# TFP5, a Peptide Derived from Cdk5 Activator p35, Protects Pancreatic β-cells from Glucose Toxicity

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# Abstract

Type 2 diabetes mellitus (T2DM) is worldwide epidemic, which challenges the health of the public. The pathological hyperactivity of cyclin-dependent kinase 5 (Cdk5) contributes to the pathogenesis of T2DM. P5, a peptide derived from the Cdk5 activator p35, shows excellent performance as a Cdk5 activity inhibitor. However, its inhibitory effect and functional regulation on Cdk5 activity needs to be confirmed. In this study, we conjugated P5 with a fluorescein isothiocyanate (FITC) tag at the N-terminus and a TAT protein transduction domain, an 11-amino acid peptide, at the C-terminus to synthesize TFP5, which was used to inhibits Cdk5 activity. We then evaluated the efficiency of TFP5 in treating T2DM. We demonstrated that TFP5 effectively penetrated pancreatic β-cells, inhibited the pathological hyperactivity of Cdk5, enhanced insulin secretion, and protected penetrated pancreatic β-cells (MIN6 cells) from apoptosis in pancreatic tissues of db/db mice (type II diabetes mice). Furthermore, we found that TFP5 reduced inflammation in pancreatic islets by reducing the expression of inflammatory cytokines, including TGF-β1, TNF-α, and IL-1β. These data indicates that the TFP5 peptide is a promising candidate for T2DM treatment.


# Impact Statement

We conjugated P5 with a Fluorescein Isothiocyanate (FITC) tag at the N-terminus and a TAT protein transduction domain containing an eleven amino acids peptide at the C-terminus to form TFP5, to explore the efficiency of TFP5 as T2DM therapy. TFP5 effectively penetrated pancreatic β-cells, inhibited the hyperactivity of Cdk5, enhanced insulin secretion, and protected pancreatic β-cells from apoptosis in pancreatic tissues of db/db mice. TFP5 also reduced inflammation in pancreatic islets by reducing the level of inflammatory cytokines, including TGF-β1, TNF-α, and IL-1β.

# Introduction

Type 2 diabetes mellitus (T2DM), with incidence increasing at an alarming rate, is associated with high morbidity and mortality and has become a global public health problem[1]. However, no effective strategies for hindering the progression of T2DM have been approved. During the progression of T2DM, the deficient insulin secretion by pancreatic b-cells and resistance to insulin in peripheral target tissues are critical pathological features, which are correlated with aberrant activation and dysregulation of multiple proteins.

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase that is activated by specific coactivators, namely, p35 and the p39 active complex[2]. Recent studies have demonstrated that Cdk5 exhibits critical hyperactivity under diabetic conditions and is involved in mitochondrial dysregulation in podocytes in diabetic nephropathy[3] and Endoplasmic reticulum stress (ER) in chronic hyperglycemia[4]. Long term hyperglycemia stimulates the formation of advanced glycation end products (AGEs) in the body. AGEs and its receptors combine to produce oxidative reaction and produce a variety of oxidative stress products, which will destroy the mitochondrial structure of the endothelial cells of both large and small vessels, as well as in the myocardium, induce apoptosis, activate nuclear transcription factor κB signaling pathway, cause cellular inflammatory response[5], In addition, islets β cells can also be damage in the similar manner. Besides, oxidative stress interferes with the phosphorylation of insulin receptor and insulin receptor

substrate, affects the activation of phosphatidylinositol 3-kinase and induces insulin resistance[6]. And in our recent studies, we have demonstrated the relevance of oxidative stress level and diabetes.

In addition, researchers have shown that aberrant Cdk5 activation can cause pancreatic b-cell failure, inhibit differentiation and neogenesis[7]. More importantly, it has recently been reported that Cdk5/p35 is an attractive candidate for disease therapy[8,9]. Therefore, we hypothesized that Cdk5/p35 is a novel therapeutic target for T2DM treatment.

In our previous studies, we demonstrated that the activity of p35, which is cleaved by the calpain protease into p25 and p10 fragments, was significantly increased under pathological high-glucose conditions and played a pivotal role in Cdk5 hyperactivation and pancreatic b-cell dysfunction[10,11].Additionally, we found that the P5 fragment of p35 (a peptide of 24 amino acid residues) shows inhibitory potential against Cdk5/p25 pathological hyperactivity[12]. Then, we found that TFP5 can inhibit Cdk5/p25 activity effectively without affecting the endogenous Cdk5/p35 or other Cdks[13,14]. The efficiency of TFP5 treatment has been demonstrated in Alzheimer's disease and Parkinson's disease mouse models[15-16]. In our previous research, we found that TFP5 could inhibit the hyperactivity of Cdk5 induced by high glucose culture, protect pancreatic cells from apoptosis, and recover insulin secretion in MIN6 cells. However, whether TFP5 works as the same way in vivo, such as diabetic animal models, is remained to be explored.

