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Suppression of STAT3 signaling promotes cellular reprogramming into insulin-producing cells induced by defined transcription factors

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ABSTRACT

Background: STAT3 has been demonstrated to play a role in maintaining cellular identities in the pancreas, whereas an activating STAT3 mutation has been linked to impaired β-cell function.
Methods: The role of STAT3 in β-cell neogenesis, induced by the exogenous expression of Pdx1, Neurog3, and Mafa, was analyzed *in vitro* and *in vivo*.
Findings: The expression of phosphorylated STAT3 (pSTAT3) was induced in both Pdx1-expressing and Mafa-expressing cells, but most of the induced β cells were negative for pSTAT3. The suppression of STAT3 signaling, together with exogenously expressed Pdx1, Neurog3, and Mafa, significantly increased the number of reprogrammed β cells *in vitro* and *in vivo*, enhanced the formation of islet-like clusters in mice, and ameliorated hyperglycemia in diabetic mice.
Interpretation: These findings suggest that STAT3 inhibition promotes cellular reprogramming into β-like cells, orchestrated by defined transcription factors, which may lead to the establishment of cell therapies for curing diabetes.
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1. Introduction

As diabetes mellitus results from the absolute or relative deficiency of insulin secretion from pancreatic β cells [8], the generation of surrogate β cells has been a target for the cure of diabetes. To date, insulin-producing cells have been generated from differentiated cell types in the pancreas, such as acinar cells and α cells [14,24,27,31]. Pancreatic acinar cells have attracted attention as a potential target that can be reprogrammed into insulin-producing cells, owing to their shared developmental origins and potential for plasticity [16]. Whereas *in vivo* reprogramming of acinar or duct cells into insulin-producing cells can be induced by humoral factors, such as cytokines and growth factors, or ectopic expression of pancreas-specific transcription factors (Pdx1,

Neurog3 [Ngn3] and Mafa) [2,24,31], more efficient methods to generate surrogate β cells would be of great value toward establishing future regenerative therapies for diabetes.

STAT3 has been demonstrated to play a role in the cellular differentiation of various cell types, including those of the immune, nervous, and endocrine systems, as well as in the maintenance of pluripotent stem cells [11,20]. We previously demonstrated that activation of STAT3 signaling is required for acinar-to-ductal transition induced by the exogenous expression of Pdx1 [19]. In addition, STAT3 was shown to induce the cellular reprogramming of exocrine (acinar or ductal) cells into an endocrine cell fate through transient cytokine treatment [2,28]. On the other hand, activating mutations in human *STAT3* have been reported to be linked to neonatal diabetes accompanied by β-cell failure [22,29], showing that the aberrant activation of STAT3 causes premature endocrine differentiation through the upregulation of *NEUROG3*.

Thus, as the proper activity of STAT3 appears to be essential for pancreas development and endocrine differentiation, we hypothesized that

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Research in context

Evidence before this study

Whereas it has been demonstrated that insulin-producing β cells can be induced from pancreatic non-β cells, generating sufficient and functional β cells remains a challenge.

Added value of this study

Here we demonstrated that STAT3 signaling is dynamically regulated during β-cell neogenesis orchestrated by the exogenous expression of pancreas-specific transcription factors. Furthermore, STAT3 inhibition increased the number of β cells and ameliorated hyperglycemia in diabetic mice.

Implications of all the available evidence

STAT3 inhibition promotes cellular reprogramming into β-like cells, orchestrated by defined transcription factors, and this may be useful for establishing cell therapies to cure diabetes.

regulating STAT3 signaling may control the reprogramming efficiency into insulin-producing cells. To address this, we developed *in vitro* and *in vivo* experimental models to investigate the status of STAT3 activity during the cellular reprogramming into β cells induced by Pdx1, Neurog3, and Mafa, which demonstrated that STAT3 activation is suppressed as the cells are reprogrammed into β cells. Furthermore, the suppression of STAT3 signaling efficiently enhanced the reprogramming efficiency into β cells induced by the defined transcription factors, and ameliorated hyperglycemia in alloxan (ALX)-induced diabetic mice. These findings support the pivotal role of STAT3 in β-cell formation, which may lead to possible future therapies for diabetes *via* this signaling pathway.

2. Experimental procedures

2.1. Cell culture

The mouse pancreatic cell line mPAC and the reporter cell line mPAC-MIP-RFP, in which RFP is expressed under the control of mouse *Insulin 1* promoter (MIP), were generated as previously described [15]. The cells were cultured in DMEM with 10% fetal bovine serum, and incubated at 37 °C in an atmosphere of 5% CO_2 in air. The STAT3 inhibitors cryptotanshinone (Selleck Chemicals, Houston, TX, USA) and BP-1-102 (Calbiochem, Billerica, MA, USA) were dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture medium in some experiments.

2.2. Animals

CAG-CAT-Pdx1^{FLAG}, *CAG-CAT-Mafa^{myc}*, and floxed-Stat3 mice were generated as previously described [13,18,26]. *CAG-CAT-Neurog3^{HA}* was constructed from *pCAG-CAT-lacZ* [1] by replacing the *lacZ* sequences with a fragment containing mouse *Neurog3*-coding sequences. The *CAG-CAT-Neurog3^{HA}* fragment was purified and microinjected into fertilized eggs of BDF1 mice (Japan SLC, Hamamatsu, Japan). *Elastase-CreERT2* transgenic mice (EC mice) [5], which express tamoxifen-activated Cre recombinase in acinar cells, were crossed with *CAG-CAT-Pdx1*; *CAG-CAT-Neurog3*; *CAG-CAT-Mafa* mice (*PNM* mice) to induce acinar-to-β reprogramming. Floxed Stat3 mice were repeatedly crossed with *EC; PNM* mice to generate *Stat3^{KO}; EC; PNM* mice. To induce Cre-mediated recombination, tamoxifen (Sigma Aldrich, St. Louis, MO, USA) was dissolved in corn oil at 20 mg/mL and injected subcutaneously

at 2 mg/10 g body weight. Rag1-deficient mice were obtained from Jackson Laboratories.

