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RESEARCH ARTICLE

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FAM3A plays crucial roles in controlling PDX1 and insulin expressions in pancreatic beta cells

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Abstract

So far, the mechanism that links mitochondrial dysfunction to PDX1 inhibition in the pathogenesis of pancreatic β cell dysfunction under diabetic condition remains largely unclear. This study determined the role of mitochondrial protein FAM3A in regulating PDX1 expression in pancreatic β cells using gain- and loss-of function methods in vitro and in vivo. Within pancreas, FAM3A is highly expressed in β, α, δ, and pp cells of islets. Islet FAM3A expression was correlated with insulin expression under physiological and diabetic conditions. Mice with specific knockout of FAM3A in islet β cells exhibited markedly blunted insulin secretion and glucose intolerance. FAM3A-deficient islets showed significant decrease in PDX1 expression, and insulin expression and secretion. FAM3A overexpression upregulated PDX1 and insulin expressions, and augmented insulin secretion in cultured islets and β cells. Mechanistically, FAM3A enhanced ATP production to elevate cellular Ca^{2+} level and promote insulin secretion. Furthermore, FAM3A-induced ATP release activated CaM to function as a co-activator of FOXA2, stimulating PDX1 gene transcription. In conclusion, FAM3A plays crucial roles in controlling PDX1 and insulin

Abbreviations: AMPK, adenosine 5'-monophosphate (AMP)-activated protein kinase; ATP, adenosine triphosphate; CaM, calmodulin; CPZ, chlorpromazine hydrochloride; ER, endoplasmic reticulum; FAM3, family with sequence similarity 3; FAM3A, family with sequence similarity 3, member A; FBS, fetal bovine serum; FFAs, free fatty acids; FOXA2, forkhead box protein A2; GSIS, glucose-stimulated insulin secretion; HBSS, Hank's balanced salt solution; HNF-3β, hepatocyte nuclear factor 3-beta; iPSCs, pluripotent stem cells (iPSCs); ITT, insulin tolerance test; KRB, Krebs-ringer bicarbonate buffer; MafA, V-maf musculoaponeurotic fibrosarcoma oncogene homolog A; NEUROD1, neuronal differentiation 1; Ngn3, neurogenin3; OGTT, oral glucose tolerance test; Pax6, paired box protein 6; PDX1, pancreas/duodenum homeobox protein 1; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate; PPAR, peroxisome proliferator-activated receptor; PPARγ, peroxisome proliferator-activated receptor γ; PTT, pyruvate tolerance test.

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expressions in pancreatic β cells. Inhibition of FAM3A will trigger mitochondrial dysfunction to repress PDX1 and insulin expressions.

KEYWORDS

FAM3A, FOXA2, mitochondria, pancreatic β cells, PDX1

1 | INTRODUCTION

According to the diabetes atlas released by International Diabetes Federation (IDF) in 2018, it was estimated that diabetes was affecting 451 million people in 2017, and causing millions of death each year worldwide.[1] Type 2 diabetes, which accounts for more than 90% of all diabetic cases, occurs when pancreatic β cells fail to compensate for insulin resistance.[2,3] Clearly, restoring pancreatic β cell functions holds the key for diabetes cure. Mitochondrial adenosine-5′-triphosphate (ATP) production plays the decisive roles in controlling insulin secretory process. After meal, glucose uptake and metabolism increases mitochondrial ATP synthesis, which closes ATP-sensitive potassium channel to open voltage-gated calcium channel and results in influx of extracellular calcium, elevating cellular calcium level to promote the exocytosis of insulin granules.[4] Chronic exposure to high levels of free fatty acids (FFAs), glucose, and inflammatory cytokines represses ATP synthesis and insulin secretion in pancreatic β cells.[5-7] Recently, released ATP was shown to regulate insulin secretion via the activation of P2 receptor (receptor for nucleotides such as ATP and uridine triphosphate (UTP)) on the plasma membrane of pancreatic β cells.[8-10] So far, the mechanism for decreased mitochondrial ATP production under obese and diabetic conditions still needs further exploration. Particularly, the role of extracellular ATP in regulating β cell functions remains largely unclear.

Family with sequence similarity 3 (FAM3) member A (FAM3A) is ubiquitously expressed among tissues of human and rodents.[11] Human and mouse FAM3A genes are located in chromosome Xq28 and chromosome X; X A7.3, respectively. Both human and mouse FAM3A proteins contain 230 amino acid residues and share high sequence similarity.[11] FAM3A is a new mitochondrial protein that enhances ATP production,[12-14] while other members of FAM3 gene family are secretory proteins.[11,14] FAM3A suppresses hepatic gluconeogenesis and lipogenesis by activating protein kinase B (Akt) and adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) pathways, and a decrease in hepatic FAM3A expression causes fasting hyperglycemia and hepatic lipid deposition.[12,13] FAM3A is a target gene of peroxisome proliferator-activated receptor gamma (PPARγ), and it mediates the beneficial effects of PPARγ's activation on liver ischemia/reperfusion injury.[15,16] Beyond liver, FAM3A also regulates the functions of vascular smooth muscular cells (VSMCs) and adipocytes by modulating ATP-Akt pathways.[17,18] FAM3A also protects neuronal cells against apoptosis triggered by oxidative and endoplasmic reticulum (ER) stress.[19,20] So far, whether FAM3A regulates pancreatic β cell functions remains unknown. It is well known that ATP plays a key role in glucose-stimulated insulin secretion (GSIS) in islet β cells. Given the role of FAM3A in promoting ATP synthesis and release in various cell types including hepatocytes, adipocytes, VSMCs, and other cells, it is of interest and importance to study whether FAM3A regulates insulin synthesis and secretion in islet β cells. Demonstrating the roles of FAM3A in the regulation of insulin expression and secretion will provide insight into the mechanism of mitochondrial dysfunction and pancreatic β cell dysfunction under diabetic condition.

Pancreas/duodenum homeobox protein 1 (PDX1) is the master transcription factor that controls pancreatic β cell maturation and function by regulating the expression of several key genes including glucokinase and insulin genes, and a decrease in PDX1 expression is the key characteristics of pancreatic β dysfunction.[21,22] Transcription factor forkhead box protein A2 (FOXA2), also known as hepatocyte nuclear factor 3-beta (HNF-3β), is also critical for maintaining pancreatic cell functions by regulating PDX1 expression.[23-27] Glucose-induced DNA methylation in the promoter region and miRNAs such as miR-141, miR-124a, and miR-342 inhibit FOXA2 expression in pancreatic β cells.[28-30] In pancreatic α cells, memin interacts with FOXA2 to repress its transcriptional activity.[31] In the islets of type 2 diabetic animals and humans, nuclear FOXA2 distribution was decreased.[32] Beyond PDX1 and FOXA2, the transcription factors neuronal differentiation 1 (NEUROD1) and V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) also play important roles in regulating pancreatic β cell functions and insulin expression.[33,34] NEUROD1, PDX1, and MafA have been shown to synergistically activate the transcription of insulin genes.[35,36] Neurogenin3 (Ngn3) is also an important transcription factor controlling mouse pancreas development and β cell maturation.[37] PDX1 could activate Ngn3 and paired box protein 6 (Pax6) to promote differentiation of pluripotent stem cells (iPSCs) into islet β cells.[38] Overall, both PDX1 and FOXA2 play important roles in regulating islet β cell functions via direct or indirect regulation of other transcription factors or genes which are important for maintaining islet β cell functions. However, the

mechanism that links mitochondrial dysfunction to FOXA2 and PDX1 repression under diabetic condition remains unclear. During our preliminary experiment, we observed that the change of PDX1 expression was consistent with that of FAM3A in the serum starvation experiment in vitro. This suggested that FAM3A might regulate insulin synthesis and secretion by affecting the expression of PDX1.

This study determined the role and mechanism of FAM3A in regulating pancreatic β cell functions. Particularly, whether FAM3A activated ATP signaling pathway to modulate the transcriptional activity of FOXA2 and PDX1 expression in pancreatic β cells were stressed.

2 | METHODS

2.1 | Animals

Male 8-12 weeks old mice on a C57BL/6J background and male 8-12 weeks old db/db mice on a C57BKS background were used. All procedures involving experimental animals were approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center.

2.2 | Antibodies

Anti-FAM3A (SAB1102488) and insulin (I2018) antibodies were purchased from Sigma-Aldrich, Inc. (St Louis, MO, USA). Antibodies for insulin (ab181547), glucagon (ab10988), pancreatic peptide (ab77192), and somatostatin (ab30788) were from Abcam. Anti-PDX1 (A3070) and anti-FOXA2 (WH096743) antibodies were purchased from Abclonal (Wuhan, China). Anti-PDX1 (ab47267) for immunofluorescence was purchased from Abcam (Shanghai, China). Anti-CaM (sc-5537) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Goat anti-mice Alexa 594 (ZSGB Biotechnology, China, ZF-0513), goat anti-rat Alexa 594 (Invitrogen, USA, A11007), donkey anti-goat Alexa 594 (Abcam, England, ab150132), donkey anti-rabbit Alexa 488 (Abcam, ab150073), donkey anti-mouse Alexa 647 (Abcam, ab150107), goat anti-rat Alexa 594 (Invitrogen, A11007), and goat anti-rabbit Alexa 488 (Invitrogen, A11034) were used in current study. Rabbit IgG was from BEIJING BIODRAGON IMMUNOTECHNOLOGIES CO., LTD, BF01001.