In this study, we focused on determining whether TFP5 could impact insulin secretion and pancreatic β-cell survival in both in vitro and in vivo. We designed a study which systematically illustrated the mechanism of how TFP5 protected pancreatic β-cells from glucose toxicity by the inhibition of Cdk5 hyperactivity through in vivo and in vitro experiments. We incubated high-glucose stressed MIN6 cells with the TFP5 peptide and found that TFP5 effectively inhibited the pathological Cdk5 hyperactivity and the secretion of insulin was reestablished in MIN6 cells. Similarly, purified pancreatic islet cells obtained from db/db mice also showed higher level of insulin secretion in TFP5 treated cells than that of cell treated by scramble peptide. Furthermore, we found that the reduction in inflammation and apoptosis was responsible for the protection of TFP5 on MIN6 and islet cells. Together, these results indicated that TFP5 may be a therapeutic candidate for T2DM therapy by reducing pathological Cdk5 hyperactivation.

# Materials And Methods

### Animals and treatment groups

All animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke/National Institute on Deafness and Other Communication Disorders (NINDS/NIDCD) (protocol is ASP: 1231-11) and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (protocol K058-KDB-10) in Bethesda, Maryland. The db/db mice and C57BL/6J mice (8-weeks-old, male) were obtained from Jackson Laboratory (Bar Harbor, ME, USA).

12 db/db mice and 12 C57BL/6J mice were used in this study. The C57BL/6J and db/db mice (Type II diabetes mice) were randomly assigned to 2 groups with equal size. Six mice in one group received TFP5 (C57BL/6J + TFP5, n=6), and other six mice received a scrambled peptide (C57BL/6J + SCB, n=6); similarly, six of the db/db mice received TFP5 (db/db + TFP5, n=6) and other six mice received the scrambled peptide (db/db + SCB, n=6). All treatments were administered by i.p. injection, and the dose and time of injection were determined on the basis of previous research[17]. All mice were housed with a 12-h light/dark cycle and had free access to standard chow diet and water[18].The timeline and dosage details are shown in Supplementary Figure S1.

## Antibodies and reagents

Anti-Cdk5 (C-8) (1:1000) antibodies were obtained from Santa Cruz Biotechnology (Dallas, Texas). Anti-cleaved Caspase-3 ($Asp^{175}$) (1:1000), anti-Caspase-3 (9662) (1:1000), anti-α-tubulin (2144) (1:1000) and anti-P35/25 (C64B10) (1:1000) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-TGF-β1, anti-TNF-α, anti-IL-1β and anti-insulin antibodies (1:500-1000) were obtained from Abcam (Cambridge, MA). Anti-β-tubulin (AC010) (1:5000) antibodies were obtained from ABclonal (Wuhan, China). Secondary horseradish peroxidase-conjugated antibodies (1:2000) were obtained from Amersham Biosciences (Piscataway, NJ). Secondary fluorescence-conjugated Oregon Green and Texas Red antibodies (Molecular Probes, Eugene OR) were used after dilution at 1:400. Collagenase XI was purchased from Sigma. A rat/mouse insulin ELISA kit was obtained from Millipore (Billerica, MA).

## Design and synthesis of TFP5

TFP5 was designed and synthesized as described previously[10,11]. Both TFP5 and the scrambled peptide (SCB) were synthesized by Peptide 2.0 (Chantilly, VA, USA). The TFP5 sequence was FITCGGGKEAFWDRCLSVINLMSSKMLQINAYARAARRAARR, and the SCB sequence was FITCGGGGGGFWDRCLSGKGKMSSKGGGINAYARAARRAARR.

## Generation of recombinant adenoviruses

We used an adenoviral vector (pAdTrack-CMV) packaging system to construct the p5 forward primer, TTTGCGGCCGCCATGGCATCAATGCAGAAGCTGATCTCAGAGGAGGACCTGATGAAGGAGGCCTTTTGGGACCG, and the reverse primer, TTTGATATCTTAGGCATTTATCTGCAGCATCTTT. The generation of the recombinant adenovirus was carried out as described previously[10].

## Cell culture and treatment

MIN6 cells (Mouse islet beta cells) were cultured in DMEM with 5 mM glucose, 1 mM sodium pyruvate and 10% fetal bovine serum (FBS) with 100U/ml penicillin G and 100 μg/ml streptomycin[10]. MIN6 cells from passages 12-25 were used in experiments. Cells were seeded in 6-well plates with $0.3\times 10^5$ cells/$cm^2$ one day before use. Cells were infected with adenovirus-p5 separately for 24 h-48 h and then exposed to TFP5 or SCB (500 nM) for 24 h. Then, cells were starved overnight with glucose-free medium, treated with different concentrations of glucose (low glucose, 5 mM, and high glucose, 25 mM) and incubated at 37°C for 24 h.

Cells were fixed for immunohistochemistry analysis or lysed for immunoprecipitation, kinase activity, and western blot analyses.

