Mitochondrial protein FAM3A suppresses hepatic gluconeogenesis and lipogenesis. This study aimed to screen drug(s) that activates FAM3A expression and evaluate its effect(s) on hyperglycemia and steatosis. Drug-repurposing methodology predicted that antidepressive drug doxepin was among the drugs that potentially activated FAM3A expression. Doxepin was further validated to stimulate the translocation of transcription factor HNF4α from the cytoplasm into the nucleus, where it promoted FAM3A transcription to enhance ATP synthesis, suppress gluconeogenesis, and reduce lipid deposition in hepatocytes. HNF4α antagonism or FAM3A deficiency blunted doxepin-induced suppression on gluconeogenesis and lipid deposition in hepatocytes. Doxepin administration attenuated hyperglycemia, steatosis, and obesity in obese diabetic mice with upregulated FAM3A expression in liver and brown adipose tissues (BAT). Notably, doxepin failed to correct dysregulated glucose and lipid metabolism in FAM3A-deficient mice fed on high-fat diet. Doxepin’s effects on ATP production, Akt activation, gluconeogenesis, and lipogenesis repression were also blunted in FAM3A-deficient mouse livers. In conclusion, FAM3A is a therapeutic target for diabetes and steatosis. Antidepressive drug doxepin activates FAM3A signaling pathways in liver and BAT to improve hyperglycemia and steatosis in obese diabetic mice. Doxepin might be preferentially recommended as an antidepressive drug in potential treatment of patients with diabetes complicated with depression.

Thus far, type 2 diabetes has become a severe public health issue affecting more than 400 million people worldwide (1). Excessive hepatic gluconeogenesis due to insulin resistance or deficiency plays crucial roles in the development of fasting hyperglycemia and diabetes (2). Nonalcoholic fatty liver disease (NAFLD) is highly associated with increased hepatic gluconeogenesis (3), and gluconeogenic inhibitors have been shown to improve NAFLD (3). Clearly, exploring new drugs that suppress hepatic gluconeogenesis independent of insulin holds great promise for treating diabetes with severe insulin resistance.

Member A of family with sequence similarity 3 (FAM3) family (FAM3A) is a new mitochondrial protein that enhances the production and release of ATP in hepatocytes (4,5). FAM3A-induced release of ATP activates a P2 receptor–calmodulin–Akt pathway independent of insulin to suppress hepatic gluconeogenesis. FAM3A also represses lipogenesis and increases lipid oxidation in hepatocytes (4,5). FAM3A expression is significantly reduced in the livers of obese mice and NALFD patients (4,5). Hepatic overexpression of FAM3A markedly improved hyperglycemia and steatosis in obese diabetic mice, while hepatic inhibition of it caused hyperglycemia and lipid deposition in normal mice (4,5). FAM3A also exerts beneficial effects on oxidative stress, endoplasmic reticulum stress, and inflammation in various cell types (6–8). Clearly, these findings have revealed that FAM3A is a promising therapeutic target for diabetes and steatosis (9).
Drug repurposing is to explore the new indications of frontline drugs beyond their original roles. As of recently, the drugs discovered by this methodology had accounted for ~30% of all drugs issued by the U.S. Food and Drug Administration (10,11). Drug repurposing represents a next-generation method of drug discovery (12,13). Although agonists of peroxisome proliferator–activated receptor γ (PPARγ) induce FAM3A expression (14,15), they have some significant side effects such as fluid retention, bone fracture, and body weight gain (16). Further screening of drugs that activate FAM3A expression will shed light on the treatment of type 2 diabetes.

We first screened drugs that potentially activated FAM3A expression among frontline drugs based on data sets in Connectivity Map (CMap) (17). Twenty-five drugs were predicted to induce FAM3A expression. Interestingly, doxepin and diphenylpyraline, which belong to histamine 1H receptor (H1R) antagonists, are tricyclic antidepressive drugs used for treating insomnia, depressive, and anxious disorders (18,19). Reference mining revealed that there was a strong association among type 2 diabetes, NAFLD, and depression (20,21). Patients with diabetes are at a higher risk of depression than healthy subjects, whereas depression is also a high risk factor of diabetes and liver injury (22–25). In a case report, therapy with doxepin induced profound hypoglycemia in patients with diabetes taking sulfonylureas (26). A single dose of doxepin produced significant hypoglycemia, which lasted up to 10 h in albino rabbits. However, chronic doxepin administration reversed the initial hypoglycemia on the 7th and 14th days and finally caused hyperglycemia on the 21st day. In the same study, long-term treatment with doxepin was shown to increase insulin sensitivity in rats (27). Collectively, these findings suggested that doxepin...
may regulate glucose metabolism, but its precise role and underlying mechanism remain to be revealed. To probe whether doxepin activated FAM3A pathways to regulate glucose and lipid metabolism under diabetic condition would provide insight into the treatment of patients with diabetes complicated with depression.