2.3 | RNA extraction and real-time PCR

Total RNA was isolated with Trizol. For FAM3A and other genes mRNA expression analysis, 1-2 μg of RNA was used to generate cDNA with High Capacity cDNA Reverse Transcription Kit following the manufacturer's protocol and followed by the PCR amplification as described previously.[13] The sequences for the primers used for real time PCR are provided in supplemental Table 1. For the detection of the expression level of miR-423-5p, Bulge-Loop real-time RT-PCR was performed as described previously.[13,39]

2.4 | siRNA transfection

For FOXA2 knockdown, MIN6 cells were treated with 50nM siRNA mixture against mouse FOXA2 mRNA (Beijing Biolino Nucleic Acid Technology Co., Ltd) for 24 hours (The same concentration of scrambled siRNAs was used as negative control). Moreover, MIN6 cells were treated with 50nM siRNA mixture against mouse FOXA2 mRNA (Beijing Biolino Nucleic Acid Technology Co., Ltd) for 6 hours, followed by infection with viruses for 24 hours (The same concentration of scrambled siRNAs was used as negative control). All the siRNA sequences were listed in supplemental Table 2.

2.5 | Fasting-refeeding model

C57BL/6J male mice were randomly divided into three groups. All animals were fed a regular chow diet until the fasting and refeeding treatment started. For fasting group, mice were fasted for 24 hours during a light and dark cycle. For the refeeding group, the mice fasted for 24 hours were refed with a normal diet for 12 hours.

2.6 | Generation of islet β cell specific FAM3A gene knockout (BKO) mice

Two Loxp sites were inserted between the exon 4 and exon 8 by Cyagen Biosciences Inc (China). FAM3A-floxed mice were crossed with Ins2-Cre transgenic mice, and the offspring were self-crossed to obtain islet β cell specific FAM3A knockout mouse which was named as BKO mouse. Ins2-Cre transgenic mice, which also named as RIP-Cre, were obtained from Cyagen Biosciences Inc (China). The RIP-Cre transgene has a 668 bp fragment of the rat insulin II promoter (RIP), nuclear-localized Cre recombinase, and a 2.1 kb fragment of the human growth hormone gene.[40,41] PCR and agarose gel electrophoresis assays were used to characterize mouse genotypes. It had been reported that Ins2-Cre mice (RIP-Cre) exhibited normal insulin secretion and glucose metabolism as flox/flox and wild-type mice,[40,42-45] which had been widely used as control mice in studies involving Ins2-triggered[42,44-49] β cell specific knockout of genes. In the current study, the flox/flox mice had been used as control mice.

2.7 | Metabolic phenotyping

OGTT (Oral Glucose Tolerance Test), ITT (Insulin Tolerance Test), and PTT (Pyruvate Tolerance Test) were performed as described previously.[13,50] To assess the effects of knockdown and knockout FAM3A in pancreas on mice's glucose tolerance and insulin secretion, OGTT was performed and about 20 μL blood was taken from the tail vein at the first 4 time points (0, 15, 30, and 60 minutes) for the determination of insulin level. Plasma insulin levels were measured using the Rat/Mouse Insulin ELISA kit (Millipore, Billerica, MA, USA).[51]

2.8 | Cell culture and treatment

HIT-T15 cells were cultured in RPMI 1640 supplemented with 10% of fetal bovine serum (FBS, Gibco, Carlsbad, CA). MIN6 cells were cultured in DMEM (high glucose) medium supplemented with 15% of FBS. HEK293 cells were cultured at 37°C in DMEM (high-glucose) medium with 10% of FBS. The cells were maintained at 37°C in a humidified atmosphere containing 95% of air and 5% of CO_2. Cells infected with 25 multiplicity of infection (MOI) of Ad-GFP or Ad-FAM3A for 24 hours. For inhibition of the P2 receptor or calcium signaling, the cells were treated with suramin (50 μM), or chlorpromazine hydrochloride (CPZ, 50 μM) for 24 hours before experimental assay. For insulin secretion, the cells were infected with adenovirus for 24 hours, followed by washed once with prewarmed Krebs-Ringer Bicarbonate Buffer (KRB) without glucose, and then incubated in KRB without glucose at 37°C in 5% of CO_2/95% of air for 1 hour. Cells were then incubated in KRB with 0, 5, and 20 mmol/L glucose, respectively, for 1 hour. After incubation, the supernatant was collected for insulin immunoassay.

2.9 | Islet isolation and culture

Ligation of the common bile duct near the liver, puncture from the common bile duct near the duodenum, then mouse pancreas was perfused with about 3-5 mL 0.5 mg/mL collagenase P (Sigma). Then the pancreas was isolated and digested with about 4-5 mL 0.5 mg/mL Collagenase P for about 10 minutes at 38°C, placed in ice to stop digestion, filtered, and centrifuged at 1500 rpm, 2 minutes at 4℃ for 3 times. The precipitate is then resuspended with Hank's Balanced Salt Solution (HBSS) and the islets were picked up under a stereoscopic microscope and the pick-up procedure must be repeated at least three times to ensure the purity of the islets. Islets were then maintained in RPMI 1640 supplemented with 10% of FBS. For GSIS experiment on BKO mouse islet, at 24 hours postisolation, islets were picked up to a low adhesion 24-well plate, 20 islets with the comparable mass were put in each well, and then the islets incubated in KRB without glucose at 37°C in 5% of CO_2/95% of air for 30 minutes. Islets were then transferred to another low adhesion 24-well plate and incubated in KRB with 5mM glucose for 1 hour, the supernatant were collected for insulin and ATP analysis and islets were transferred to another low adhesion 24-well plate and incubated in KRB with 20mM glucose for 1 hour. After incubation, the supernatant was collected for insulin and ATP analysis and islets were lysed for insulin, ATP and protein analysis. In addition, in order to evaluate the effect of FAM3A on the GSIS of WT mouse islet, at 24 hours postisolation, the islets were infected with 25 MOI of Ad-FAM3A or Ad-GFP for 24 hours, and then insulin secretion was determined as above. Three multiple holes were made for each condition, and the average value was taken for calculation. For determination of relative ATP and insulin level in the medium and cells, the ATP and insulin content values were first normalized to the protein mount in the same sample, and then normalized to the control values.

2.10 | ATP content determination

ATP content was assessed by bioluminescence method using ATP-Lite Assay Kit (Vigorous Biotechnology Beijing Co., Ltd) as described previously.[13] For determination of relative ATP level in the medium and cells, the ATP content values were first normalized to the protein mount in the same sample, and then normalized to the control values.

2.11 | Determination of free cellular calcium level

Cells seeded on coverslips were infected with Ad-GFP or Ad-FAM3A for 24 hours, and then loaded with 1 μM Fura-2 AM for 30 minutes, followed by analyzing Ca^{2+} level under Olympus ix71 fluorescence microscope as described previously.[12] Meanwhile, cells were perfused with Tyrode's solution in different concentration of glucose (G0, G5, G20) or KCl. For inhibition of the P2 receptor, L-type calcium channel or calcium signaling, the cells were treated with suramin (50 μM) or CPZ (50 μM) for 1 hours before experimental assay.

2.12 | Luciferase reporter assay

The promoter region of mouse PDX1 gene (−2900bp~+100bp) was cloned into the pGL3-Basic

vector. PDX1 promoter was cotransfected with FOXA2 expression plasmid or pEGFP-C3 in MIN6 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 Gao Yuansheng) was also co-transfected with each condition as a transfection control. At 6 hours post-transfection, change fresh medium, then luciferase activity of Firefly and Renilla luciferase activities were then measured using the Dual-Luciferase Reporter Assay (Promega) after 24 hours. In addition, in order to evaluate the effect of FAM3A and CaM on the activation of PDX1 by FOXA2, at 6 hours post-transfection, change fresh medium, then the cells were infected with 25 MOI of Ad-FAM3A（or Ad-CALM2）or Ad-GFP , then luciferase activity were measured as above.

2.13 | Extraction of mitochondrial/cytosolic fractions

Mitochondrial and cytosolic fractions were isolated from MIN6 and HIT-T15 cells using the Mitochondria/Cytosol Fractionation Kit (Pierce) according to the manufacture's protocol. In brief, cells were homogenized in Mito-Cyto extraction buffer provided by the Kit, and then the lysate was centrifuged at 800 *g* for 5 minutes twice to pellet the nucleus and cell debris. The supernatant was collected and centrifuged at 10 000 *g* for 10 minutes to pellet mitochondria. The supernatant was cytosol fraction. The pellets were resuspended with lysis buffer and washed three times to clean contaminated cytoplasmic proteins.

2.14 | Nuclear and cytoplasmic protein extraction

Nuclear and cytoplasmic fractions were extracted from MIN6 cells according to the manufacturer's instructions using a nuclear extraction kit (Thermo, Prod#78833). The extracted cytosolic fraction and nuclear fraction were analyzed by immunoblotting. β-actin and LaminB1 were used as loading control for cytosolic and nuclear fraction, respectively.

2.15 | Immunoprecipitation and immunoblotting

The immunoprecipitation was performed using Protein G Agarose bead as described previously.[52] For immunoblotting analysis, cells and pancreas were washed twice with ice-cold PBS and total cell lysates were obtained in Roth lysis buffer (50 mM HEPES, 150 mM NaCl, 1% of TritonX-100, 5 mM EDTA, 5 mM EGTA, 20 mM NaF, pH7.4). The concentration of total protein was determined using the BCA protein assay method. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis with antibodies. The protein bands were detected by enhanced chemiluminescent reaction according to the manufacturer's instructions.

2.16 | Immunofluorescent and immunohistochemical staining

Five micrometer pancreas sections were treated with EDTA (pH 8.0) in 95°C for 15 minutes for antigen retrieval. After washed with PBS for 3 times, slides were incubated in 3% of H_2O_2 containing 0.2% of Triton X-100 for 10 minutes. After washing with PBS for 3 times, slides were incubated with corresponding antibody or IgG overnight at 4°C (The detailed information including various primary antibody dilutions was provided in supplemental Table 3). At the second day, secondary antibodies (1:200) were added in, and the slides were incubated for 1 hours at room temperature. The nucleus was stained with DAPI for 10 minutes, and 50% of glycerol in PBS was used to mount the glass slides with coverslips. For immunofluorescent staining, the images were visualized using Confocal Laser Scanning Microscope.