### Mouse pancreatic islet isolation and culture

Mouse pancreatic islet isolation was carried out using the method described by Li et al [19]. Each pancreas was perfused with a 3-ml injection of collagenase XI solution (1000 U/ml in 1× HBSS) via the common bile duct. Then, the pancreas was removed, and 2 ml of collagenase XI solution was used for digestion at 37°C for 20 min with 2-3 brief shakes of the tube by hand. Digestion was stopped when putting the tube on ice, then added 25 ml of washing solution (1 mM $CaCl_2$ into 1× HBSS) and centrifugated at 290 g for 30 s at 4°C. After two time's washing, the resuspended solution was filtered through a 7-μm nylon cell strainer, and the isolated islets were picked up using a manually operated pipette. To allow cell recover, the hand-picked islets (purity >95%) were cultured overnight in DMEM containing 10% FBS, 100 U/mL penicillin, and streptomycin (HyClone) in a humidified atmosphere of 5% $CO_2$ and 95% air at 37°C and then cultured in RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine, 11 mM glucose, 10% (vol/vol) fetal calf serum, and 1% penicillin/streptomycin.

### Insulin secretion test

β-cells were treated with TFP5 and SCB for 24 h and washed twice with basal Krebs-Ringer's solution bicarbonate HEPES buffer (5 mM glucose, 124 mM NaCl, 5.6 mM KCl, 2.5 mM CaCl2, 20 mM HEPES at pH 7.4, and 0.5% BSA). Cells were then incubated with glucose-free KRBH for overnight starvation. After the medium was discarded, cells were incubated in either basal KRBH containing low glucose (5 mM) or KRBH containing high glucose (25 mM) for another 2 h. The supernatant was then collected to measure the secreted insulin. Insulin released into the supernatant and insulin remaining in cells was measured with LINCO ELISA kits, and insulin secretion was expressed as the concentration of insulin per million cells secreted every 2h.

### Western blot analysis

Western blot analysis was performed as described previously[10]. Briefly, cells were harvested by scraping and then lysed in ice-cold lysis buffer. The protein content was determined using a BCA protein assay (Pierce, Rockford, Illinois). An equal amount of total protein (20 mg of protein/lane) was resolved using 4-20%, 15%, or 8% SDS-polyacrylamide gel electrophoresis and blotted onto a PVDF membrane. The membrane was incubated in blocking buffer for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. The membrane was then washed and incubated with goat anti-mouse or goat anti-rabbit IgG (H+L)-HRP conjugated secondary antibodies (Amersham Biosciences, 1: 2500) for 2 h at room temperature. Western blots were analyzed using an enhanced chemiluminescence kit (Pierce) following the manufacturer's instructions. Here, we need to mention that some blots were cut prior to hybridization with antibodies and were only exposed the area of the blot which we needed, so that it may look as if they were cropped. We have provided both of original images showing half-length and full-length membranes, with membrane edges visible (please see the supplementary materials 2).

### In vitro Cdk5 kinase assays

Kinase assays were performed as previously described[20]. Cdk5 was immunoprecipitated overnight from the supernatants of lysed cells using the polyclonal C-8 antibody at 4°C, and immunoglobulin isolation was performed with protein A-Sepharose beads for 2h at 4°C. Kinase assays were performed in the same lysis buffer used for immunoprecipitation supplemented with 1 mM DTT, 0.1 mM ATP and 0.185 MBq [$\gamma$-$^{32}$P] ATP with 20 mg of histone H1 as the substrate. The phosphorylation assay was performed at a final volume of 50 ml and incubation at 30°C for 60 min. The reaction was stopped by the addition of 10% SDS sample buffer and then heated at 95°C for 5 min. Samples were separated by SDS-PAGE, the gels were stained with Coomassie blue, destained, dried, and exposed via autoradiography.

### Immunofluorescence

MIN6 cells were treated with TFP5, SCB or adenovirus-p5 on glass coverslips. After 24 h, cells were starved overnight with glucose-free medium, treated with different concentrations of glucose (5 mM or 25 mM) and incubated at 37°C for 24 h. Cells were then washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, and permeabilized with buffer (25 mM Tris, pH 7.4; 150 mM NaCl; and 0.1% Triton X-100) for 15 min. The coverslips were incubated overnight at 4°C with primary antibodies. All antibodies were diluted in PBS with 1% Triton-X-100. After three washes with PBS, the coverslips were incubated with fluorescein goat anti-mouse IgG or Texas Red goat anti-rabbit IgG for 1 h at room temperature, followed by three times' washing with PBS. Cell nuclei was counterstained with Hoechst 33342 (Sigma).

Paraffin-embedded mouse pancreatic tissue was cut into 3 μm sections and deparaffinized with xylene and ethanol. Sections were then placed in sodium citrate solution for antigen retrieval, blocked with goat serum for 1 h, and incubated with primary antibodies overnight at 4°C. After sections were incubated with Texas Red goat anti-rabbit or anti-mouse IgG for 1 h at room temperature, nuclei was counterstained with Hoechst 33342. Fluorescent images were obtained with a Zeiss LSM-510 laser-scanning confocal microscope, and images were managed with Adobe Photoshop.

### Statistical analysis

All data analyses presented in figures are based on three replicates of experimental results. A two-tailed Student's test was performed for the comparation between two groups, with statistical significance determined as $p \leq 0.05$. The data are presented as the standard means ± SEM. Histology and immunofluorescence images were generated using Photoshop and quantified using Image J software.