This study aimed to determine whether doxepin activated FAM3A signaling pathways to improve hyperglycemia and steatosis in obese diabetic mice.

**RESEARCH DESIGN AND METHODS**

**Drug Repurposing Based on the CMap Database**

The traditional drug screening is to finding small molecules that can dock with a candidate target for one disease, which is mainly using docking algorithms for protein targets and small molecules based on their structures. Recently, drug repurposing has come to represent one class of novel strategies for drug screening, which aims to find new use for existing drugs and provides efficient solutions to the above problems in the traditional strategy. Because gene expression profiles directly reflect the biological activity of drugs, they show great potential in drug purposing. The CMap database (17) shows great potential to find drugs or drug combinations affecting candidate diseases or candidate genes. Here, we identified the drugs that upregulated FAM3A at least twofold as potential functional activators of FAM3A based on data sets in CMap v2.0. The flowchart for drug screening was as shown in Fig. 1A.

**Animal Treatment**

Eight- to ten-week old male wild-type (WT) and FAM3A−/− mice on a C57BL/6 background were used. FAM3A−/− mice were generated using TALEN technology in our previous study (6). Mice were fed on high-fat diet (HFD) (Medicience, Jiangsu, China) for 12 weeks (4). The mice were daily treated with doxepin (5 or 10 mg/kg body wt) or vehicle (sterile 0.9% NaCl) by intraperitoneal injection. db/db mice were treated with 10 mg/kg body wt doxepin (cat. no. DER-C13084200; Dr. Ehrenstorfer). FAM3A−/− mice were treated with doxepin at 5 mg/kg body wt. Animal experiments were conducted in accordance with the Guide for the Care and Use of the Laboratory Animals of Peking University.

**Cell Culture and Treatment**

HepG2 cells or mouse hepatocytes were treated with various drugs (diphenpyraline 30 μmol/L, doxepin 30 μmol/L, chlorzoxazone 10 μmol/L, aspirin 10 μmol/L, ampyrone 50 μmol/L) for 48 h, respectively. Rosiglitazone (12.5 μmol/L) was used as a positive control. All cell lines were routinely tested for mycoplasma presence using a PCR detection kit (MP0035; Sigma-Aldrich).

**Confocal Analyses of FOXO1 and HNF4α Nuclear Exclusion**

Cells treated with drugs were permeabilized with 0.2% Triton X-100/0.5% BSA, followed by washing with PBS. The coverslips were blocked in 1% BSA for 30 min at 37°C. The coverslips were incubated with anti-FOXO1 or anti-hepatic nuclear factor 4α (HNF4α) antibodies at 4°C overnight and then washed with PBS, followed by detecting with goat anti-rabbit Alexa Fluor 594 (cat. no. ZF-0516; ZSGB-BIO). Mounted coverslips were imaged and cells were visualized by fluorescence microscopy using a confocal laser scanning microscope. The procedure for gluconeogenesis assay in cultured hepatocytes has previously been described (28).

**Western Blotting**

Cells or tissues were lysed in radioimmunoprecipitation assay lysis buffer containing protease inhibitors and 40–100 μg proteins was separated by SDS-PAGE. The membranes were incubated with primary antibodies overnight at 4°C, followed by washing with TBS and incubating with a horseradish peroxidase–conjugated secondary antibody for 2 h. Proteins were visualized with enhanced chemiluminesence technique. Anti–phosphorylated (p)Akt antibody (phosphorylation at Ser473 site) (cat. no. 2880), anti-Akt antibody (9272), anti-pFOXO1 antibody (9461), and anti-FOXO1 antibody (2880) were purchased from Cell Signaling Technology. Anti-G6Pase antibody (sc-25840) and anti-PPARγ antibody (sc-7273) were purchased from Santa Cruz Animal Health. Anti-FAM3A antibody (SAB1102488) was purchased from Sigma-Aldrich. Anti-FAS antibody (ab22759) was purchased from Abcam. Anti-HNF4α antibody (A2085) was purchased from Bclonal. Anti–PEPCK antibody (BS6870) was purchased from Bioworld. Anti–β-actin antibody (B1029) was purchased from Biodragon.

**RNA Extraction and Real-time PCR Assays**

Total RNA from HepG2 cells or tissues was prepared using TRizol (Invitrogen) in accordance with the manufacturer’s recommendations. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (TransGen Biotech). All PCR primers are listed in Supplementary Table 1.