2.17 | Statistical analysis

Data were presented as mean ± S.E.M. Statistical significance of differences between groups was analyzed by *t* test or by one-way analysis of variance (ANOVA) when more than two groups were compared.

3 | RESULTS

3.1 | Pancreatic islet FAM3A expression is correlated with insulin expression under physiological and diabetic conditions

To determine the potential role of FAM3A in regulating pancreatic β cell functions, its expression distribution within pancreas was first determined. Immunohistochemical and immunofluorescent staining revealed that FAM3A protein is highly enriched in islet through mouse and human pancreas (Supplemental Figure 1A-C). In contrast, no significant FAM3A expression was detected in other parts beyond islet in human and animal pancreases (Supplemental Figure 1A-C). Then, the distribution of FAM3A expression within

islet was further determined. Immunofluorescent staining revealed that FAM3A is highly expressed in pancreatic β cells of mouse islets (Figure 1A). Moreover, FAM3A protein is also expressed in other endocrine cells of islets including α, δ, and pp cells in pancreas (Figure 1B-D). The current study was focused on probing the roles of FAM3A in the regulation of insulin expression and secretion in pancreatic β cells. Thus, whether FAM3A was involved in the regulation of insulin expression under physiological and diabetic conditions was determined. Pancreatic islet FAM3A expression was significantly reduced in fasting status, but restored after refeeding in mice as evidenced by immunohistochemical staining and immunoblotting assays (Figure 2A,B). The pancreatic FAM3A mRNA level was similarly reduced in fasting status, and restored after refeeding in mice (Figure 2C). Insulin expression was similarly changed as FAM3A in fasting-refeeding mouse pancreas (data not shown). Pancreatic islet FAM3A expression was correlated with blood glucose and serum insulin levels in fasting-refeeding mice (Figure 2D,E). In cultured HIT-T15 cells, the expression levels of FAM3A, PDX1, and insulin genes (Ins1 and Ins2) were reduced in serum-starvation status, and restored after re-supply of serum (Figure 2F,G). Subcellular location analysis indicated that FAM3A protein is located in mitochondrial fraction of MIN6 cells (Supplemental Figure 2A) and HIT-T15 cells (data not shown) in basal and overexpression conditions. In the pancreatic islets of db/db mice, FAM3A and insulin staining densities were reduced when compared with that of db/m mice as evidenced by immunofluorescent staining assay (Figure 3A,B), indicating that FAM3A expression was reduced in islet β cells under obese diabetic condition. We had previously demonstrated that miR-423-5p had the highest expression level in pancreas among mouse tissues, and it directly targeted and inhibited FAM3A expression in hepatocytes.[13] So far, miR-423-5p is the only factor that has been revealed to directly repress FAM3A expression.[14] So, whether chronic exposure to high concentrations of glucose and FFAs modulated miR-423-5p expression to affect FAM3A expression in pancreatic β cells was determined. In MIN6 cells, chronic exposure to high levels of glucose, insulin, and palmitate upregulated miR-423-5p level and repressed FAM3A expression (Figure 3C-F). In contrast, oleate upregulated FAM3A expression in MIN6 cells with little effect on miR-423-5p expression

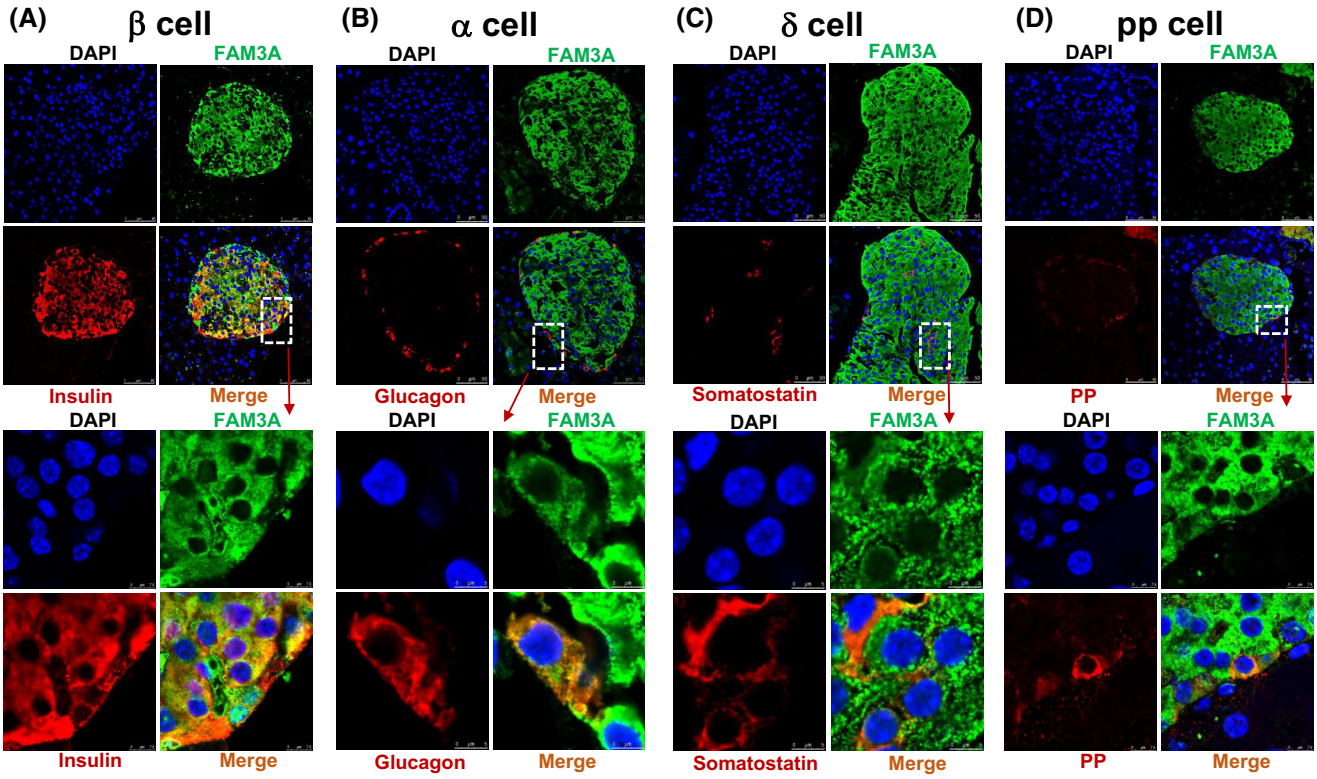

FIGURE 1 FAM3A is expressed in endocrine cells of mouse islets. A, Immunofluorescent staining revealed that FAM3A and insulin are co-localized in β cells of mouse islets. B, Immunofluorescent staining revealed that FAM3A is expressed in α cells of mouse islets. C, Immunofluorescent staining revealed that FAM3A is expressed in δ cells of mouse islets. D, Immunofluorescent staining revealed that FAM3A is expressed in pp cells of mouse islets. Insulin, biomarker for β cells; glucagon: biomarker for α cells; somatostatin, biomarker for δ cells. PP, pancreatic peptide, biomarker for pp cells. The images were the representatives of at least 3 independent staining