To induce β-cell ablation, alloxan (ALX; Sigma Aldrich) was intravenously injected into the mice (70 mg/kg body weight). Diabetic mice that displayed severe hyperglycemia (>500 mg/dL) for at least 2 consecutive days were used for further experiments and were injected with purified adenovirus directly into the splenic lobe of the pancreas. To induce STAT3 inhibition, BP-1-102 (3 mg/kg in 0.5% DMSO in PBS) was administered daily into the mice *via* oral gavage for 10 days.

Mice were housed on a 12-h light/dark cycle in a controlled climate. The study protocol was reviewed and approved by the Animal Care and Use Committee of Juntendo University. Mice were housed on a12-h light/dark cycle, and fed a standard rodent food.

2.3. Preparation of adenoviruses

Recombinant adenoviruses expressing Pdx1 (Ad-Pdx1), Neurog3 (Ad-Ngn3), Mafa (Ad-Mafa), and a polycistronic adenoviral vector (Ad-PNM) carrying Pdx1-2A-Neurog3-2A-Mafa were generated as described previously [15]. As each adenovirus used in this study carries green fluorescent protein (GFP), adenovirus-infected cells are labeled with green fluorescence. An adenovirus expressing only GFP was used as a control (Ad-Ctrl). Recombinant adenoviruses expressing a dominant-negative form of STAT3 (STAT3-DN) or a constitutively active form of STAT3 (STAT3-CA) [10] were prepared using the AdEasy system (kindly provided by Dr. Vogelstein, Johns Hopkins Cancer Center) [9]. High titer adenovirus (>10^8 infectious units per mL) was obtained by repeated infection into HEK293 cells and purified with Virakit (Virapure, San Diego, CA, USA).

2.4. Western blotting

Whole-cell protein extracts were isolated using RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitor cocktail (Thermo Scientific). Ten micrograms of total proteins was loaded and fractionated by SDS-PAGE, transferred to nitrocellulose membranes (Merck Millipore, Darmstadt, Germany), and probed with primary antibodies against pSTAT3, total STAT3 (rabbit, 1:1000; Cell Signaling Technology), and GAPDH (rabbit, 1:1000; Cell Signaling Technology). Immunoreactivity was visualized using SuperSignal West Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.5. Immunostaining

Immunocytochemical staining of mPAC-MIP-RFP cells and immunohistological staining of mouse pancreata were performed as previously described [15,19]. The primary antibodies used were the following: rabbit anti-phospho-STAT3 (pSTAT3) (1:100; Cell Signaling Technology, Danvers, MA, USA), guinea pig anti-insulin (1:5; Dako, Carpinteria, CA, USA), mouse anti-FLAG epitope tag (1:200; kindly provided by Yokomizo. T [23]), mouse anti-Myc tag (1:10000; Cell Signaling Technology, Danvers, MA, USA), mouse anti-HA tag (1:200; Cell Signaling Technology), rabbit anti-Ucn3 (1:500; Phoenix Pharmaceuticals, Burlingame, CA, USA), mouse anti-Ki-67 (1:100; BD Pharmingen, San Diego, CA, USA), rabbit cleaved caspase-3 antibody (1:100; Cell Signaling Technology, Danvers, MA, USA), and guinea pig anti-Pdx1 (1:500; kindly provided by Michael German). Slides were imaged using a Keyence BZ-8100 fluorescence microscope (Osaka, Japan), a Zeiss LSM780 confocal laser scanning microscope (CLSM; Carl Zeiss, Jena, Germany), or a Leica TCS SP5 confocal laser scanning microscope (Wetzlar, Germany).

2.6. Statistical analyses

Statistical analyses were performed using SPSS 18.0 for Windows software (SPSS Inc., Chicago, IL, USA). Comparisons of two samples were performed by the unpaired two-tailed *t*-tests. Multiple groups were analyzed by one-way ANOVA with a multiple comparison test. A *p*-value of <0.05 was considered to indicate a statistically significant difference between two groups. All data are presented as the mean ± SE.

3. Results

3.1. Expression of defined transcription factors activates the phosphorylation of STAT3 in mPAC cells

STAT3 has been shown to play a role in cell-fate decisions in the pancreata of mice and humans [6,19,22]. To confirm STAT3 activation during the reprogramming process, a polycistronic adenoviral vector expressing Pdx1, Neurog3, and Mafa (Ad-PNM) [15] was infected into mouse pancreatic mPAC cells, which exhibit pancreatic progenitor-like characteristics and differentiate into insulin-producing cells by Neurog3-expressing adenovirus or Ad-PNM infection [7] [15], and phosphorylation of STAT3 (pSTAT3) at Tyr705 was analyzed by immunoblotting and immunostaining. Western blotting for pSTAT3 in mPAC cells resulted in a significant increase in pSTAT3 levels in Ad-PNM-infected mPAC cells compared with control mPAC cells, with the highest expression levels of pSTAT3 observed 48 h after Ad-PNM infection (Fig. 1A, B, and S1A). Consistent with the immunoblot findings, immunocytochemical staining demonstrated that the pSTAT3 protein was clearly detected in some nuclei (28.2% ± 7.3%) of Ad-PNM-infected cells, which are marked as GFP-expressing cells, whereas <0.5% of nuclei were positive for pSTAT3 in control cells infected with Ad-Ctrl (Fig. 1C, D and Fig. S1B and C). These findings show that the exogenous expression of Pdx1, Neurog3, and Mafa induces STAT3 activation in a pancreatic cell line *in vitro*.