## Results

### TFP5, an Cdk5 inhibitor derived from p35, effectively inhibited pathological Cdk5 hyperactivity.

As demonstrated in our previous studies, the small truncated peptide from p35 (residues 154-279), also known as Cdk5 inhibitory peptide (CIP), is a highly effective and specific inhibitor of Cdk5/p25 activity[12]. To

develop a smaller, more permeable, and easier-to-detect inhibitory peptide of p35/p25, we conjugated P5 with a FITC tag at the N-terminus and a TAT protein transduction domain containing an 11 amino acid peptide at the C-terminus of P5 to synthetize TFP5 (Fig 1A-B). Expression of p5 (using adenovirus with GFP and p35 in MIN6 cells were observed by fluorescence microscopy (Fig 1C, a and b). We also observed the expression of TFP5 and SCB in both the MIN6 cells when treated with TFP5 or SCB (Fig 1D, a and b), and the C57BL/6J mouse pancreas when injected with TFP5 or SCB (Fig 1D, c and d) under fluorescence microscopy. After performing in vitro kinase assays, we found that the viral-infected p5 was a more effective inhibitor than CIP (Fig 1E). We then compared the inhibitory effect of viral-infected p5 with TFP5 through the in vitro kinase assays, and the results indicated that TFP5 inhibited Cdk5 activity as efficiently as infection with p5 (Fig 1E).

### TFP5 effectively inhibited pathological Cdk5 overactivation in vitro and in vivo

First, the effect of TFP5 on pathological Cdk5 overactivation was investigated. We found that the high glucose group showed higher p25 and Cdk5 expression than the low glucose group in vitro (Fig 2A, upper and middle panel, lanes 1 and 2). The statistical analysis of p25 was shown in Fig 2B. TFP5 treatment significantly decreased the expression of p25 and Cdk5 under high glucose conditions (Fig 2A, upper and middle panel, lanes 2 and 3). Consistent with p25 expression profiles, Cdk5 activity was significantly increased under high glucose condition (Fig 2E, lane 2) compared to that under low glucose condition (Fig 2E, lane 1), while cells treated by TFP5 and p5 exhibited significantly reduced Cdk5 activity (Fig 2E, lanes 3 and 4). In addition, we isolated the islets from mice of each group and performed western blot assay. We found that p25 and Cdk5 expression were significantly increased in islets of db/db mice (Fig. 2C, upper and middle panel, lanes 1 and 2). The statistical analysis of p25 was shown in Fig 2D. However, TFP5 exposure could significantly reduce the expression of p25 and Cdk5 (Fig 2C, upper and middle panel, lanes 2 and 3). Similarly, Cdk5 activity was significantly increased in db/db mice (Fig 2F, lane 2, compared to control mice lane 1and 4), while TFP5 treated db/db mice exhibited significantly reduced Cdk5 activity compared with db/db mice treated with scramble peptide. (Fig 2F, lanes 3 compared to lane 2).

### TFP5 reestablished insulin secretion from pancreatic β-cells

Then, we explored the therapeutic efficiency of TFP5. Interestingly, we found that the reduced insulin secretion from the MIN6 cells treated with high glucose (Fig 3A, lane 2 compared to the control) was restored when cells were treated with TFP5 or p5 (Fig 3A, lanes 3 and 4 compared to lane 2). Furthermore, insulin gene expression and insulin secretion were detected in the cultured cell lysates and supernatants of pancreatic islet cells isolated from different groups of mice. We found that both insulin gene expression and insulin secretion were reestablished in pancreatic islet cells after TFP5 treatment in db/db mice (Fig 3B and C, lane 3 compared to lane 2). These data demonstrate that TFP5 can effectively reestablish insulin secretion from the toxicity of glucose damaged pancreatic β-cells.

### TFP5 might protect pancreatic β-cells from high glucose-induced apoptosis

Next, in order to investigate the effect of TFP5 exposure on the apoptosis of pancreatic β-cells, we performed western blot and immunohistochemistry assay to detect the expression of a specific apoptosis marker,

caspase 3. MIN6 cells were treated with TFP5 peptide under high-glucose conditions. MIN6 cells underwent apoptosis when exposed to high glucose levels. However, apoptosis can be attenuated when cells were co-infected with CIP[12]. Therefore, we hypothesized that TFP5 might protect pancreatic β-cells against high glucose-induced apoptosis. To determine whether TFP5 treatment had the same protective role as CIP, MIN6 cells were treated with TFP5 or SCB. After 24 h, cells were starved overnight in glucose-free medium and then treated with 5 mM or 25 mM glucose for 24 h. Results showed that TFP5 exposure decreased the expression level of cleaved caspase 3 in MIN6 cells, and the total caspase 3 level remained the same in cells treated with high glucose (Fig 4A and B). In addition, these results were confirmed in the db/db mouse model. We found that elevated cleaved caspase 3 expression induced by high glucose exposure was decreased significantly upon TFP5 peptide administration (Fig 4C and D). These results indicate that TFP5 might protect pancreatic β-cells from high glucose-induced apoptosis.