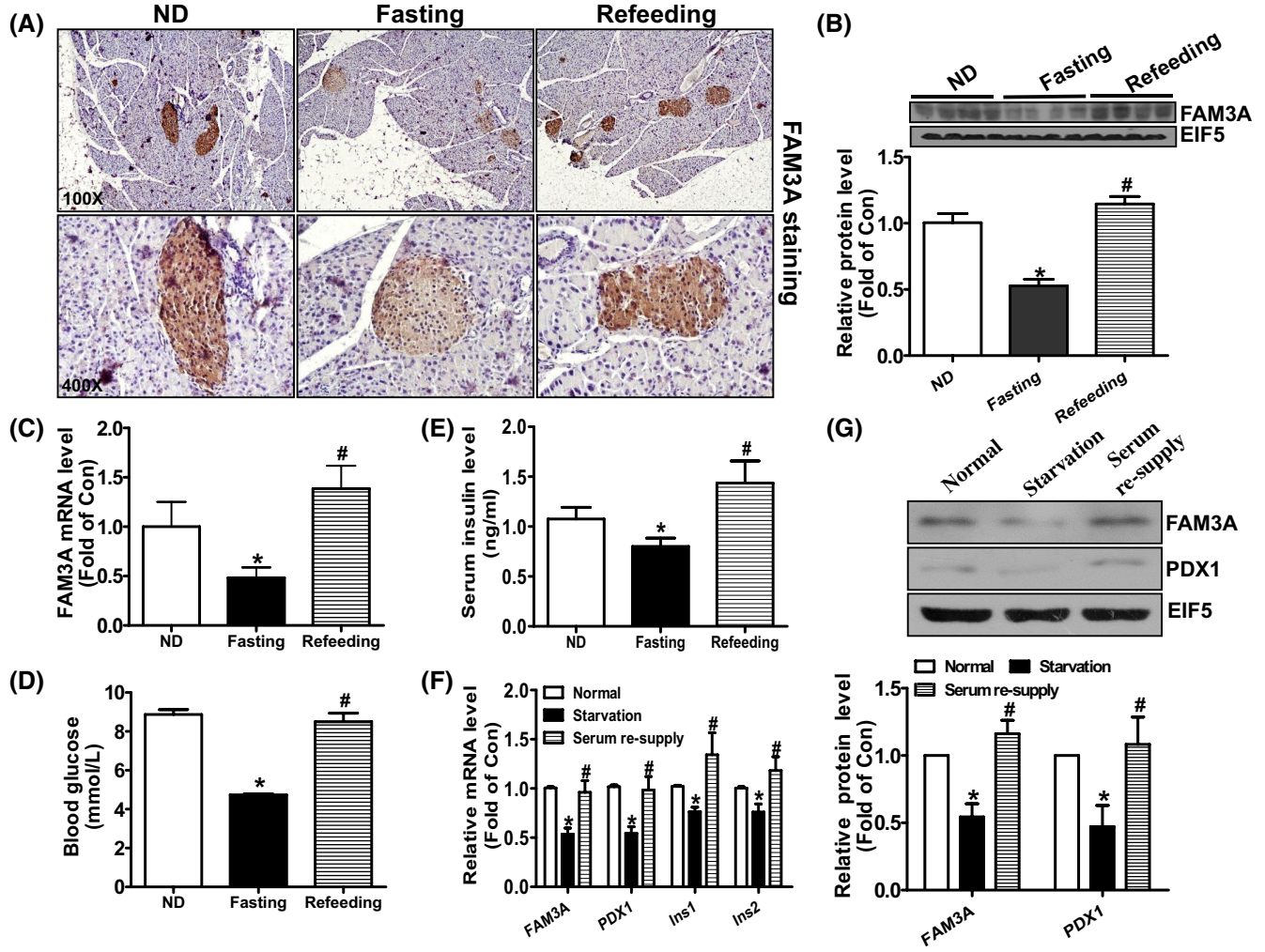

FIGURE 2 Pancreatic FAM3A expression is reduced in fasting status, and restored after refeeding. A, Immunohistochemical staining revealed the changes in FAM3A protein in fasting and refeeding mouse pancreases. B, Immunoblotting confirmed that changes in FAM3A protein in fasting and refeeding mouse pancreases. C, Changes in FAM3A mRNA level in fasting and refeeding mouse pancreases. D, Blood glucose levels of mice. E, Serum insulin levels of mice. ND, mice were fed on normal diet; Fasting, mice were fasted for 24 hours; Refeeding, mice were fasted for 24 hours, followed by 12-hour refeeding. N = 4 for immunoblotting assays in panel B, and N = 6-8 for other assays, $*P < .05$ versus ND group of mice, $\#P < .05$ versus fasting group of mice. F, Serum starvation reduced the mRNA levels of FAM3A, PDX1, and insulin genes, but restored after re-supply of serum in HIT-T15 cells. G, Serum starvation reduced the protein levels of FAM3A and PDX1, but restored after re-supply of serum in HIT-T15 cells. Representative gel images were shown in upper panel, and quantitative data in lower panel. Normal, cells were cultured in normal medium; Starvation, cells were cultured in medium without serum for 24 hours; Serum re-supply, cells were cultured in serum-free medium for 12 hours, followed by culturing in normal medium for another 12 hours. N = 3-5, $*P < .05$ versus normal group of cells, $\#P < .05$ versus starvation group of cells

(Figure 3E,F). miR-423-5p overexpression repressed, whereas miR-423-5p inhibition upregulated FAM3A expression in MIN6 cells (Figure 3G-J). With the change in FAM3A expression, miR-423-5p overexpression or inhibition decreased or increased the mRNA levels of PDX1 and insulin genes, and ATP levels in MIN6 cells (Supplemental Figure 2B-D). Collectively, FAM3A is highly expressed in pancreatic islet β cells, and its expression is correlated with insulin expression under physiological and diabetic conditions.

3.2 | FAM3A gene deficiency markedly impaired insulin secretion and glucose tolerance in mice

To directly evaluate the roles of FAM3A in insulin expression and secretion, β cell specific FAM3A knockout mice (BKO mice) were generated by crossing FAM3A-Loxp mice with Ins2-Cre mice (Supplemental Figure 3A,B). Although there is concern that Cre may impact β cell functions, it had also been reported that Ins2-Cre mice (RIP-Cre) exhibited

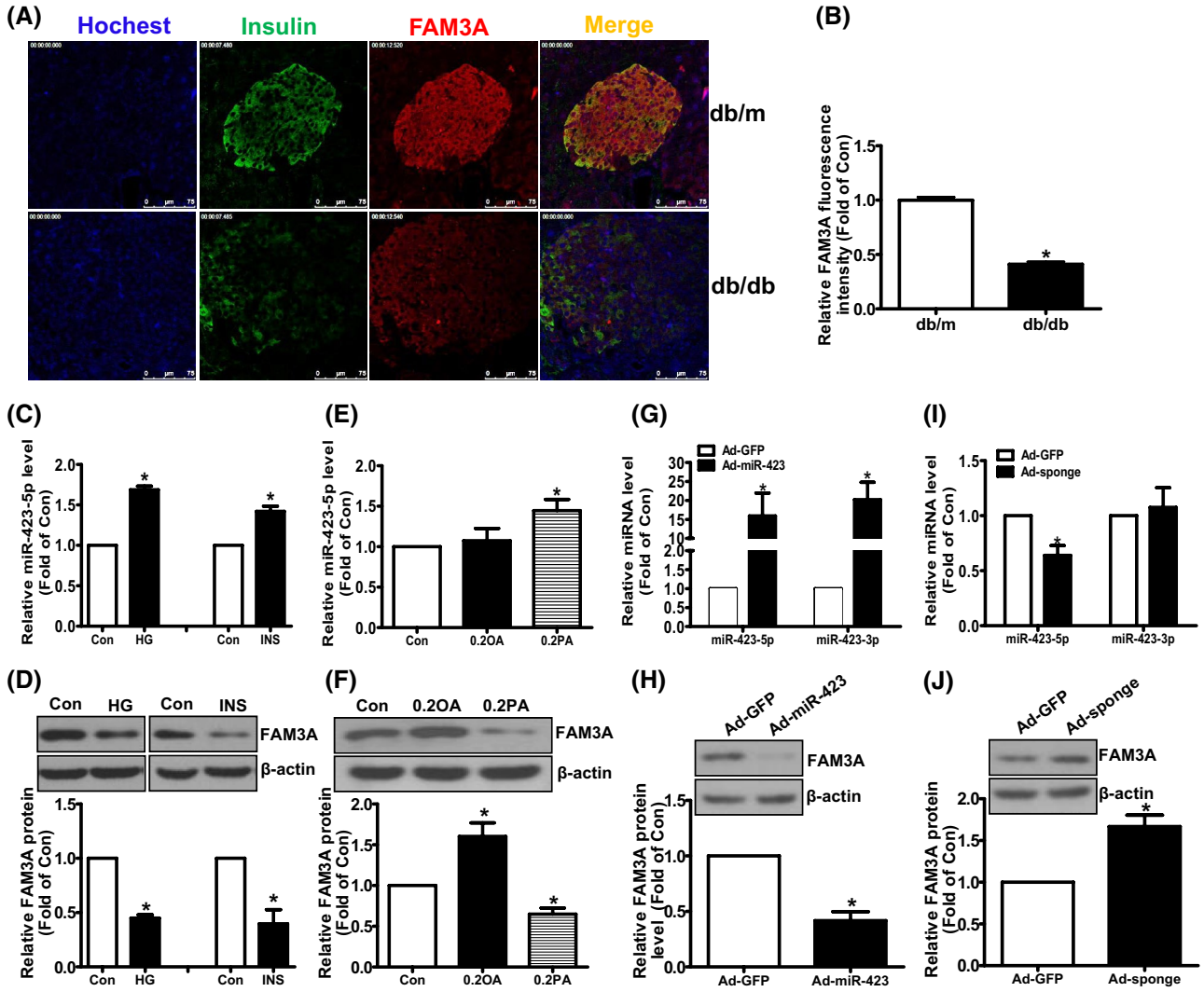

FIGURE 3 FAM3A expression was reduced in pancreatic islets of db/db mice. A-B, Confocal Microscopy assay revealed that islets of db/db mouse exhibited a lower FAM3A staining intensity than that of db/m mice. The representative images were shown in (A) and the quantitative data in (B). The quantitative data was obtained from at least 3 consecutive slides from 5 mouse pancreas. In each slide, the average staining intensity of all the islets were monitored. *$P < .05$ versus db/m mouse. C, Glucose and insulin upregulated miR-423-5p expression in MIN6 cells. D, Glucose and insulin reduced FAM3A expression in MIN6 cells. E-F, Different effects of palmitate and oleate on miR-423-5p and FAM3A expression in MIN6 cells. MIN6 cells were treated with 33.3 mM glucose, 200 nM insulin, 0.2 mM oleate, or 0.2 mM palmitate for 24 hours before experimental assays, respectively. HG, 33.3 mM glucose; INS, 200 nM insulin; 0.2OA, 0.2 mM oleate; 0.2PA, 0.2 mM palmitate. N = 3-5, *$P < .05$ versus control cells. G-H, miR-423 overexpression repressed FAM3A expression in MIN6 cells. Cells were infected with 25MOI of viruses for 24 hours before experimental assay. I-J, Inhibition of miR-423-5p upregulated FAM3A expression in MIN6 cells. Ad-sponge, MIN6 cells infected with Ad-miR-423-5p-sponge. N = 3-6, *$P < .05$ versus Ad-GFP-treated control cells

normal insulin secretion and glucose metabolism as flox/flox (Loxp) and wild-type mice,[40,42-45] and Loxp mice had been widely used as control mice in studies involving RIP-Cre-triggered β cell specific knockout of genes.[42,44-49] Our preliminary experiments revealed that there was no significant difference in OGTT between Loxp mice and RIP-Cre mice (Supplemental Figure 3C), so the Loxp mice had been used as control mice in the current study. BKO mice had similar body weight as control mice during the growth (Supplemental Figure 3D). BKO mice exhibited markedly impaired glucose tolerance from 8 week old when compared with control mice. OGTT were performed at the age of 8, 12, 16, 20, and 24 weeks old, respectively, and the OGTT data of 12 and 20 weeks were shown in Figure 4A,B. Importantly, BKO mice exhibited markedly blunted insulin secretion after glucose load (Figure 4C,D). BKO mice had similar insulin and blood glucose levels as control mice in fasting status (Figure 4A-D). Immunofluorescent staining assays indicated the decrease of FAM3A and insulin proteins in BKO mouse pancreatic islets (Figure 4E). Moreover, isolated BKO mouse islets exhibited similar morphology as control mouse islets under light microscopy (Supplemental Figure 3E). Cell