**ATP Measurement**

ATP was assayed by bioluminescence assay using an ATP Assay Kit (Vigorous Biotechnology Beijing Co., Ltd.). For determination of intracellular and extracellular ATP content, the absolute ATP values were measured and normalized to protein content and then normalized to control values (4,29). Intracellular ATP was referred to ATP content within the cells, and extracellular ATP meant ATP content in the medium of cultured cells.

**Metabolic Phenotype Analyses**

Oral glucose tolerance tests (OGTTs), insulin tolerance tests (ITTs) and pyruvate tolerance tests were performed as previously described (4,5,29). Generally, ITTs were performed after 4.5 weeks of treatment and PTT were performed after 5 weeks of treatment.

**Metabolic Cage Analyses**

Mice treated with drugs for 6 weeks were individually housed in sealed chambers and maintained at 22°C to
monitor metabolic activity. Animals were acclimated to the chambers for 24 h before data collection. Calorimetry was measured for 1 day. Mice were provided with HFD diet as mentioned above. Activity monitoring and detection of animal location were performed with infrared sensor pairs arranged in strips for horizontal and vertical activity, detecting every ambulatory movement. The sum of the spontaneous physical activity and the high-frequency activity (equivalent of breathing activity) represents the total activity. Energy expenditure (EE) was calculated using the Comprehensive Laboratory Animal Monitoring System. In detail, EE = (3.815 + (1.232 • RQ)) • oxygen consumption • 1.44.

**Cold Stress Experiments**
Mice were placed in an artificial climate box at 4°C with a free diet. The core body temperatures were measured at the start of the experiments and every 1 h using a rectal probe (ThermoWorks). The infrared thermography was performed with an infrared camera (T640; FLIR) after mice were placed in a 4°C cold room for 4–5 h (30).

**Luciferase Activity Assay**
HepG2 cells were plated into sixwell plates and incubated for 24 h before plasmid transfection. Mouse FAM3A-luciferase reporter was constructed previously (14) (2 μg) or pGL3-basic luciferase reporter (2 μg) was cotransfected with human HNF4α plasmid (2 μg, NM_000457) or GFP plasmid (2 μg) as control into cells using VigoFect transfection reagent (Vigorous Biotechnology Beijing Co., Ltd.). Luciferase activity was measured using the Dual-Luciferase Reporter Assay kit (Promega).

**Chromatin Immunoprecipitation**
The protocol for chromatin immunoprecipitation was described in our previous study (28). The DNA was extracted using a microextraction method with phenol, chloroform, and isoamyl alcohol. The same volume of DNA eluted in bidistilled water was used for real-time PCR assays. The relative change of doxepin group compared with non–drug-treated group was calculated as follows, where Ct is cycle threshold:

\[
\text{Doxepin group: } 2^{\Delta\Delta Ct} = \frac{2^{\Delta Ct(\text{Input})} - 2^{\Delta Ct(\text{IgG})}}{2^{\Delta Ct(\text{Input})} - 2^{\Delta Ct(\text{IgG})}} \\
\text{Nondrug group: } 2^{\Delta\Delta Ct} = \frac{2^{\Delta Ct(\text{Input})} - 2^{\Delta Ct(\text{IgG})}}{2^{\Delta Ct(\text{Input})} - 2^{\Delta Ct(\text{IgG})}}
\]

The primer sequences for the chromatin immunoprecipitation assays are listed in Supplementary Table 2.

**Cell Treatment With HNF4α Antagonist BI6015**
Cells were treated with diphenylpyraline 30 μmol/L or doxepin 30 μmol/L in the absence or presence of HNF4α antagonist BI6015 (cat. no. 93987-29-2, 20 μmol/L for HepG2 cells or 60 μmol/L for primary mouse hepatocyte; Cayman Chemical) with the same volume of DMSO as control.

**Cells Neutral Lipid Stains**
HepG2 cells plated on coverslips were treated with FFAs (0.1 mmol/L oleic acid and 0.2 mmol/L palmitic acid) in the absence or presence of drugs. Cells were washed with PBS twice after 24-h treatment and fixed with 4% paraformaldehyde for 10 min. We diluted the LipidTOX neutral lipid stain (Invitrogen) 1:1,000 in buffer to make a 1× solution. For every coverslip, 100 μL solution is required for 30 min at room temperature. After nuclear staining with DAPI, coverslips were mounted on glass slides using 50% glycerol in PBS. Mounted coverslips were imaged and cells were visualized by fluorescence microscopy using a confocal laser scanning microscope. For quantitative determination of triglyceride (TG) content in cells, treated hepatocytes were lysed in lysis buffer (TG determination kit, cat. no. E1013; Applygen Technologies, Beijing, China), and TG content was measured according to the manual of the kit. The TG content (μmol) was normalized to the protein content in the same sample (μmol/mg protein).