### TFP5 protected pancreatic β-cells by reducing inflammation

To further explore the potential protection mechanisms induced by TFP5 exposure in MIN6 cells and db/db mice. As before, MIN6 cells were treated with TFP5 or SCB. After 24 h, cells were starved overnight in glucose-free medium and then treated with 5 mM or 25 mM glucose for 24 h. Given that inflammation is one of the main pathologic features of T2DM, we used western blot analysis to detect the effects of TFP5 on the level of inflammatory factors. Results showed that TGF-β1, TNF-α, and IL-1β expression levels were significantly increased in cells treated with high glucose, but was decreased significantly upon TFP5 peptide exposure (Fig 5A). The increased expression of cytokines in MIN6 cells was significantly reduced upon TFP5 treatment (Fig 5B-D). These results indicated that TFP5 might protect MIN6 cells from high glucose-induced inflammation. In addition, these results were confirmed in the db/db mouse model. Injection paradigm was as described above in db/db mice. Purified pancreatic islet tissue was processed for western blotting to detect the effects of TFP5 on inflammatory factors. We found that TGF-β1, TNF-α, and IL-1β expression levels were significantly increased in the pancreatic islets of SCB treated db/db mice but were significantly decreased in db/db mice treated with TFP5 (Fig 5E). The increased expression of cytokines in the db/db mice was significantly reduced by 30%-50% upon TFP5 treatment (Fig 5F-H).

### The inhibition of inflammation by TFP5 in pancreatic islets

To confirm the function of TFP5 in inhibiting inflammation, immunofluorescence staining was performed to detect the expressions of inflammatory cytokines in pancreatic tissue (Fig 6A). It showed that the expressions of TGF-β1, TNF-α, and IL-1β were significantly increased in pancreatic islet of db/db treated with SCB mice, while the expression of inflammatory cytokines was decreased by 50% in the pancreatic tissues of db/db mice treated with TFP5 (Fig 6B-D).

## Discussion

T2DM accounts for more than 90% of adult diabetes cases and severely affects health and life worldwide. Insulin resistance is the major cause of chronic hyperglycemia in diabetic patients. Currently, therapeutic interventions targeting T2DM are largely limited to control of hyperglycemia. Therefore, exploring promising therapies for T2DM is extremely urgent.

Recent findings strongly suggest that Cdk5-mediated phosphorylation, such as PPARgamma and sirt1 phosphorylation, may be involved in the pathogenesis of insulin resistance in diabetes[21]. Here, we found that TFP5 protects β-cells from glucose toxicity and may act as a therapeutic candidate for T2DM therapy.

T2DM and Alzheimer's diseases are degenerative diseases, and their incidence continues to increase globally[22]. Although insights into the pathogenesis of these diseases are limited, similarities in the pathological changes of brain neurons and pancreatic β-cells have been reported[23-25]. Previous studies have indicated that neuronal dysfunction in AD patients (Alzheimer's disease patients) is partly linked to hyperactivity of multifunctional Cdk5 kinase. It has been proposed that under neurotoxic conditions, p35, the normal regulator of Cdk5, was cleaved into p25 and a p10 fragment by calpain (a calcium-dependent protease)[26,27]. Cdk5 complexed with p25 was stabilized, hyperactivated, and involved in the hyperphosphorylation of tau, causing inflammation and amyloidosis, and eventually leading to neuronal death[16,28]. Thus, it is reasonable to speculate that regulating Ckd5/p35 is a promising pathway for T2DM therapy.

In previous studies, Cdk5 and p35 have been identified in pancreatic islets and β-cells and have been shown to play important roles in insulin secretion[29-31]. We hypothesized that the model of activation of Cdk5/p25 in neurons was the same in stressed pancreatic b-cells and was critical for reduced insulin secretion and increased apoptosis rates. Our previous studies have shown that long-term exposure of MIN6 cells to a high concentration of glucose could trigger p25 expression, which caused Cdk5 hyperactivity, induced apoptosis, and inhibited insulin secretion[11,12]. These results indicated that aberrant Cdk5 activity was involved in the pathogenesis of T2DM. Our data also demonstrated that the Cdk5 inhibitory peptide CIP could specifically inhibit Cdk5/p25 activity, protect b-cells from apoptosis, and restore insulin secretion in MIN6 cells without affecting the activity of endogenous p35 or other kinase[8]. These data suggested that inhibition of Cdk5 pathological hyperactivity might be an efficacious approach to T2DM therapy. Although several potent chemical inhibitors of Cdk5 have been identified and studied[10,32,33], most of these inhibitors compete for ATP binding. Therefore, these compounds lack specificity and induce serious toxic side effects, since all kinases are equally dependent on ATP binding. As a therapeutic agent, although CIP specifically inhibits Cdk5/p25 activity, it is not a small molecule, cannot penetrate cells, and has side effects. P5, a much smaller peptide derived from CIP, was previously identified as a more effective Cdk5/p25 inhibitor than CIP[33]. However, it requires a carrier for transfection into cells. To overcome the low transfection rates and carcinogenicity of viral vector transfection, we used TFP5 in this study. TFP5 is a modified p5 that readily penetrates cells and shows an inhibitory effect equal to that of virally infected p5 on Cdk5/p25-induced pathology of stressed MIN6 cells in vitro and db/db pancreas tissues in vivo following i.p. treatment. In our previous studies, we confirmed that the application of TFP5 could effectively reduce the blood glucose and weight of the db/db mice[17].