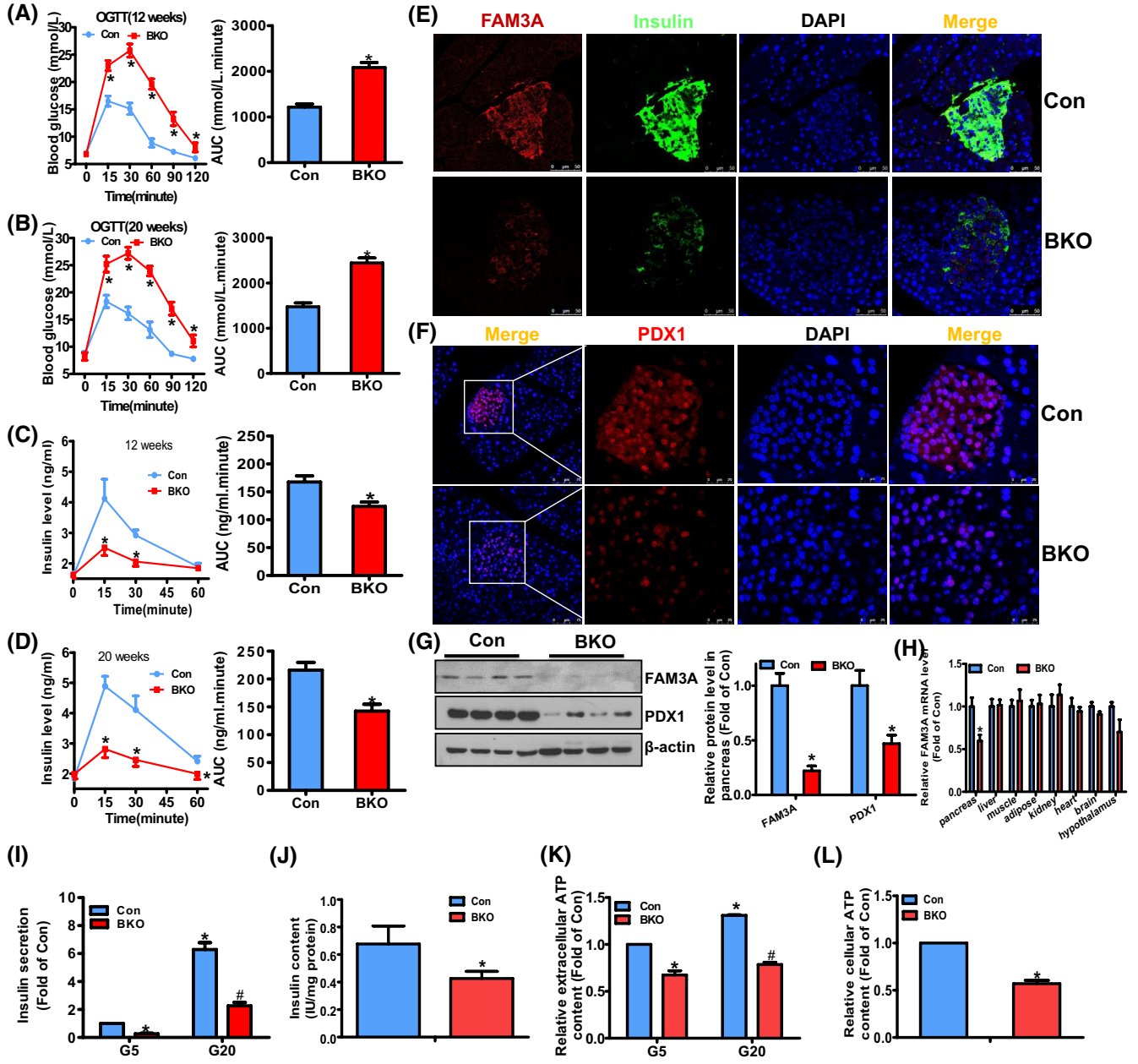

FIGURE 4 β cell-specific knockout of FAM3A markedly impaired insulin secretion and glucose tolerance. A-B, Glucose tolerance tests of 12- and 20-week male mice, respectively. Glucose data were shown in left panel, AUC data in right panel. C-D, Insulin secretion of 12- and 20-week male mice, respectively. Serum insulin data were shown in left panel, and AUC data in right panel. Con, control mice (FAM3A-flox/flox mice); BKO, β cell-specific FAM3A gene knockout mice. N = 10, $*P < .05$ versus control mice. E, Knockout of FAM3A reduced insulin protein level in pancreatic islets as evidenced by immunofluorescent staining. Images were obtained by confocal microscopy. F, Knockout of FAM3A reduced PDX1 expression in pancreatic islets as evidenced by immunofluorescent staining. Images were obtained by confocal microscopy. G, PDX1 protein level was reduced in BKO mouse pancreas than in control mouse pancreas. Representative gel images were shown in left panel, and quantitative data in right panel. N = 6, $*P < .05$ control mice. H) FAM3A mRNA level in various tissues of control and BKO mice (22 weeks old). N = 8-12, $*P < .05$ versus control mice. I, FAM3A-deficient mouse islets exhibited impaired insulin secretion in the presence of low or high glucose challenge. J, FAM3A-deficient mouse islets had lower insulin content than control mouse islets. K, FAM3A-deficient mouse islets exhibited impaired ATP release in the presence of low or high glucose challenge. L, FAM3A-deficient mouse islets had lower ATP content than control mouse islets. G5, 5 mM glucose; G20, 20 mM glucose. N = 6, $*P < .05$ versus control islets under G5 stimulation, # versus control mouse islets under G20 stimulation

composition analysis further revealed that BKO mouse islets exhibited similar cell composition of β, α, and δ cells as control islets (Supplemental Figure 4A,B). However, although BKO mouse islets had similar glucagon cells as control mouse islets, they had decreased glucagon expression when compared with control mouse islets (Supplemental Figure

4C). In support, BKO mouse had reduced serum glucagon level in fasting status when compared with control mice (Supplemental Figure 4D). FAM3A deficiency reduced PDX1 protein level in mouse islets as indicated by immunofluorescent staining assays (Figure 4F). Western blotting confirmed that FAM3A protein was reduced by about 70% in BKO mouse pancreas (Figure 4G), which was consistent with the fact that β cell accounted for 60-70% of mouse islets. Western blotting assays also confirmed that FAM3A deficiency reduced PDX1 expression in mouse pancreas (Figure 4G). Moreover, BKO mice showed significantly decreased FAM3A mRNA level in pancreas but not in other tissues including brain and hypothalamus (Figure 4H). These findings confirmed the specific knockout of FAM3A gene in pancreatic β cells. Isolated BKO mouse islets exhibited blunted insulin secretion in the presence of low and high glucose stimulation when compared with control mouse islets (Figure 4I). BKO mouse islets had lower insulin content than control mouse islets (Figure 4J). Moreover, BKO mouse islets also exhibited decreased ATP secretion and ATP content when compared with control mouse islets (Figure 4K,L). BKO mice exhibited age-dependent slight increase in global insulin sensitivity (Supplemental Figure 5A-C) and hepatic glucose production (Supplemental Figure 5D-F).

3.3 | FAM3A promoted insulin expression and secretion via the upregulation of PDX1 expression

Because FAM3A deficiency reduced PDX1 expression, and impaired insulin expression in vivo, the underlying mechanism(s) was further determined in cultured β cell lines and primary islets. FAM3A overexpression upregulated the mRNA levels of PDX1 and insulin genes (Figure 5A,B), and augmented insulin and ATP secretion in MIN6 cells in the absence or presence of glucose challenge (Figure 5C,D). FAM3A overexpression increased ATP but not insulin content in MIN6 cells (Figure 5E,F). Moreover, FAM3A elevated cellular Ca^{2+} levels, but was inhibited by P2 receptor antagonist (suramin) in MIN6 cells (Figure 5G). In primary islets of normal mice, FAM3A overexpression also enhanced insulin secretion in response to low or high glucose challenge (Figure 5H). Insulin content in mouse islets was not increased after FAM3A overexpression (Figure 5I). FAM3A also enhanced ATP production and secretion in islets (Figure 5J,K). FAM3A overexpression upregulated the mRNA levels of PDX1 and insulin genes in normal mouse islets (Figure 5L). In support, BKO mouse islets had lower PDX1 and insulin gene mRNA levels than control mouse islets (Figure 5M). FAM3A mRNA and protein levels were reduced by about 60% in BKO mouse islets when compared with control mouse islets (Figure 5M,N).

