as energy homeostasis, calcium metabolism, and cell proliferation, by modulating ATP production and secretion.

In summary, we present novel data indicating that miR-423-5p targets the FAM3A-ATP-Akt pathway to disturb hepatic glucose and lipid metabolism. Under the obese condition, activation of hepatic NFE2 plays important roles in promoting gluconeogenesis and lipogenesis by inducing miR-423-5p to repress the FAM3A-ATP-Akt pathway (Fig. 8*I*). Inhibiting the hepatic NFE2/miR-423-5p axis may represent a potential strategy for the treatment of type 2 diabetes and NAFLD.

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Author Contributions. W.Y. wrote the manuscript. W.Y., J.W., and Z.C. researched data and contributed to discussion. W.Y., J.W., Z.C., Q.C., and J.Y. designed the study and revised and edited the manuscript. J.C. and Y.M. provided the technical assistance. L.C., Y.C., B.G., Y.G., and Q.C. contributed to discussion and reviewed and edited the manuscript. L.S., L.D., and J.L. provided human liver samples and contributed to discussion. J.Y. is the guarantor of this work and had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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