Furthermore, the potential mechanism of the effect of TFP5 on T2DM treatment was also explored. Interestingly, we found that inflammation, which is a critical pathogenic factor of pancreatic islet dysfunction, was significantly inhibited in T2DM. Compelling evidence showed that the apoptosis of pancreatic cells was associated with inflammation and that targeting inflammation was a potential

approach to the treatment of T2DM[34]. The cell damage dynamic changed in different stages according to the development of diseases (degeneration). TFP5 can rescue cells by attenuating the inflammatory response, rescuing cell from apoptosis, and increasing insulin secretion. Thus, inhibition of inflammation-related apoptosis of pancreatic cells is a potential mechanism of TFP5 in the treatment of T2DM. This restoration process might be considered that TFP5 might have a regeneration function for T2DM.

Admittedly, there are some limitations in this study. The results reflect only short-term TFP5 exposure. Therefore, long-term exposure to TFP5 is required to assess its effects in vivo in the future exploration, including the effect on blood biochemical indicators, such as the levels of insulin and glycosylated hemoglobin. The molecular mechanism of the effects of TPF5 on inflammation during T2DM treatment should also be investigated.

In this study, we explored the inhibitory function of TFP5 on the aberrant activation of Cdk5/p25 and the regulation of insulin secretion in vivo and in vitro. We demonstrated that TFP5 can protect MIN6 cells from high glucose-induced apoptosis, reduce inflammatory stress in vitro and attenuate diabetes in db/db mice by reducing the hyperactivation of Cdk5 in vivo. These data suggest that future studies of TFP5 can provide information on the mechanisms of drug acting as a means of gaining further insight into the etiology of diabetes.

# Declarations

### Ethics approval and consent to participate

It is confirmed that the study was carried out in compliance with the ARRIVE guidelines. All animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke/National Institute on Deafness and Other Communication Disorders (NINDS/NIDCD) (protocol is ASP: 1231-11) and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (protocol K058-KDB-10) in Bethesda, Maryland. The db/db mice and C57BL/6J mice (8-weeks-old males) were obtained from Jackson Laboratory (Bar Harbor, ME, USA).

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary materials.

### Competing interests

The authors declares no potential conflicts of interest.

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### Authors' contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; SYL, SLC contributed to conception and design, data acquisition, drafting the article, final approval of the version to be published; HYL, XZ, LB contributed to conception and design, data analysis and interpretation, drafting the article;JE, BL, XML, GQZ, XB drafted the article or critically revising it for important intellectual content; YLZ, HCP drafted the article or critically revising it for important intellectual content.

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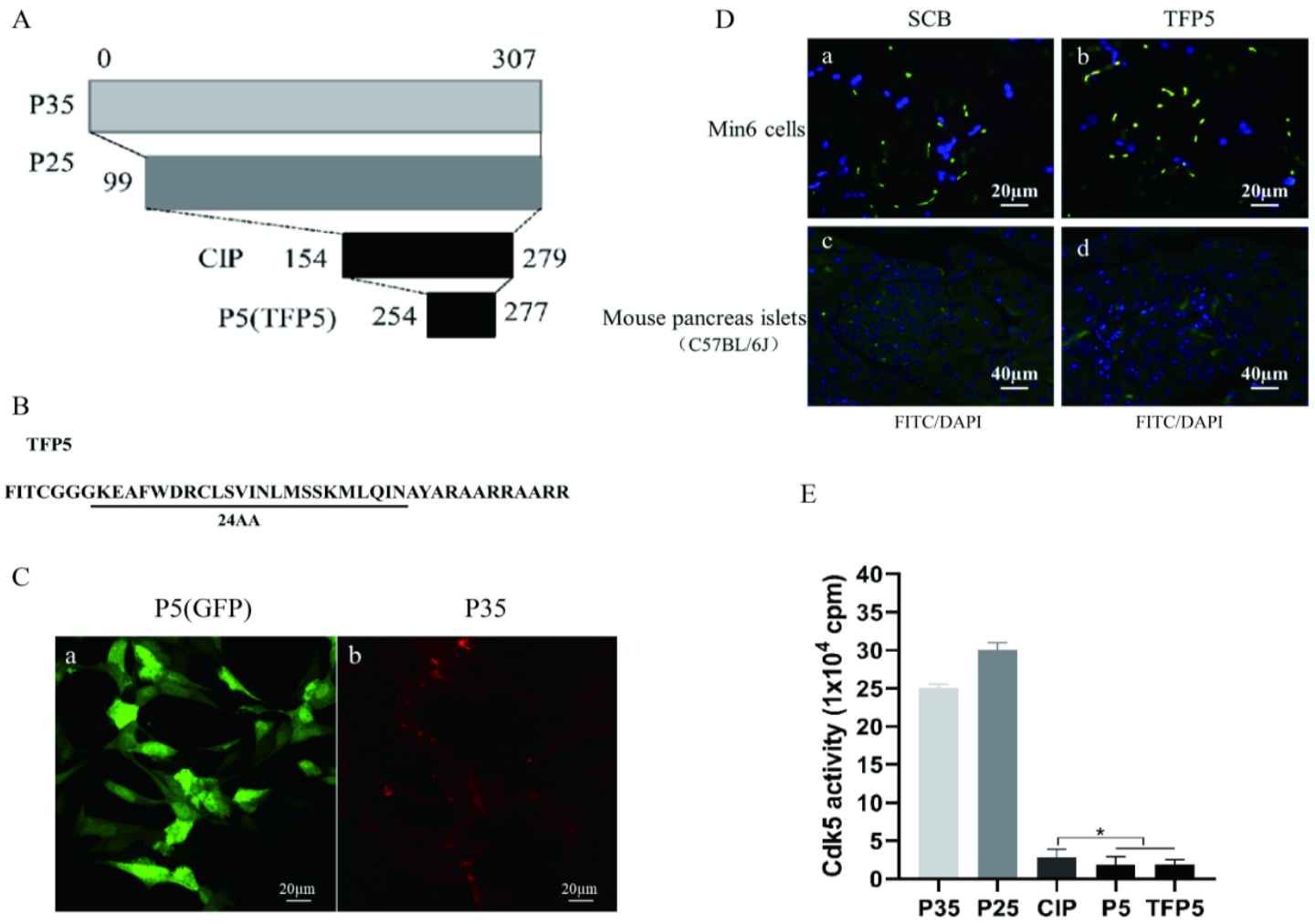
# Figures