3.2. Expression of Pdx1 or Mafa induces STAT3 phosphorylation in mPAC cells

Next, mPAC cells were infected with an adenoviral vector carrying a single transcription factor out of the three transcription factors (Pdx1, Neurog3, or Mafa), to clarify which transcription factor actually activates STAT3. Western blot analysis of pSTAT3 demonstrated that mPAC cells infected with Ad-Pdx1 or Ad-Mafa had significantly increased levels of pSTAT3, compared with cells infected with the control adenovirus (Ad-Ctrl), whereas the expression levels of pSTAT3 in Ad-Ngn3-infected mPAC cells were comparable with those of Ad-Ctrl (Fig. 1E and F). Consistent with the Western blot data, immunocytochemical analysis resulted in a robust increase in pSTAT3 levels in mPAC cells infected with Ad-Pdx1 or Ad-Mafa, but not with Ad-Ngn3 (Fig. S2A and B).

3.3. Phosphorylation of STAT3 is suppressed during reprogramming of mPAC cells into insulin-producing cells

To further investigate the activation status of STAT3 during the reprogramming process of mPAC cells into insulin-producing β-like cells, we infected Ad-PNM into mPAC-MIP-RFP cells, in which red-fluorescent protein (RFP) was expressed under the control of the mouse *insulin 1* promoter (MIP) [15]. Immunofluorescence images demonstrated that RFP-expressing cells were detected in mPAC-MIP-RFP cells from 48 h after Ad-PNM infection, and clearly observed 72 h after the infection (Fig. S3), and pSTAT3-positive cells were detected almost exclusively in Ad-PNM- infected cells (Fig. 2A). The percentage of pSTAT3-positive cells among GFP/RFP double- fluorescent β-like cells was 7.1%, which was significantly decreased compared with 17.0% in GFP-positive/ RFP-negative cells, which were infected with Ad-PNM

but not reprogrammed into insulin-producing cells (Fig. 2A, B). These findings observed in mPAC cells suggest that STAT3 activation is enhanced by the exogenous expression of Pdx1 or Mafa, and then suppressed while mPAC cells get reprogrammed into insulin-producing cells.

3.4. Inhibition of STAT3 signaling promotes the reprogramming of mPAC cells into insulin-producing cells

Based on the *in vitro* findings described above, we hypothesized that the downregulation of STAT3 signaling may affect the reprogramming efficiency of mPAC cells into insulin-producing cells. To address this, STAT3 signaling was activated or suppressed by constructing adenoviruses expressing a dominant-negative form (Ad-STAT3DN) and a constitutively active form of STAT3 (Ad-STAT3CA) [10], and reprogramming efficiencies were quantified in mPAC cells 72 h after infection with Ad-STAT3DN or Ad-STAT3CA. When Ad-STAT3DN was infected into mPAC-MIP-RFP cells together with Ad-PNM, the phosphorylation of STAT3 was robustly suppressed in RFP-expressing cells (Fig. S4A and B), and the number of RFP-expressing cells was significantly increased compared with control cells infected with Ad-PNM and Ad-Ctrl (Fig. 2C and D). On the other hand, constitutive activation of STAT3 with Ad-STAT3CA caused a >80% decrease in the number of RFP-expressing cells (Fig. 2C and D), with a significant increase in pSTAT3-positive cells (Fig. S4A and B), suggesting that STAT3 activation negatively regulates the formation of β-like cells induced by Ad-PNM. To further confirm the effect of STAT3 inhibition in the reprogramming efficiency, mPAC cells were treated with the STAT3 inhibitors, cryptotanshinone or BP-1-102 [25,30], together with Ad-PNM, which resulted in a significant increase in the number of RFP-expressing cells by both STAT3 inhibitors (Fig. 2E and F), with a significant decrease in pSTAT3-positive cells (Fig. S5A and B). To rule out the possibility that the increased number of RFP-positive cells might result from increased proliferation or decreased apoptosis, immunostaining against Ki67 and cleaved caspase-3 was performed in mPAC-MIP-RFP cells treated with Ad-PNM, together with Ad-STAT3DN or STAT3 inhibitors, which demonstrated that none of the RFP-positive cells were stained with anti-Ki67 or anti-cleaved caspase-3 antibodies (Figs. S6 and S7). Furthermore, when STAT3 inhibitors were added 48 h after Ad-PNM infection and fluorescence images were captured 24 h later, there was no significant difference in the number of RFP-positive cells between STAT3 inhibitor-treated cells and control cells (Fig. S8). Taken together, these findings suggest that the downregulation of STAT3 activity promotes the cellular reprogramming of mPAC cells toward β-like cells, rather than increasing the proliferation of or decreasing the apoptosis of β-like cells.