**Statistical Analyses**
The results are presented as the mean ± SD. Statistical significance of differences between groups was analyzed by t test or ANOVA. P values <0.05 were considered statistically significant.

**Data and Resource Availability**
All data generated or analyzed during this study are included in in this published article and its Supplementary Data.

**RESULTS**

**Screening Drugs That Potentially Activate FAM3A Expression**
We screened the CMap data for drugs that potentially upregulate FAM3A expression by at least twofold. As a result, 25 drugs were predicted to activate FAM3A expression (Fig. 1A). Among these predicted drugs, doxepin and diphenylpyraline are antidepressive drugs. Given the strong association between type 2 diabetes and depression (20,21), doxepin and several other drugs were selected for further evaluating their effects on FAM3A expression in hepatocytes. Although aspirin was not among the predicted targets, it had also been selected for further validation in hepatocytes because our previous study revealed that PGE2-EP2 axis repressed FAM3A expression in vascular smooth muscular cells (31). Moreover, rosiglitazone was used as a positive control because FAM3A was a target gene of PPARγ (14).

**Doxepin Induced FAM3A Expression to Suppress Gluconeogenesis and Lipogenesis in Cultured Hepatocytes**
H1R mRNA is widely expressed in metabolic tissues with the highest expression in hypothalamus (Supplementary Fig. 1A). In HepG2 cells, chloroxazone, doxepin, diphenylpyraline, ampyrone, and aspirin activated FAM3A promoter activity, whereas glibenclamide and clofibrate failed to do so (Supplementary Fig. 1B). Treatment with chloroxazone, doxepin, diphenylpyraline, ampyrone, and aspirin increased FAM3A mRNA and protein levels, pAkt level, and
ATP production in HepG2 cells (Supplementary Fig. 1C–F). Given that doxepin exhibited the strongest effects on inducing FAM3A expression, ATP production, and Akt phosphorylation than other drugs in HepG2 cells (Supplementary Fig. 1B–E), had a higher prediction score than diphenylpyrrole, and was also previously shown to likely regulate glucose metabolism (26, 27), this study was focused on demonstrating the roles and mechanisms of doxepin in regulating FAM3A signaling pathway and glucose/lipid metabolism. Dose and time course experiments confirmed that doxepin induced FAM3A expression in HepG2 cells (Fig. 1B and C). Doxepin induced FOXO1 phosphorylation, promoted FOXO1 nuclear exclusion, and repressed gluconeogenic gene expression and gluconeogenesis in HepG2 cells (Fig. 1D–F). Doxepin reduced TG deposition induced by free fatty acids (FFA) in HepG2 cells (Fig. 1G and H). Doxepin similarly upregulated FAM3A expression, induced Akt phosphorylation, stimulated ATP production and FOXO1 phosphorylation, and inhibited gluconeogenic gene expression in primary mouse hepatocytes (Fig. 2A–C). Notably, doxepin stimulated FOXO1 nuclear exclusion and repressed gluconeogenesis in a P2 receptor–dependent manner in mouse hepatocytes (Fig. 2D and E). Moreover, doxepin reduced TG deposition induced by FFA in mouse hepatocytes (Fig. 2F and G).

**Doxepin Treatment Attenuated Steatosis, Hyperglycemia, and Obesity in Obese Diabetic Mice**

For evaluation of the effects of doxepin on glucose and lipid metabolism, HFD and db/db mice were treated with doxepin for 1 month. Doxepin markedly improved glucose intolerance at 2 and 4 weeks posttreatment (Fig. 3A–F). Doxepin treatment also ameliorated insulin resistance and suppressed hepatic glucose production of HFD mice (Fig. 4A and B). Consistently, doxepin treatment reduced serum insulin levels (Fig. 4C). Doxepin at both doses gradually and significantly reduced body weight of HFD mice. After 4 weeks of treatment, the body weight of HFD mice was almost reduced to that of normal mice (Fig. 4D). Doxepin began to reduce fasting blood glucose levels at 1 week posttreatment (Fig. 4E). Doxepin treatment reduced the weight of liver and epididymal adipose tissues and adipose–to–body weight ratio of HFD mice (Fig. 4F and G). Doxepin treatment increased EE and reduced weight of whole body, liver, and adipose tissues in db/db mice (Fig. 5D–G). Oil red O staining and quantitative analyses revealed that doxepin treatment reduced TG content with little effect on CHO content in livers of db/db mice (Fig. 5H and I). In contrast, doxepin treatment had little effect on serum TG and CHO levels in db/db mice (Fig. 5J).