3.4 | FAM3A upregulated PDX1 expression via the activation of CaM-FOXA2 pathway

Given the critical roles of PDX1 in regulating insulin expression and secretion, the mechanism of FAM3A-induced PDX1 expression was further pinpointed. Because FAM3A modulated ATP production to elevate cellar Ca^{2+} level in P2 receptor-dependent manner, whether FAM3A induced PDX1 expression through P2 receptor-calmodulin (CaM) pathway was further evaluated. Antagonism of P2 receptor using suramin or CaM using CPZ blocked FAM3A-induced PDX1 upregulation in MIN6 cells (Figure 6A,B). In support, CaM overexpression also upregulated PDX1 protein expression in MIN6 cells (Figure 6C). Moreover, FAM3A overexpression increased CaM protein level in pancreatic β cells (Figure 6D). Consistently, CaM overexpression upregulated the mRNA levels of PDX1 and insulin genes in mouse islets (Figure 6E) and β cell lines (data not shown). These findings suggested that FAM3A activated PDX1 expression through the ATP-P2 receptor-CaM pathway. Generally, CaM interacts its target proteins to exert the biological functions. To further determine the potential mechanism for CaM-induced PDX1 transcription, CoIP plus mass spectrometry was performed to identify the potential protein(s) that interacted with CaM in MIN6 cells. Three CaM genes, named CALM1, CALM2, and CALM3, respectively, are present in mammalians, and all of them encode one identical CaM protein.[53] Human CALM2 gene with 6-His tag was overexpressed in MIN6 cells using adenoviruses constructed in our previous study.[54] After silver staining, several potential protein bands including the indicated one immunoprecipitated by both anti-CaM and anti-His antibodies were analyzed by mass spectrometry (Figure 7A). Interestingly, FOXA2 is among the potential target proteins identified for the indicated band by mass spectrometry (Mass spectrometry data were shown in supplemental Table 4). Given the crucial role of FOXA2 in regulating PDX1 expression in pancreatic β cells,[23,55] whether it interacted with CaM was further determined. CoIP plus immunoblotting assay confirmed that CaM directly interacted with FOXA2 (Figure 7B). CaM overexpression augmented the transcriptional activity of FOXA2 on mouse PDX1 gene promoter in MIN6 and HK293 cells (Figure 7C,D). FAM3A overexpression increased the nuclear distribution of CaM with little effect on its cytosolic distribution as evidenced by analyses of cytosolic and nuclear fractions in MIN6 cells (Figure 7E), which was consistent with that FAM3A overexpression increased total CaM protein level in MIN6 cells (Figure 6D). Importantly, CoIP plus immunoblotting assay further revealed that FAM3A overexpression enhanced CaM-FOXA2 interaction (Figure 7F). Consistent with increased CaM nuclear distribution and CaM-FOXA2 interaction, FAM3A overexpression augmented the transcriptional activity of FOXA2 on PDX1 gene promoter in MIN6 cells (Figure 7G). To further validate

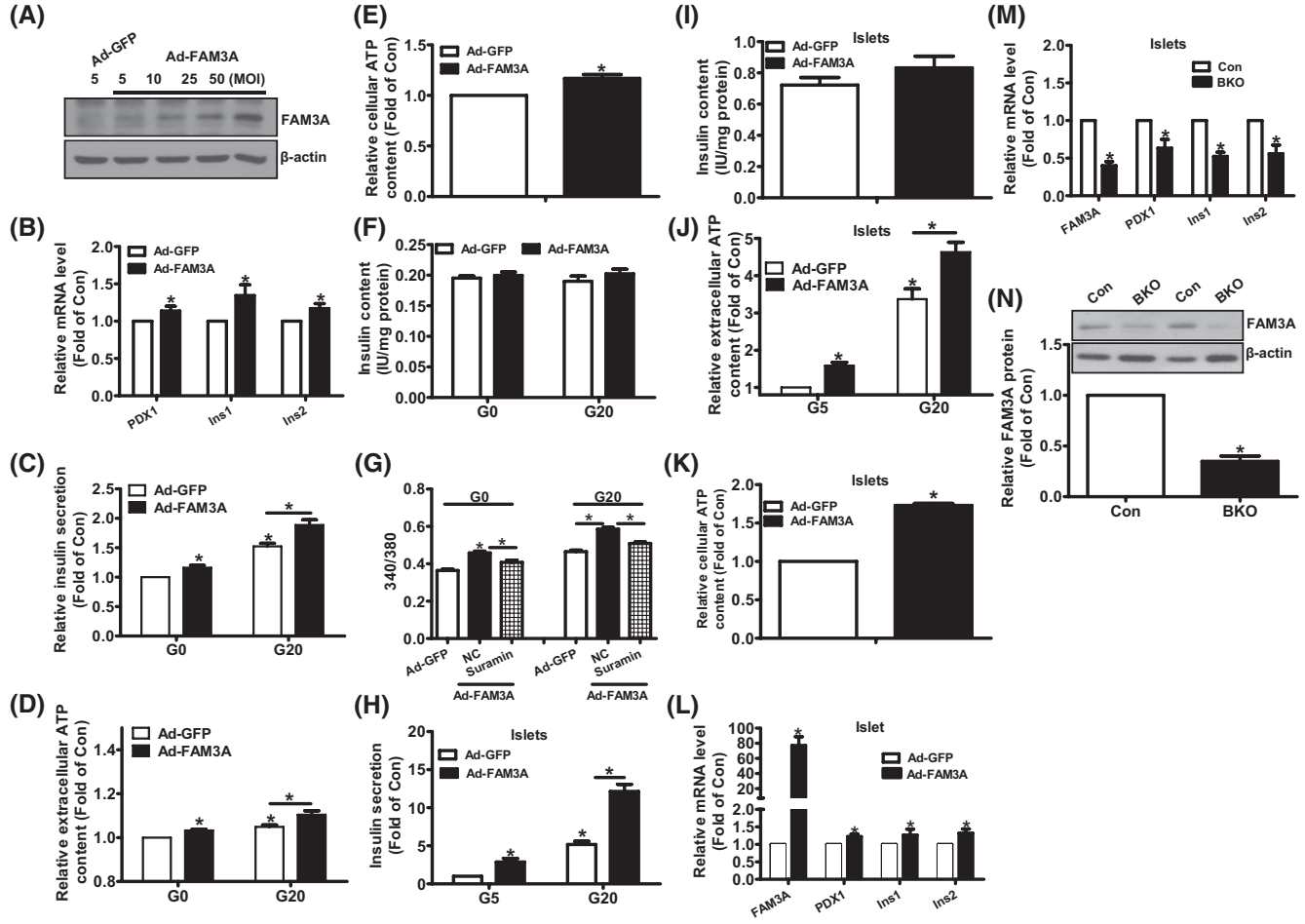

FIGURE 5 FAM3A overexpression augmented insulin secretion in MIN6 cells. A, Characterization of Ad-FAM3A infection efficacy in MIN6 cells. FAM3A protein level was determined at 24 hours post infection. 25MOI of viruses was chosen for the study. B, FAM3A overexpression upregulated the mRNA levels of PDX1 and insulin genes in MIN6 cells. C-D, FAM3A overexpression augmented insulin (C) and ATP (D) secretion with or without glucose presence. E-F, FAM3A overexpression on insulin and ATP content in MIN6 cells. G, FAM3A overexpression increased cellular calcium levels, but was inhibited by suramin. N = 4-5, $*P < .05$ versus control cells or between two indicated groups of cells. H-I, FAM3A overexpression on insulin secretion (H) and insulin content (I) in islets of C57BL/6J mice. J-K, FAM3A overexpression on extracellular (J) and intracellular (K) ATP levels in mouse islets. L, FAM3A overexpression increased the mRNA levels of PDX1 and insulin genes in mouse islets. M, The mRNA levels of PDX1 and insulin genes were decreased in FAM3A-deficient islets when compared with control islets. N = 4-6, $*P < .05$ versus control islets or between two indicated groups of islets. N, FAM3A protein is reduced in BKO mouse islets when compared with control mouse islets. About 150 islets were performed for western blotting assays. N = 3, $*P < .05$ versus control islets

the roles of FOXA2 in FAM3A-induced PDX1 upregulation in β cells, its expression was inhibited by siRNA transfection in MIN6 cells. Silencing of FOXA2 by siFOXA2 transfection reduced the mRNA levels of PDX1 and insulin genes in MIN6 cells, further confirming that FOXA2 directly regulated the expression of PDX1 gene (Figure 7H). Importantly, knockdown of FOXA2 blunted CaM- and FAM3A-induced PDX1 upregulation in MIN6 cells (Figure 7I,J). In vivo nuclear distribution of CaM and FOXA2 were reduced in BKO mouse islet cells when compared with control mouse islet cells (Figure 8A), further confirming the in vitro observations that FAM3A activated CaM to activate FOXA2 in β cells. Collectively, these findings revealed that FAM3A activated ATP-Ca^{2+}-CaM-FOXA2 pathway to induce PDX1 gene transcription in pancreatic β cells.

4 | DISCUSSION

Beyond our previous demonstration that FAM3A suppresses hepatic gluconeogenesis and lipogenesis by activating Akt and AMPK pathway independent of insulin,[12,13] the current study further revealed that FAM3A also plays critical roles in controlling insulin expression and secretion in pancreatic β cells. FOXA2 is critical for pancreas development by controlling the expression of PDX1.[56] It has