3.5. STAT3 deficiency promotes acinar-to-β reprogramming in vivo

To further assess the role of STAT3 signaling in cellular reprogramming into insulin-producing cells *in vivo*, we developed a mouse model to induce acinar-to-β reprogramming by crossing *Elastase-CreERT2* mice (*EC* mice) with *CAG-CAT- Pdx1, CAG-CAT- Ngn3*, and *CAG- CAT- Mafa* mice, which exogenously express PDX1, NEUROG3, and MAFA, respectively (Fig. 3A). When *Elastase-CreERT2; CAG-CAT-Pdx1; CAG-CAT- Ngn3; CAG- CAT- Mafa* (*EC-PNM*) mice are treated with tamoxifen, the floxed sequence is removed by Cre recombinase, and the CAG promoter induces the expression of PDX1, NEUROG3 and MAFA in acinar cells (Fig. 3A). It is noted that the Flag-, Myc-, or HA-tagged sequence is attached to each transgene, so that the exogenous expression of each transcription factor can be distinguished from the endogenous expression of the genes (Fig. 3A). Immunostaining against pSTAT3 and each tagged sequence demonstrated that the phosphorylation of STAT3 was induced in the pancreata of *EC; CAG-CAT-Pdx1* and *EC; CAG-CAT-Mafa* mice, but not in *EC; CAG-CAT-Ngn3* mice (Fig. S9A), which is consistent with the *in vitro* data in mPAC cells (Fig. 1E, F, and
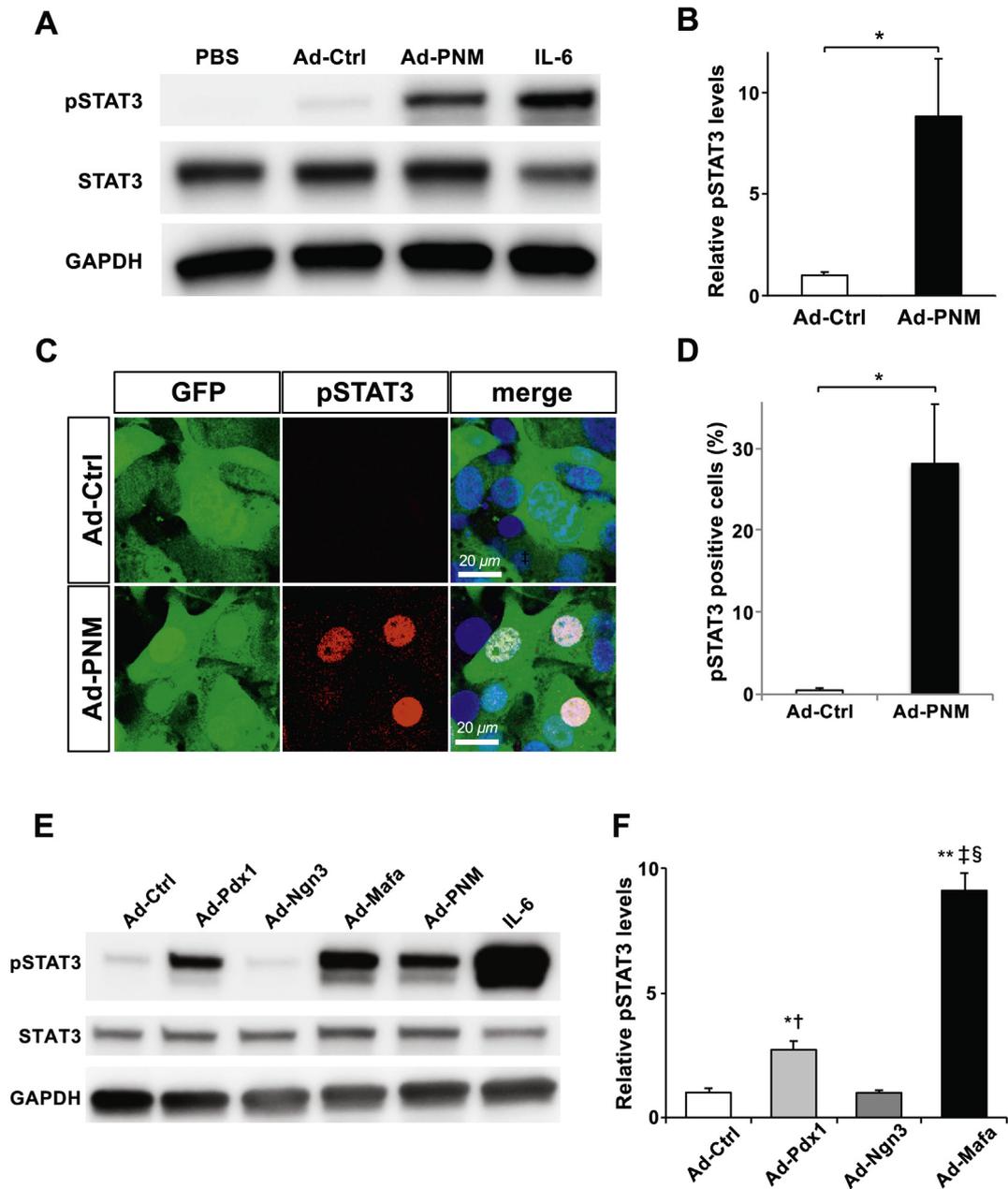
Fig. 1. Expression of Pdx1 and/or Mafa induces phosphorylation of STAT3 in mPAC-MIP-RFP Cells. (A) mPAC cells were infected with a polycistronic adenoviral vector expressing Pdx1, Neurog3 and Mafa (Ad-PNM) or a control adenovirus expressing GFP (Ad-Ctrl). Protein levels of phosphorylated STAT3 (pSTAT3), total STAT3, and GAPDH were assessed by Western blot 48 h after adenoviral infection. IL-6-treated cells were used as positive control. (B) Quantification for Western blots shown in A. The expression levels were normalized to those in Ad-Ctrl-infected cells. *, $p < .05$ ($n = 5$ in each group). (C) Immunocytochemical staining for pSTAT3 was performed in mPAC cells 48 h after adenoviral infection. pSTAT3-positive cells (red) were observed predominantly in GFP-expressing cells (green). Scale bars, 20 μm. (D) The ratio of pSTAT3-positive cells to GFP-expressing cells was quantified. *, $p < .05$ ($n = 3$ in each group). (E) mPAC cells were infected with Ad-Ctrl, Ad-Pdx1, Ad-Neurog3 (Ad-Ngn3), Ad-Mafa, or Ad-PNM, and protein levels of pSTAT3 were assessed by Western blotting. (F) Quantification of Western blot bands shown in E. Expression levels were normalized to those in Ad-Ctrl-infected cells. *, $p < .05$ and ** $p < .01$ *versus* Ad-Ctrl, †, $p < .05$ and ‡, $p < .01$ *versus* Ad-Ngn3, §, $p < .01$ *versus* Ad-Pdx1 ($n = 9$ in each group).

Fig. S2). In addition, immunostaining against pSTAT3, FLAG-tag, and insulin in the pancreata of *EC; PNM* mice demonstrated that most of FLAG-tag-positive (Pdx1-expressing) cells were positive for pSTAT3 but negative for insulin at 3 days after tamoxifen injection. Furthermore, most Myc-tag-positive (Mafa-expressing) cells were positive for pSTAT3 (Fig. 3B and Fig. S9B), suggesting that STAT3 can be activated by either Pdx1 or Mafa. On the other hand, most FLAG-tag/insulin double-positive cells were negative for pSTAT3 a week later (Fig. 3B). These results suggested that STAT3 activation is enhanced by exogenous Pdx1 or Mafa, and is then suppressed during acinar-to-β reprogramming, which is also consistent with the *in vitro* data in mPAC cells (Fig. 2A and B).