**Doxepin Repressed Gluconeogenic/Lipogenic Gene Expression in Obese Mouse Livers**

In HFD mouse livers, doxepin treatment increased the mRNA and protein levels of FAM3A (Fig. 6A and B). In contrast, doxepin treatment had little effect on FAM3A mRNA level in white adipose (epididymal adipose), muscle, pancreas, and heart tissues of HFD mice (Supplementary Fig. 5A–D). Doxepin treatment reduced FAM3A expression in the brain of HFD mice (Supplementary Fig. 5E and F). In humans, it has been reported that doxepin treatment inhibits the reintake of norepinephrine and increases its circulating level (32). In HFD mice, doxepin treatment increased the circulating level of norepinephrine (Supplementary Fig. 5G). Doxepin treatment reduced serum AST activity with little effect on alanine aminotransferase activity (Supplementary Fig. 5H), suggesting that doxepin improves liver function of obese mice. Doxepin treatment reduced the mRNA levels of PEPCK, glucose-6-phosphatase (G6Pase), SREBP1, and fatty acid synthase (FAS) in HFD mouse livers (Fig. 6C). Doxepin treatment increased Akt phosphorylation with decreased protein levels of PEPCK, G6Pase, and FAS in HFD mouse livers (Fig. 6D). Doxepin treatment increased hepatic ATP content in HFD mouse livers (Fig. 6E). Doxepin treatment also upregulated the mRNA and protein levels of FAM3A and UCP1 in BAT of HFD mice, supporting the increased thermogenic function of BAT (Figs. 4I and 6F and G). Similarly, doxepin treatment increased Akt phosphorylation with reduced protein levels of PEPCK, G6Pase, and FAS in db/db mouse livers (Fig. 6H). Doxepin treatment had little effect on PPARγ expression in the livers of HFD and db/db mice (Fig. 6D and H). Doxepin treatment increased ATP content in db/db mouse livers (Fig. 6I).

**Doxepin Treatment Failed to Ameliorate Hyperglycemia and Steatosis of FAM3A<sup>−/−</sup> Mice Fed on HFD**

For further determination of whether the beneficial effects of doxepin on obesity, hyperglycemia, and steatosis were...
dependent on FAM3A, FAM3A−/− mice were fed on HFD for 3 months and then daily treated with doxepin for 1 month. FAM3A deficiency was confirmed in liver and pancreas by immunohistochemical staining (Supplementary Fig. 6). Doxepin treatment failed to reduce the body weight or fasting blood glucose levels, improve glucose intolerance or insulin resistance, or suppress hepatic glucose production in FAM3A−/− mice (Fig. 7A–E). Doxepin treatment also failed to ameliorate fatty liver and affect serum lipid levels of FAM3A−/− mice (Fig. 7F–H). Doxepin failed to affect ATP content and the mRNA levels of PEPCK, G6Pase, SREBP1, and FAS in FAM3A−/− mouse livers (Supplementary Fig. 7A and B). In cultured FAM3A−/− mouse hepatocytes, doxepin failed to stimulate ATP production and Akt phosphorylation and repressed glucose production and FFA-induced TG deposition (Fig. 7I–L). In support, doxepin failed to promote FOXO1 nuclear exclusion in P2 receptor−dependent manner in mouse hepatocytes (Supplementary Fig. 7C). Overall, FAM3A deficiency abolished doxepin’s beneficial effects on dysregulated glucose and lipid metabolism.

Doxepin Activated HNF4α to Induce FAM3A Transcription

For further probing of the mechanism(s) of drug-induced FAM3A transcription, whether it activated FAM3A expression by inhibiting H1R was first evaluated. The result
indicated that H1R silencing failed to affect FAM3A expression and Akt phosphorylation in the absence or presence of doxepin and diphenylpyraline treatment in HepG2 cells (Supplementary Fig. 8A–D), indicating that they induced FAM3A expression in an H1R-independent manner.