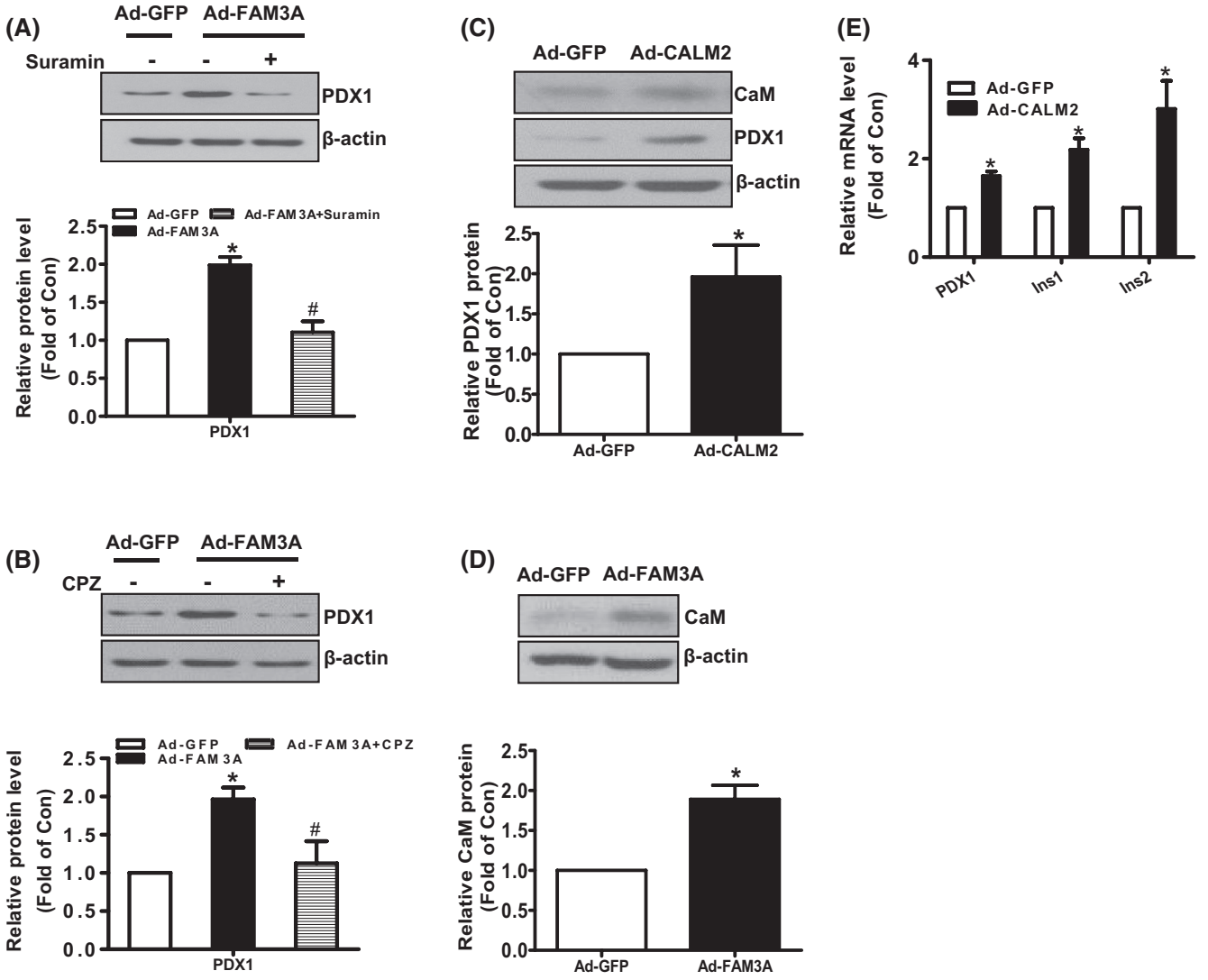

FIGURE 6 FAM3A upregulated PDX1 expression through P2 receptor-CaM pathway. A-B, Inhibition of P2 receptors (A) and CaM (B) blunted FAM3A-induced PDX1 upregulation in MIN6 cells. Suramin, P2 receptor inhibitors; CPZ, CaM inhibitor. N = 3-5, $*P < .05$ versus Ad-GFP group of cells, $\#P < .05$ versus Ad-FAM3A group of cells. C, CaM overexpression increased the protein level of PDX1 in MIN6 cells. D, FAM3A overexpression increased CaM protein level in MIN6 cells. E, CaM overexpression increased mRNA levels of PDX1 and insulin genes in mouse islets. N = 3-5, $*P < .05$ versus Ad-GFP group of cells

been revealed that FOXA2 also play crucial roles in regulating PDX1 expression, insulin gene expression and insulin secretory process in mature pancreatic β cells.[25,26,55,57] Under diabetic condition, FOXA2 and PDX1 expression are reduced in pancreatic β cells[58] In the past decades, it has been wildly accepted that intracellular ATP plays a decisive roles in controlling insulin secretion process by closing ATP-sensitive K^+-channel and opening L-type Ca^{2+} channel to elevate cellular Ca^{2+} levels.[59,60] More recently, extracellular ATP has also been shown to be involved in the regulation of insulin secretion.[9,10] Pretreatment with P2 receptor antagonist PPADS and suramin significantly attenuated GSIS in rat islets.[9] In the current study, we revealed that new mitochondrial protein FAM3A enhances ATP production and release in pancreatic β cells. Released extracellular ATP plays important roles in FAM3A-induced increase in cellular Ca^{2+} levels and insulin secretion. Notably, FAM3A-induced ATP release elevates cellular Ca^{2+} level to stimulate CaM translocation into the nucleus. Nuclear CaM functions as a novel co-activator to augment FOXA2's transcriptional activity on PDX1 gene in pancreatic β cells. These findings established a novel action model of ATP/Ca^{2+}/CaM/FOXA2 axis in regulating PDX1 and insulin gene expressions, and provided a clearer map for explaining the inhibition of FOXA2 and PDX1 in pancreatic β cells under obese condition. Under obese condition, an increase in circulating levels of saturated FFAs and/or glucose activates miR-423-5p to repress FAM3A expression and impair

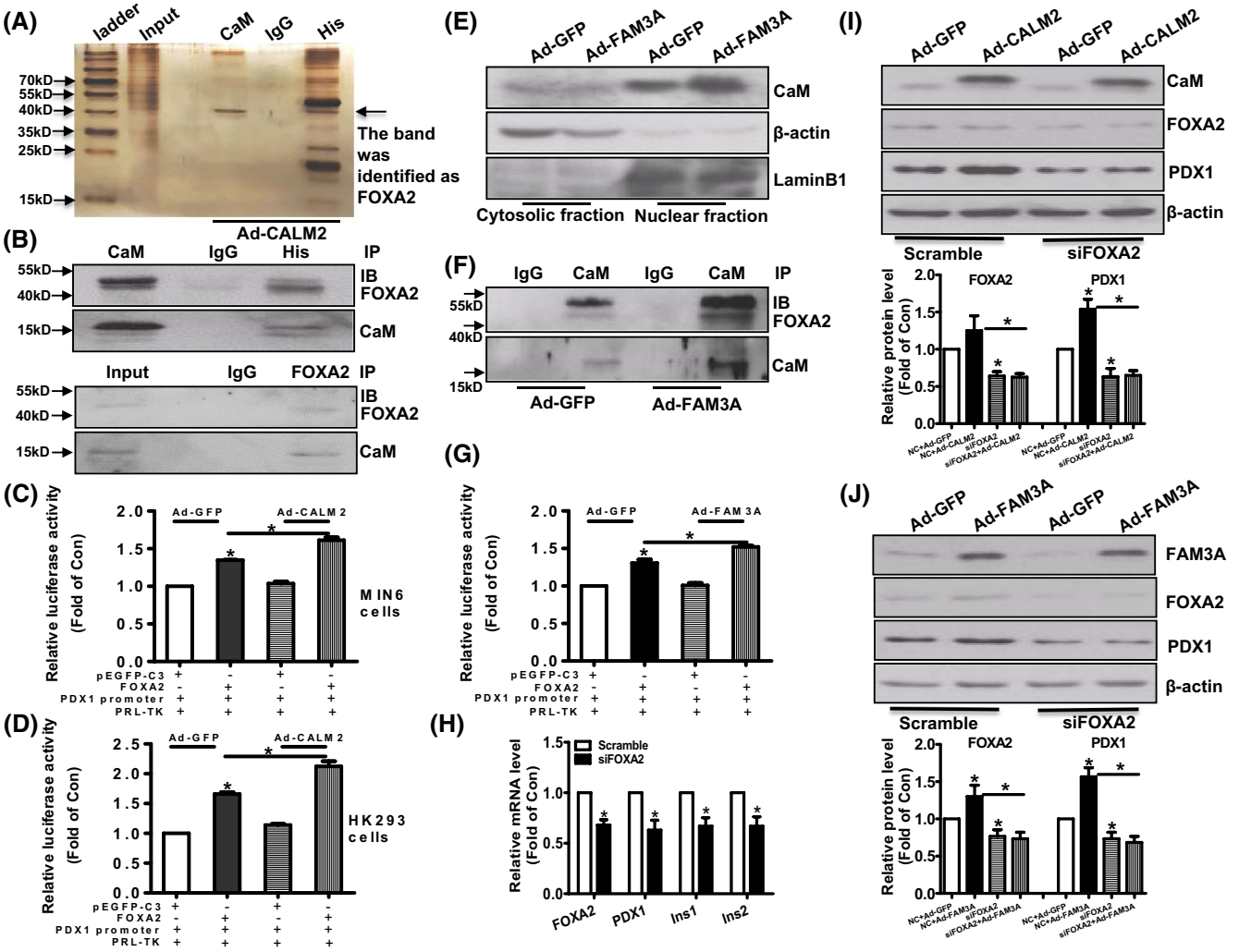

FIGURE 7 FAM3A activated PDX1 expression via the activation of FOXA2. A, CaM interacted with FOXA2 as identified by CoIP plus mass spectrometric analysis. MIN6 cells were infected with Ad-CALM2 for 24 hours, then CoIP was performed using anti-CaM and anti-His antibodies. In the silver staining gel image, the indicated band was potentially identified as FOXA2. B, CoIP + immunoblotting assay confirmed that CaM interacted with FOXA2. C-D, CaM augmented the transcriptional activity of FOXA2 on mouse PDX1 gene promoter in MIN6 (C) and HK293 (D) cells. N = 4, $*P < .05$ versus control cells or between indicated two groups of cells. E, FAM3A overexpression increased the nuclear distribution of CaM as evidenced cytosolic/nuclear fraction extraction in MIN6 cells. β actin and LaminB1 were used as biomarkers for the cytosolic and nuclear fractions, respectively. F, FAM3A overexpression increased CaM-FOXA2 interaction in MIN6 cells as confirmed by CoIP + immunoblotting. G, FAM3A overexpression augmented the transcriptional activity of FOXA2 on mouse PDX1 gene promoter in MIN6 cells. N = 4, $*P < .05$ versus control cells or between indicated two group of cells. H, Silencing of FOXA2 reduced PDX1 and insulin gene expressions in MIN6 cells. Cells were transfected with 50 nM siFOXA2 or scramble for 24 hours before experimental assays. N = 6, $*P < .05$ versus control cells. I-J, silencing of FOXA2 blunted CaM (I) and FAM3A (J) induced upregulation of PDX1 in MIN6 cells. Cells were transfected with 50 nM siFOXA2 or scramble for 6 hours, followed by infection with viruses for 24 hours. N = 3-5, $*P < .05$ versus control cells or between indicated two group of cells