Next, floxed Stat3 mice [26] were crossed with *EC-PNM* mice to induce acinar-to-β reprogramming under Stat3 deficiency. Immunostaining for insulin and the FLAG-tag in the pancreata of *Stat3^{flox/flox}* (*Stat3^{KO}*); *EC; PNM* and *Stat3^{flox/+}* (*Stat3^{Het}*); *EC; PNM* mice demonstrated a 2.9-fold increase in the number of reprogrammed β cells derived from acinar cells in the pancreata of *Stat3^{KO}; EC; PNM* mice compared with *Stat3-^{Het}; EC; PNM* mice (Fig. 3C, D, and Fig. S11A). As all the induced insulin-producing cells were positive for FLAG-tag, HA-tag, and Myc-tag in the pancreata of *EC; PNM* mice (Fig. 3B and Fig. S10), the combined expression of Pdx1, Neurog3, and Mafa appears to be essential for inducing acinar-to-β reprogramming. Furthermore, the number of islet-like clusters, consisting of three or more insulin-positive cells, were robustly
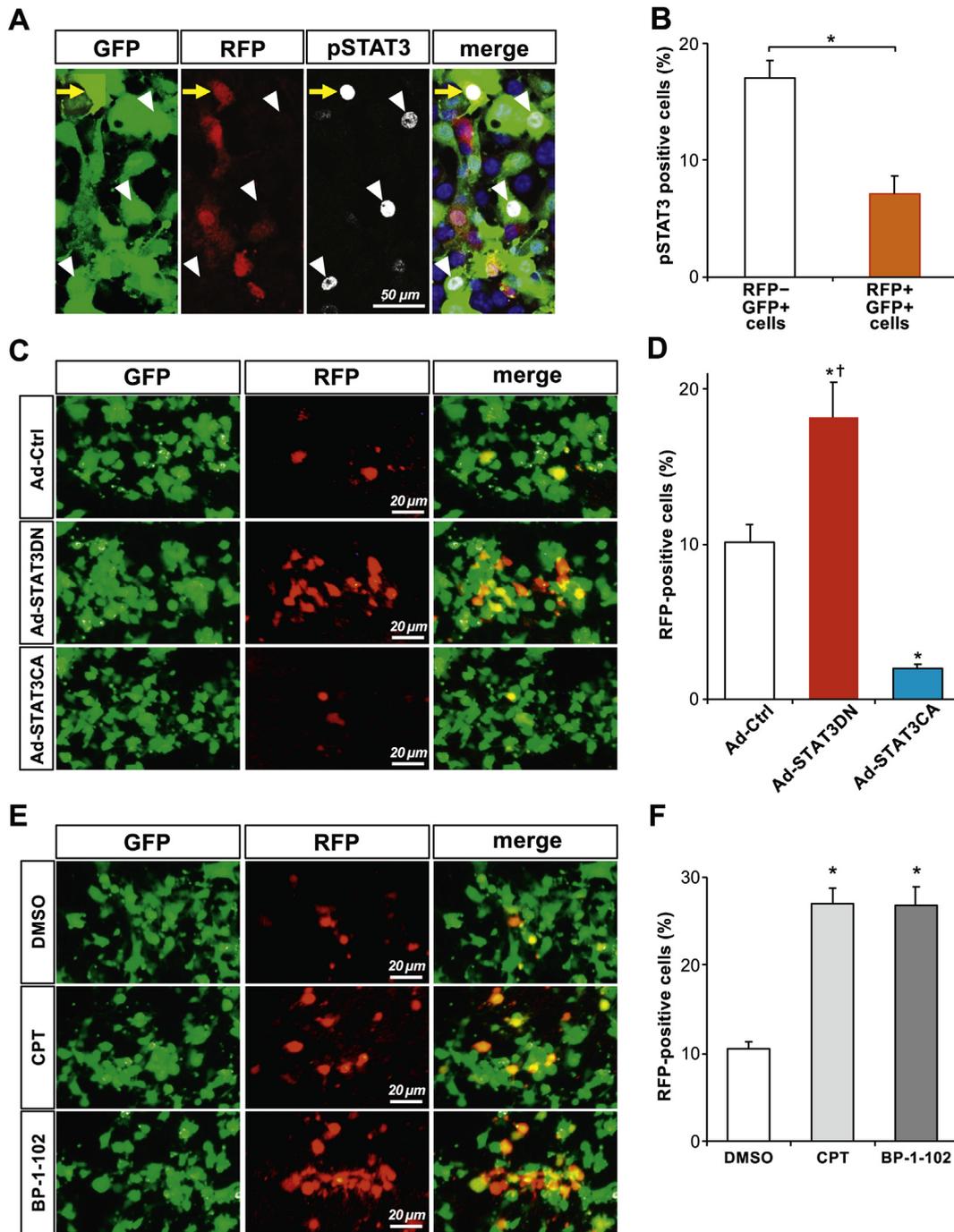
Fig. 2. Phosphorylation of STAT3 is suppressed in insulin-producing cells. (A, B) Immunocytochemical staining for pStat3 (white) in mPAC-MIP-RFP cells 3 days after Ad-PNM infection. Red fluorescent protein (RFP) is expressed under the control of the mouse insulin 1 promoter in mPAC-MIP-RFP cells. The yellow arrows indicate pSTAT3/RFP double-positive cells, and white arrowheads indicate pSTAT3-positive/RFP-negative cells. The percentage of pSTAT3-positive cells among RFP-negative/GFP-positive cells or RFP/GFP double-positive cells was quantified (B). *, $p < .05$ ($n = 4$ in each group). (C, D) The mPAC-MIP-RFP cells were infected with Ad-PNM, together with an adenovirus expressing a dominant-negative form of STAT3 (Ad-STAT3DN) or a constitutively active form of STAT3 (Ad-STAT3CA), and fluorescence images of mPAC-MIP-RFP cells were captured 72 h after Ad-PNM infection. As a control, Ad-Ctrl expressing only GFP was used instead of Ad-STAT3DN or Ad-STAT3CA. The percentage of RFP-positive cells among GFP-expressing cells was quantified. *, $p < .05$ *versus* Ad-Ctrl, †, $p < .01$ *versus* Ad-STAT3CA. (E, F) The mPAC-MIP-RFP cells were infected with Ad-PNM, and treated with cryptotanshinone (CPT; 1 μM), BP-1-102 (10 μM), or DMSO. The percentage of RFP-positive cells among GFP-expressing cells was quantified. *, $p < .05$ *versus* DMSO.