Then, the potential binding sites for important transcription factors in human and rodent FAM3A gene promoters were analyzed. The analysis revealed a potential binding site highly specific for HNF4α in mouse, rat, and human FAM3A gene promoters (Supplementary Fig. 9). Given that HNF4α is a hepatocyte-enriched nuclear transcription factor that plays important roles in regulating glucose and lipid metabolism, and its loss-of-function mutations cause maturity-onset diabetes of the young type 1 (MODY1) in humans (33), whether doxepin activated it to induce FAM3A transcription was determined. First, whether HNF4α directly regulated FAM3A expression was evaluated. As a result, HNF4α overexpression upregulated the mRNA and protein levels of FAM3A, increased ATP production, and activated Akt in HepG2 cells (Supplementary Fig. 10A–C). Antagonism of HNF4α using its specific inhibitor BI6015 repressed FAM3A expression and ATP secretion in the absence or presence of drugs in the HepG2 cells (Supplementary Fig. 10D and E). HNF4α expression in primary mouse hepatocytes is about sevenfold higher than that in HepG2 cells (Supplementary Fig. 10F). In mouse hepatocytes, antagonism of HNF4α also inhibited drug-induced FAM3A upregulation and Akt activation (Fig. 8A). Drug treatment increased nuclear HNF4α distribution with little effect on its total expression (Fig. 8B). In support, doxepin treatment increased nuclear HNF4α but was blocked by BI6015 as evidenced by confocal imaging (Supplementary Fig. 11). Furthermore, doxepin treatment enhanced the binding of HNF4α to FAM3A gene promoter in mouse hepatocytes (Fig. 8C) and augmented the activation of HNF4α on mouse FAM3A gene promoter (Fig. 8D). Inhibition of HNF4α reversed doxepin-induced suppression on glucose production and TG deposition in mouse hepatocytes (Fig. 8E–H).

Figure 3—Administration of doxepin improved glucose intolerance in HFD mice. HFD mice were treated with saline or doxepin for 4 weeks, and OGTTs were performed every 2 weeks. A and B: OGTT before drug treatment. OGTT data shown in panel A and area under the curve (AUC) data shown in panel B. C and D: OGTT after 2-week drug treatment. OGTT data shown in panel C and area under the curve data shown in panel D. E and F: OGTT after 4-week drug treatment. OGTT data shown in panel E and area under the curve data shown in panel F. N = 8–10. *P < 0.05 vs. HFD mice treated with saline. NC, negative control (mice treated with saline).
BAT has higher expressions of FAM3A and UCP1 but lower expression of HNF4α than liver tissue of normal mice (Supplementary Fig. 12A). Doxepin treatment increased the expressions of HNF4α, FAM3A, and UCP1 in BAT (Fig. 6F and G and Supplementary Fig. 12B) but not in white adipose tissue of obese mice (Supplementary Figs. 5A and 12C). In HFD mouse livers, doxepin treatment increased nuclear HNF4α distribution, while it decreased cytosolic HNF4α distribution, without significant effect on its total protein level (Supplementary Fig. 12D). Because doxepin is mainly excreted from the kidney (32), doxepin treatment on renal functions was evaluated in obese mice. Doxepin treatment tended to reduce 24-h protein excretion (Supplementary Fig. 13A). Doxepin increased nitrogen concentration with little effect on creatinine and N-acetyl-β-glucosaminase levels in urine (Supplementary Fig. 13B–D). Doxepin had little effect on 24-h excretion of Na⁺, K⁺, Ca²⁺, and Cl⁻ in urine (Supplementary Fig. 13E–H). Overall, doxepin treatment did not impair renal functions of db/db mice.

**DISCUSSION**

Antidepressive drug doxepin was among the drugs that were predicted to activate FAM3A expression. Given that doxepin had the strongest effects on inducing FAM3A expression and Akt phosphorylation among the tested drugs in hepatocytes, and doxepin was previously shown to likely exert beneficial roles in glucose metabolism
its effects on an FAM3A-ATP-Akt pathway, hyperglycemia, and steatosis were evaluated in cultured hepatocytes and obese mouse models. However, it should be noted that the roles of other predicted drugs on FAM3A expression and glucose/lipid metabolism also deserved further exploration. In 2010, doxepin was approved by the U.S. Food and Drug Administration at very low doses for insomnia (3–6 mg at night) (34). Generally, the moderate doses of doxepin for depression is 75–300 mg/day (18). In animal experiments, the dose of doxepin was used up to 50 mg/kg with a median effective dose (ED$_{50}$) of 5–10 mg/kg in the treatment of diseases such as neuropathic pain (35). Our experimental data did not indicate doxepin had hepatocytotoxicity in vitro (data not shown) or in vivo. In cultured human and mouse hepatocytes, doxepin suppressed gluconeogenesis and lipogenesis by activating an FAM3A signaling pathway. In HFD and db/db mice, doxepin at doses of 5–10 mg/kg exhibited significant effects on improvement of gluconeogenesis, hyperglycemia, and steatosis. In both HFD and db/db mice, doxepin enhanced EE and improved obesity. In obese mouse livers, doxepin treatment repressed gluconeogenic and lipogenic gene expressions. In FAM3A-deficient mice fed on HFD, the beneficial effects of doxepin on hyperglycemia, steatosis, and obesity were abolished. Doxepin also failed to increase ATP content and Akt phosphorylation and repress gluconeogenic/lipogenic gene expression in FAM3A-deficient mouse livers. Doxepin treatment upregulated FAM3A expression in liver and BAT but reduced its expression in the brain of HFD mice. Thus far, the roles of central FAM3A in the regulation of glucose and lipid metabolism remain unknown, and doxepin treatment...
reduced its expression in diabetic mouse brain. Collectively, the roles of central FAM3A in doxepin’s beneficial effects in dysregulated glucose and lipid metabolism still remain unknown. Overall, our findings revealed that chronic treatment with doxepin improved steatosis, hyperglycemia, and obesity of obese mice by mainly activating FAM3A signaling pathways in liver and BAT. Amelioration of hyperglycemia subsequently improved insulin resistance and hyperinsulinemia.