ATP production, inhibiting Ca^{2+}-CaM-FOXA2-PDX1 axis to cause pancreatic β cell dysfunction. Clearly, inhibition of FAM3A-CaM-FOXA2 axis links mitochondrial dysfunction to PDX1 repression and pancreatic β cell dysfunction under obese condition. It has long been known that saturated fatty acid such as palmitate exerts deleterious effects, whereas unsaturated fatty acid such as oleate has beneficial effects on pancreatic β cell functions.[61,62] Activation of miR-423-5p to repress FAM3A signaling pathway provides a novel explanation for palmitate-induced nuclear exclusion of FOXA2 in pancreatic β cells.[32] Moreover, activation of FAM3A expression is also a novel mechanism for explaining oleate's beneficial effects on pancreatic β cell functions. Given the critical roles of intracellular ATP in stimulating insulin exocytosis,[4] it should also play important roles in FAM3A's effects on insulin secretion in pancreatic β cells beyond extracellular ATP because FAM3A modulates both intracellular and extracellular ATP content.

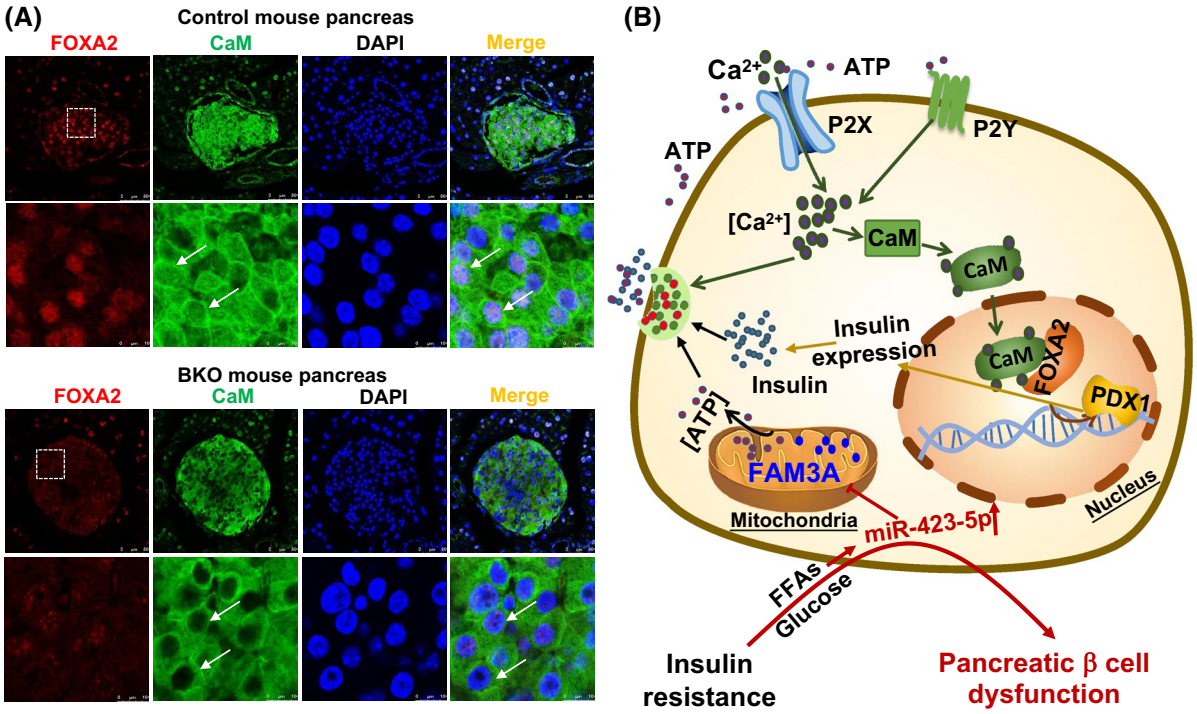

FIGURE 8 FAM3A is a crucial regulator of pancreatic β cell functions. A, Immunofluorescent staining revealed that nuclear CaM and FOXA2 distributions were reduced in BKO mouse islet when compared with control mouse islet. Representative nucleus had been indicated by white arrows. B, Proposed model of FAM3A in regulating pancreatic β cell functions. FAM3A controls insulin secretion by modulating ATP production to maintain Ca^{2+} homeostasis in pancreatic β cells. Particularly, FAM3A stimulates insulin gene expression by activating ATP-CaM-FOXA2-PDX1 signaling axis. Under insulin resistant condition, chronic exposure to high levels of glucose and/or FFAs will activate miR-423-5p to repress FAM3A expression and inhibit mitochondrial ATP production, resulting in pancreatic β cell dysfunctions and diabetes. Inhibition of FAM3A by miR-423-5p activation is a novel mechanism for glucotoxicity- and lipotoxicity-triggered mitochondrial dysfunctions in pancreatic β cells. ATP, adenosine triphosphate; CaM, calmodulin; FAM3A, FAM3 gene family member A; FFAs, free fatty acids; FOXA2, forkhead box protein A2; P2X, P2X receptors; P2Y, P2Y receptors; PDX1, pancreas/duodenum homeobox protein 1

Regarding the roles of FAM3A in maintaining β cell functions, several issues should also be noted. Although FAM3A overexpression upregulated the mRNA levels of insulin genes in primary mouse islets and β cell lines, it had little effect on insulin content, which we thought should be due to the higher insulin secretion rate after FAM3A overexpression than control cells. In case of FAM3A overexpression, cellular insulin content is the balance between increased insulin synthesis and higher insulin secretion rate when compared with control cells. Actually, it had been previously reported that leucine activated glucokinase and ATP synthase to increase insulin secretion, resulting in decreased insulin content in cultured islets.[60] The RIP-Cre transgene has a 668 bp fragment of the rat insulin II promoter, nuclear-localized Cre recombinase, and a 2.1 kbp fragment from the human growth hormone gene (hGH).[41] hGH is a good marker for immunohistochemical detection of gene expression and has been used in a number of transgenic studies, and it has been reported that permanent expression of hGH in β cells does not alter the differentiation and functions of endocrine cells.[63] Moreover, there is also concern that RIP-Cre may affect GSIS in mice. Some studies indeed showed that RIP-Cre showed impaired glucose tolerance and GSIS because some transgenic lines using Ins2 and PDX1 promoter fragments triggered Cre expression not only in islet β cells, but also in the brain.[64,65] However, other researches also showed that RIP-Cre does not affect gene express in the hypothalamus and other tissues.[66,67] At present, some study used RIP-Cre mice as controls.[68] However, a number of studies had also shown that RIP-Cre have comparable glucose tolerance tests and insulin secretion as Loxp mice, and Loxp mice and even wild-type mice had been widely used as control mice[40-48,69-71] in study involving β cell specific knockout of genes triggered by RIP-Cre. In addition, although FAM3A-deficience impairs while FAM3A overexpression increases insulin secretion in primary mouse islets and β cell lines under basal glucose concentration, BKO mice has comparable insulin and blood glucose levels in fasting status as control mice. We speculated that this might be due to some unknown compensational mechanism(s) such as changes in glucagon expression and secretion, insulin clearance and insulin sensitivity at the basal condition.

Similarly, although isolated FAM3B (PANDER)$^{-/-}$ islets exhibited blunted insulin secretion in response to glucose concentrations ranging from 0mM to 30mM when compared with control islets, PANDER$^{-/-}$ mice had comparable insulin and glucose levels in fasting status as control mice.[72] This phenomenon had been further explained by decrease in insulin clearance in PANDER$^{-/-}$ mice when compared with control mice.[72] Moreover, decreased glucagon expression and secretion likely partially antagonize the hyperglycemic effect of FAM3A deficiency in pancreatic β cells in fasting condition. However, the mechanism for decreased glucagon expression in pancreatic α cells of BKO mouse islets still remains unknown at present. FAM3A overexpression induced significant but slight increase in PDX1 and insulin mRNAs in vitro, which we thought should be due to the high basal expression levels of these genes in β cells or islets. Actually, the mRNA levels of PDX1 and insulin genes were reduced by about 50% in FAM3A-deficient islets.

In summary, FAM3A plays important roles in regulating insulin secretion by modulating ATP production to maintain Ca^{2+} homeostasis in pancreatic β cells. Particularly, FAM3A-induced ATP release also controls insulin gene expression by activating CaM-FOXA2-PDX1 signaling axis. Under diabetic condition, inhibition of FAM3A signaling axis by glucose- and FFA-induced miR-423-5p activation is an important new mechanism for mitochondrial dysfunction, PDX1 repression, and pancreatic β cell dysfunction (Figure 8B). Activating FAM3A signaling transduction in pancreatic β cells represents a new strategy for treating type 2 diabetes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

W. Yang, Y. Chi, Y. Meng, and Z. Chen researched data and contributed to the discussion. W. Yang, Z. Chen, and Y. Meng wrote the manuscript. R. Xiang and H. Yan provided the technical assistance in experimental procedure and animal experiments. W. Yang, Y. Chi, Y. Meng, Z. Chen, and J. Yang designed the study, and revised/edited manuscript. Dr. Jichun Yang is the guarantor of this work and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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