increased in the pancreata of *Stat3*^{KO}*; EC; PNM* mice (Fig. 3E and Fig. S11A). It is noted that the increased number of reprogrammed β-like cells in the pancreata of *Stat3*^{KO}*; EC;PNM* mice were positive for urocortin 3 (Ucn3), a marker of mature β cells (Fig. S11B and C). Thus, the suppression of STAT3 signaling not only increases the number of β-like cells but also induces the formation of islet-like cell clusters expressing Ucn3, suggesting that STAT3 inhibition promotes β-cell maturation.

3.6. STAT3 inhibitor BP-1-102 promotes acinar-to-β reprogramming and ameliorates hyperglycemia in diabetic mice

Both the *in vitro* and *in vivo* experiments described above have shown the beneficial effects of STAT3 inhibition in generating insulin-producing cells. To further investigate whether the reprogrammed β-like cells induced by the exogenous expression of defined transcription factors, together with STAT3 inhibition, can ameliorate hyperglycemia
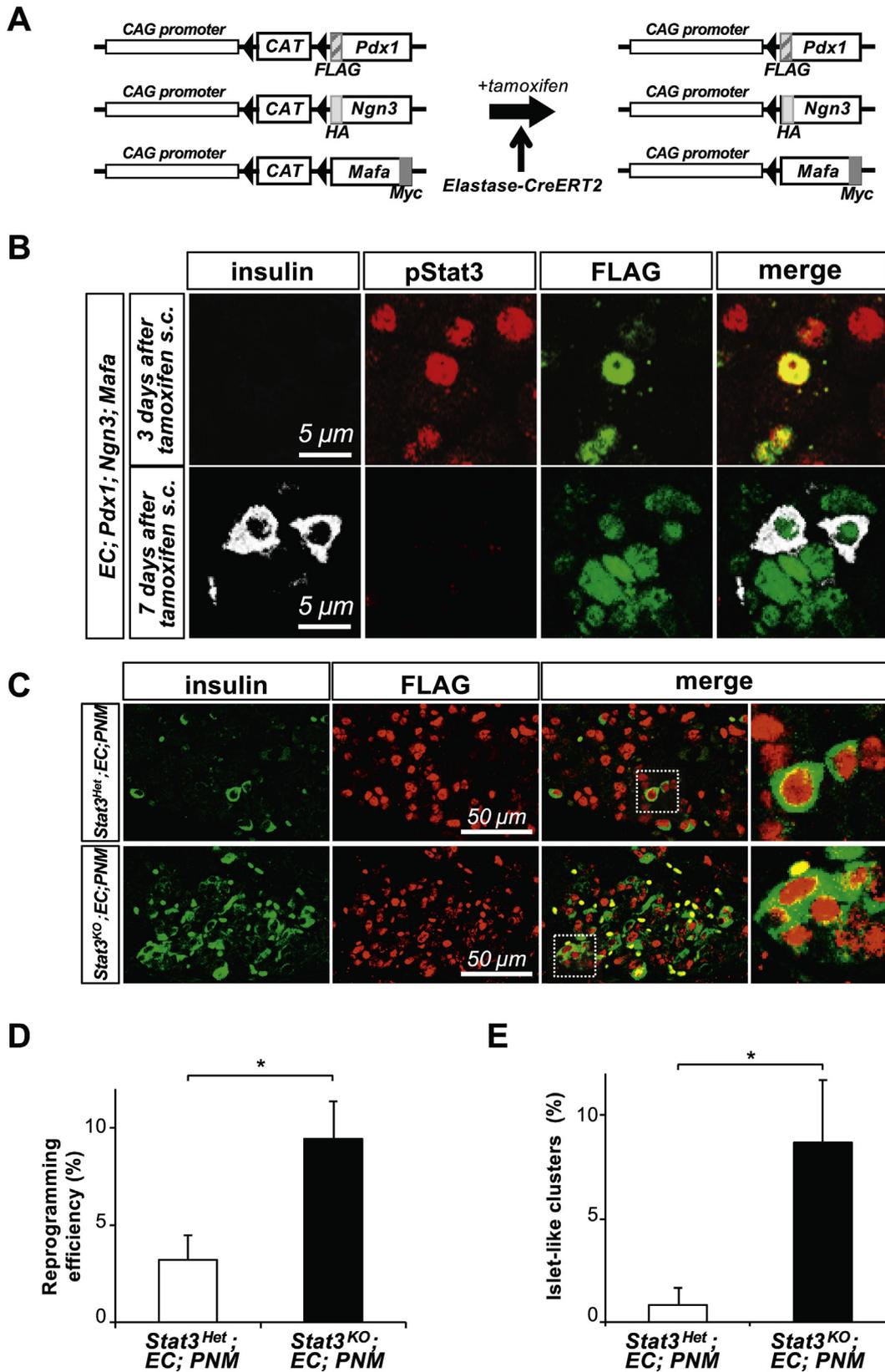
Fig. 3. Stat3 deficiency increases acinar-to-beta reprogramming *in vivo*. (A) Schematic representation of the transgenes and their Cre-mediated recombination in *Elastase-CreERT2; CAG-CAT-Pdx1; CAG-CAT-Neurog3; CAG-CAT-Mafa* (*EC;PNM*) mice. Before recombination, the transcription of Pdx1, Neurog3 and Mafa is blocked by the floxed STOP cassette. When the mice are treated with tamoxifen, the floxed sequence is removed by Cre recombinase, and the CAG promoter activates the expression of Pdx1, Neurog3 and Mafa. (B) Immunostaining for insulin (white), pSTAT3 (red) and FLAG-tagged Pdx1 (green) in the pancreas of *EC;PNM* mice 3 days or 7 days after tamoxifen administration. Scale bars, 5 μm. (C) Immunostaining for insulin (green) and FLAG-tagged Pdx1 (red) was performed in the pancreata of Stat3-deficient *EC;PNM* (*Stat3^{KO};EC;PNM*) mice and control mice (*Stat3$^{flox/+(Het)}$; EC; PNM*) that were sacrificed one week after tamoxifen injection. Islet-like clusters were observed in the pancreata of *Stat3^{KO};EC;PNM* mice. Scale bars, 50 μm. (D, E) *Stat3^{KO}; EC; PNM* and control mice at the age of 2–6 months were treated with tamoxifen, and sacrificed 7 days after the tamoxifen injection to count the percentage of reprogrammed-β cells among FLAG-positive cells (D), and the percentage of islet-like-clusters consisting of three or more insulin-positive cells among total reprogrammed β cells (E). *, $p < .05$, n = 4 in each group.