Long-term use of some antidepressants might be associated with increased risk of type 2 diabetes in depressive patients (36). However, it had also been reported that there is no increased incidence of diabetes in depressive patients receiving antidepressants (37). Andersohn et al. (38) found that various tricyclic and tetracyclic antidepressants differently impacted the risk of type 2 diabetes. For example, amitriptyline, clomipramine, nortriptyline, and trimipramine increased, whereas imipramine and mianserin reduced, the risk of diabetes. Doxepin did not increase the incidence of diabetes in depressive patients (38). Another study confirmed that amitriptyline and nortriptyline increased, whereas doxepin did not increase, the incidence of diabetes in depressive patients (39). Our

Figure 6—Effect of doxepin treatment on the expressions of metabolic genes in mouse liver and BATs. A: Doxepin treatment increased FAM3A mRNA level in HFD mouse livers. B: Doxepin (10 mg/kg) treatment increased FAM3A protein level in HFD mouse livers. Representative gel images shown in upper panel and quantitative data in lower panel. C: Effect of doxepin treatment on the mRNA levels of gluconeogenic and lipogenic genes in HFD mouse livers. D: Effect of doxepin (10 mg/kg) on the protein levels of gluconeogenic and lipogenic genes in HFD mouse livers. Representative gel images shown in upper panel and quantitative data in lower panel. E: Doxepin treatment increased hepatic ATP content in HFD mouse livers. F: Doxepin treatment increased the mRNA levels of FAM3A and UCP1 in BAT of HFD mice. N = 6–10. *P < 0.05 vs. HFD mice treated with saline. G: Doxepin treatment increased the protein levels of FAM3A and UCP1 in BAT of HFD mice. N = 4. *P < 0.05 vs. HFD mice treated with saline. H: Doxepin treatment increased FAM3A protein level and reduced gluconeogenic/lipogenic protein levels in db/db mouse livers. Representative gel images shown in upper panel and quantitative data in lower panel. I: Doxepin treatment increased hepatic ATP content in db/db mouse livers. N = 5–9. *P < 0.05 vs. db/db mice treated with saline. Con, control; NC, negative control (mice treated with saline).
findings revealed that doxepin exerted a beneficial effect on fatty liver, hyperglycemia, and obesity in FAM3A-deficient mice fed on HFD. FAM3A−/− mice were fed on HFD for 3 months and then treated with doxepin (5 mg/kg). A: Doxepin treatment failed to reduce the body weight of FAM3A−/− mice. B: Doxepin treatment failed to ameliorate fasting hyperglycemia of FAM3A−/− mice. C: Doxepin treatment failed to ameliorate glucose intolerance in FAM3A−/− mice. OGTT data shown in upper panel and area under the curve (AUC) data shown in lower panel. D: Doxepin treatment failed to ameliorate insulin resistance in FAM3A−/− mice. ITT data shown in upper panel and area under the curve data shown in lower panel. E: Doxepin treatment failed to suppress hepatic glucose production in FAM3A−/− mice. PTT data shown in upper panel and area under the curve data shown in lower panel. F and G: Doxepin treatment failed to attenuate fatty liver in FAM3A−/− mice. Representative oil red O staining images shown in panel F and quantitative determination of TG and CHO content in panel G. H: Doxepin had little effect on serum TG and CHO levels, N = 7–10. *P < 0.05 vs. FAM3A−/− mice treated with saline. I–K: Doxepin failed to increase ATP production (I), activate Akt (J), and suppress glucose production (K) in FAM3A−/− mouse hepatocytes. L: Doxepin failed to reduce FFA-induced lipid deposition in FAM3A−/− mouse hepatocytes. The quantitative data of TG content was displayed as numbers under the images. In panels I–L, the data were collected from three to five independent experiments using different FAM3A−/− mice. Con, control; NC, negative control (mice treated with saline); pro, protein.