in diabetic mice, immune-deficient *Rag1*$^{-/-}$ mice were injected with ALX, infected with Ad-PNM, and treated with the STAT3 inhibitor BP-1-102 or DMSO as a control. Immunofluorescence images demonstrated that insulin-producing cells were detected from 3 days after Ad-PNM infection in the mice treated with DMSO or BP-1-102 (Fig. S12A). Whereas some GFP-expressing cells were positive for pSTAT3, no insulin/pSTAT3 double-positive cells were observed in the mice treated with Ad-PNM (Fig. S12B). As shown in Fig. 4A and B, whereas there was no significant difference in daily blood glucose levels between the groups until 5 days after Ad-PNM infection, BP-1-102 significantly ameliorated hyperglycemia from 6 days after the adenoviral infection. In addition, immunostaining for insulin resulted in a significant increase in the number of insulin-producing cells in mice treated with BP-1-102 compared with vehicle (Fig. 4C and D), suggesting that STAT3 inhibition by BP-1-102 ameliorates hyperglycemia by enhancing acinar-to-β reprogramming.

4. Discussion

Whereas several studies demonstrated that non-β cells can change their cell fates into insulin-producing cells by the exogenous expression of Pdx1, Neurog3, and Mafa [3,4,12,15,31], additional research is anticipated for identifying methods to increase the reprogramming efficiency into β cells for the cure of diabetes [17]. In the present study, both *in vitro* and *in vivo* experimental models demonstrated that the suppression of STAT3 signaling significantly enhanced the cellular reprogramming into β cells, accompanied by an increased number of islet-like clusters *in vivo*.

Our *in vitro* experiments also demonstrated that the activation of STAT3 signaling robustly inhibits the cellular reprogramming of mPAC cells into insulin-producing cells. These findings are consistent with the phenotypes of neonatal diabetes caused by activating mutations in *STAT3* [22,29]. Saarimaki-Vire et al. recently reported the activating
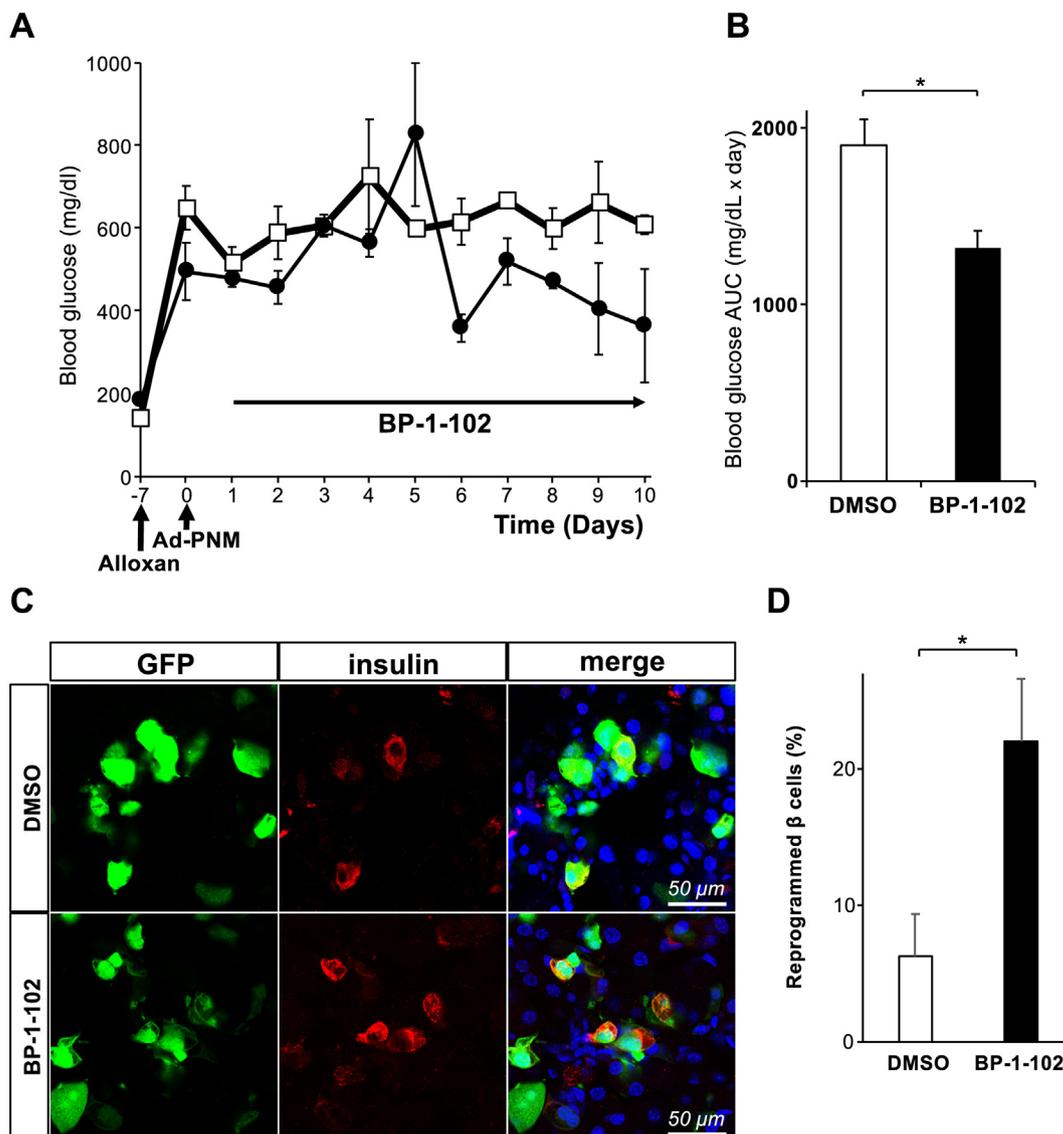
Fig. 4. STAT3 inhibitor ameliorates hyperglycemia and promotes acinar-to-β reprogramming *in vivo*. (A, B) Improvement of blood glucose levels in diabetic mice after administration of the STAT3 inhibitor BP-1-102. Rag1-deficient mice were administered ALX to induce β-cell ablation at the age of 10 weeks, and were then injected with an adenovirus carrying Pdx1-Neurog3-Mafa (Ad-PNM) 7 days after ALX administration. Blood glucose levels were monitored every day through tail vein blood (A), and the mice were sacrificed 10 days after adenoviral injection. Arrows indicate the timing of injection. Area under the curve (AUC) of glucose levels between 7 and 10 days was calculated (B). *, $p < .05$; $n = 3$ in each group. (C) Immunostaining for insulin (red) was performed in the pancreata of Ad-PNM-treated mice. Some of the green-fluorescent adenovirus-infected cells were positive for insulin. (D) The number of reprogrammed insulin-producing cells was significantly increased in the mice treated with BP-1-102 compared with control mice. *, $p < .05$; n = 4 in each group.