The common side effects of doxepin include dizziness, headache, drowsiness, restlessness, confusion, gastrointestinal upset, nausea, increased appetite, and other diseases have been reported in humans (42). Moreover, doxepin-induced increase in circulating norepinephrine
level will cause vasoconstriction, which may potentially trigger hypertension and cardiovascular diseases in case of long-term usage (43). In potential clinical use in treating patients with diabetes and with depression, although doxepin avoids the side effects of PPAR such as obesity, its own side effects should also be taken into consideration.

Loss-of-function mutations of HNF4a will cause MODY1 and late-onset type 2 diabetes in humans (33,44). Moreover, single nucleotide polymorphisms in human HNF4a gene increase the risk of type 2 diabetes (45). HNF4a activity was decreased in obese mouse livers (46), and knockout or inhibition of hepatic HNF4a resulted in lipid deposition, glucose intolerance, and insulin resistance (47–49). In hepatocytes, nonphosphorylated FOXO1 associates with the DNA-binding domain of HNF4a to interfere with its ability to activate target gene transcription, while pFOXO1 dissociates from HNF4a to reverse its repressive effects on HNF4a transcriptional activity (50). In the current study, we demonstrated that doxepin activated HNF4a to induce FAM3A transcription. On one hand, doxepin activated a HNF4a-FAM3A-Akt pathway to suppress hepatic gluconeogenesis by inactivating FOXO1. Although HNF4a activation could induce gluconeogenic gene expression (51), this effect is likely overwhelmed by FOXO1 inactivation in case of doxepin-induced activation of FAM3A-Akt signaling pathway in hepatocytes. On the other hand, dissociation of

Figure 8—Doxepin enhanced the binding of HNF4a with FAM3A gene promoter. A: Inhibition of HNF4a blocked drug-induced FAM3A upregulation and Akt phosphorylation in primary mouse hepatocytes. B: Drug effect on the cytosolic and nuclear distribution of HNF4a protein level in mouse hepatocytes. Lamin-B1 and β-actin were used as biomarkers for nuclear and cytosolic fractions, respectively. C: Doxepin enhanced the binding of HNF4a to FAM3A gene promoter in mouse hepatocytes. D: Doxepin augmented HNF4a activation on the promoter activity of mouse FAM3A gene in HepG2 cells. E: Inhibition of HNF4a reversed doxepin-induced suppression on glucose production in primary mouse hepatocytes. F and G: Inhibition of HNF4a antagonized the beneficial effects of doxepin on FFA-induced lipid deposition in mouse hepatocytes. Representative confocal images shown in panel F and quantitative data of TG content in panel G. N = 3–5. *P < 0.05 vs. control cells or between two indicated groups; #P < 0.05 vs. cells treated with drugs. H: Proposed model of doxepin in ameliorating diabetes and steatosis. In hepatocytes, doxepin treatment stimulated the translocation of HNF4a from the cytoplasm into the nucleus, where it bound to FAM3A gene promoter and induced its transcription, finally activating an FAM3A signaling pathway to suppress gluconeogenesis and lipogenesis. In BAT, doxepin treatment activated the expressions of HNF4a, FAM3A, and UCP1, increasing EE. In summary, doxepin activated FAM3A signaling pathways in liver and BAT to ameliorate hyperglycemia, steatosis, and obesity. Con, control.
FOXO1 from HNF4α triggered by an FAM3A-Akt pathway may increase HNF4α transcriptional activity on lipid metabolic genes, likely contributing to the amelioration of steatosis. So far, the mechanism of doxepin-induced translocation of HNF4α from the cytoplasm into the nucleus remains unrevealed. Because the promoter of mouse UCP1 gene contains several potential binding sites for HNF4α (prediction data not shown), it is likely that doxepin activates HNF4α to induce the expressions of both FAM3A and UCP1 in BAT. Moreover, given that norepinephrine stimulated UCP1 expression in BAT (52), increase in circulating norepinephrine level should also contribute to doxepin-induced UCP1 expression in BAT.

In summary, FAM3A is a novel target for the treatment of diabetes and fatty liver. Antidepressive drug doxepin exerts beneficial effects on hyperglycemia and steatosis by activating FAM3A signaling pathways in liver and BAT of obese diabetic mice (Fig. 8F). In clinical treatment of patients with diabetes complicated with depression, doxepin might be preferentially recommended as an antidepressive drug. However, cautions should also be taken to avoid hypoglycemia if insulin/insulin secretagogues and doxepin are potentially prescribed together to patients with diabetes and with depression.

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