mutation STAT3^K392R in a patient with neonatal diabetes, and an *in vitro* study using the patient-derived iPS cells demonstrated that aberrant STAT3 signaling caused premature endocrine differentiation through induced *NEUROG3* expression [22]. On the other hand, our *in vitro* data showed that STAT3 activation inhibits reprogramming into β cells regardless of sustained NEUROG3 expression (Fig. 2C and D). Taken together, these findings suggest that STAT3 signaling controls β-cell differentiation in both NEUROG3-dependent and NEUROG3-independent manners (Fig. S4).

In contrast to the negative effects of STAT3 activation on the cellular reprograming into β cells described above, it has been reported that STAT3-dependent NEUROG3 induction is required for the cellular reprogramming of acinar or ductal cells into insulin-producing cells [2,28]. This difference can be explained by the different timing of NEUROG3 expression in our study, which was exogenously induced regardless of STAT3 activation. Considering these studies together, STAT3 activation may have two sequential and distinct effects in the pancreas, namely, the induction of NEUROG3 and then inhibition of the cellular reprogramming into β cells. In other words, the precise timing and level of STAT3 activation is thought to be essential for controlling cellular reprogramming of non-β cells into insulin-producing cells in the pancreas. Small-molecule STAT3 inhibitors, such as cryptotanshinone and BP-1-102, can be used for the tight regulation of STAT3 activity, as these chemicals were shown to enhance β-cell formation *in vitro* and *in vivo* (Figs. 2E, F, and 4A–D).

Our *in vitro* and *in vivo* studies demonstrated that STAT3 is phosphorylated in Pdx1-expressing or Mafa-expressing cells, but not in Neurog3-expressing cells (Fig. 1E, F, and Figs. S2A, B and S9A), or rather, STAT3 phosphorylation tended to be suppressed in Neurog3-expressing cells compared with control cells (Fig. S2B). As STAT3 phosphorylation is likely to be more highly induced by Ad-Pdx1 or Ad-Mafa compared with Ad-PNM (Fig. 1D and Fig. S2B), it is possible that exogenous Neurog3 negatively regulates STAT3 phosphorylation induced by Pdx1 and Mafa in Ad-PNM-treated cells. In addition, it is noted that pSTAT3 was not detected in GFP-negative cells surrounding Pdx1-expressing or Mafa-expressing cells (Fig. S2A). These findings suggest that the exogenous expression of Pdx1 or Mafa appears to activate STAT3 in a cell autonomous manner at least in our *in vitro* experiments, although the underlying molecular mechanisms by which STAT3 is activated during the cellular reprogramming remains to be elucidated.

The small molecule STAT3 inhibitor BP-1-102 as well as genetic deletion of Stat3 significantly increased the efficiency of β-cell formation *in vivo*, and BP-1-102 ameliorated hyperglycemia in chemically induced diabetic mice (Fig. 4A–D). A number of STAT3 inhibitors have been developed for the treatment of patients with cancer, and some of them are tested in clinical trials [21]. As STAT3 deletion has been shown to result in no apparent phenotype during normal pancreas development [6,19], we expect that targeting STAT3 signaling will also assist in the development of novel therapeutic strategies for the cure of diabetes with few safety concerns.

Author contributions

M.M., T.Mi., T.K., S.S., L.S., M.H., and T.Ma. performed the experiments. T.Mi. and H.W. supervised the study. M.M., T.Mi. ad H.W. wrote the paper. Y.N., Y.F. and T.Ma. contributed to the interpretation of the data.

Conflict of interest

There is no conflict of interest on this study.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.09.035.

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