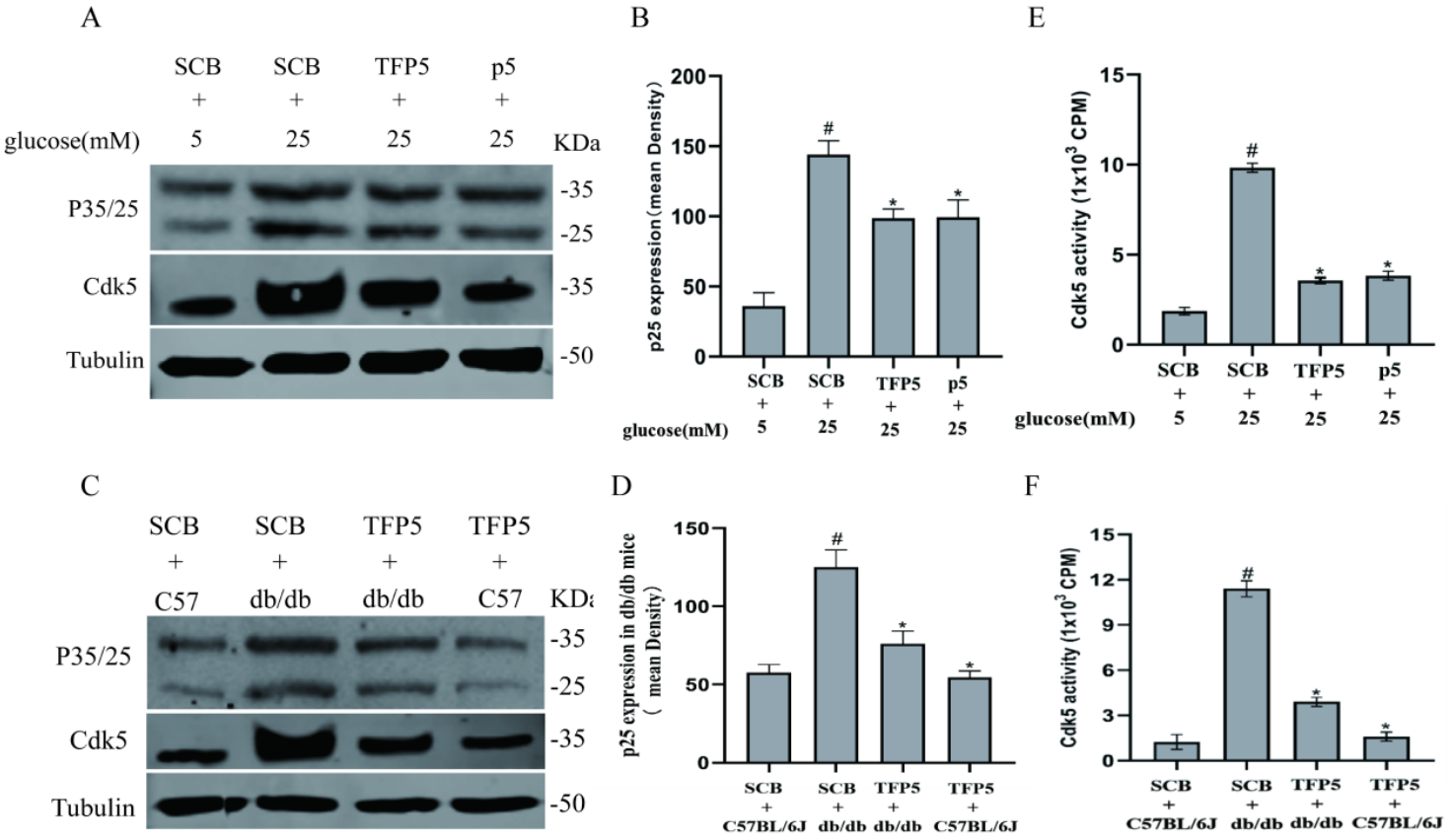
Figure 1




## Figure 1

**Figure 1. TFP5, derived from p35, effectively inhibited Cdk5 activity in high glucose-treated MIN6 cells.** (A) The schematic diagram of p35 truncated peptides shows the 24-residue peptide TFP5 or p5, spanning p35 residues Lys254–Ala277. (B) The TFP5 peptide was conjugated with a FITC tag at the N-terminus and a TAT protein transduction domain peptide at the C-terminus to ease entry into cells. (C) Fluorescence microscopy observed that expression of p5 adenovirus with GFP and p35 (with anti-p35 antibody) in MIN6 cells. (D) Fluorescence microscopy also showed TPF5 and SCB expression in MIN6 cells and mouse pancreatic tissue (n=3). Both TFP5 (500 nM) and SCB (500 nM) were incubated with MIN6 cells or TFP5 (200 μM) and SCB (200 μM) were i.p. injected into C57BL/6J mice. TFP5 and SCB expression in both MIN6 cells and pancreatic tissue. (E) Inhibitory effects of TFP5, CIP, and P5 on Cdk5 activity in stressed MIN6 cells (compared with the CIP group, $*p < 0.05$).

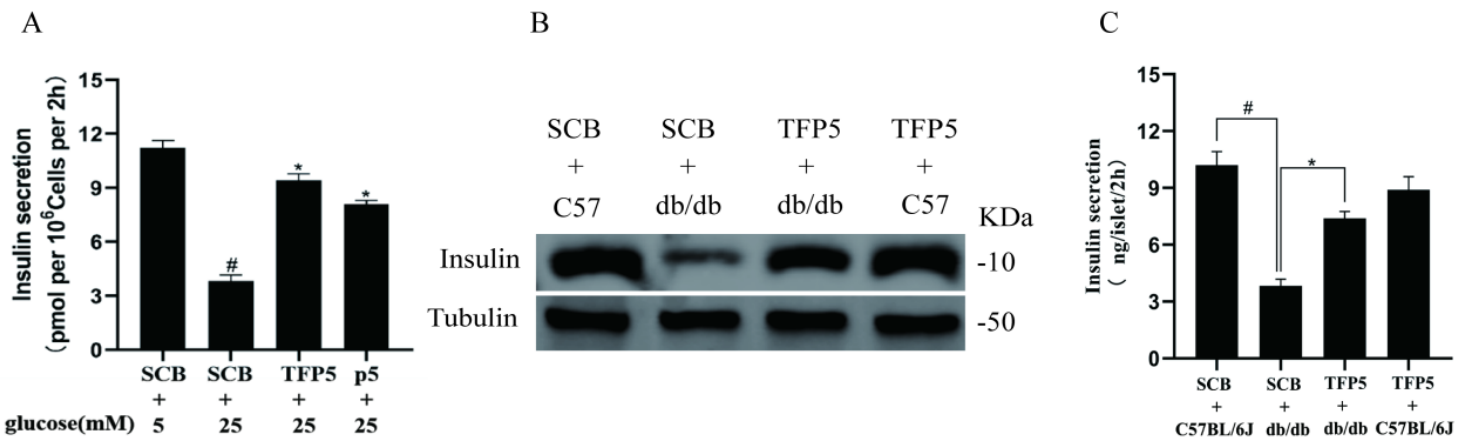
Figure 2




## Figure 2

**Figure 2. TFP5 reduced the hyperactivation of Cdk5 *in vitro* and *in vivo*.** MIN6 cells were exposed to TFP5 or SCB or infected with adenovirus-p5. After 24 h, cells were starved overnight with glucose-free medium and then treated with 5 mM or 25 mM glucose for 24 h. (A) Expression levels of Cdk5, p35, and p25 in MIN6 cells was determined by Western blot analysis. (B) The statistical analysis of p25 expression levels in MIN6 cells of Figure (A). (C) Cdk5, p35, and p25 expression levels in pancreatic islets obtained from db/db mice and C57BL/6J mice as determined by Western blot analysis. (D) The statistical analysis of p25 expression levels in db/db mice of Figure. (E) Quantification of Cdk5 activity in each group of cells. The data represent the mean ± SEM of four experiments (compared with the 5+SCB group, #$p < 0.05$; compared with the 25+SCB group, *$p < 0.05$). (F) Quantification of Cdk5 activity in pancreatic islets in the mice of each group (n=3) (compared with the SCB+C57BL/6J group, #$p<0.05$; compared with the SCB+db/db group, *$p < 0.05$).

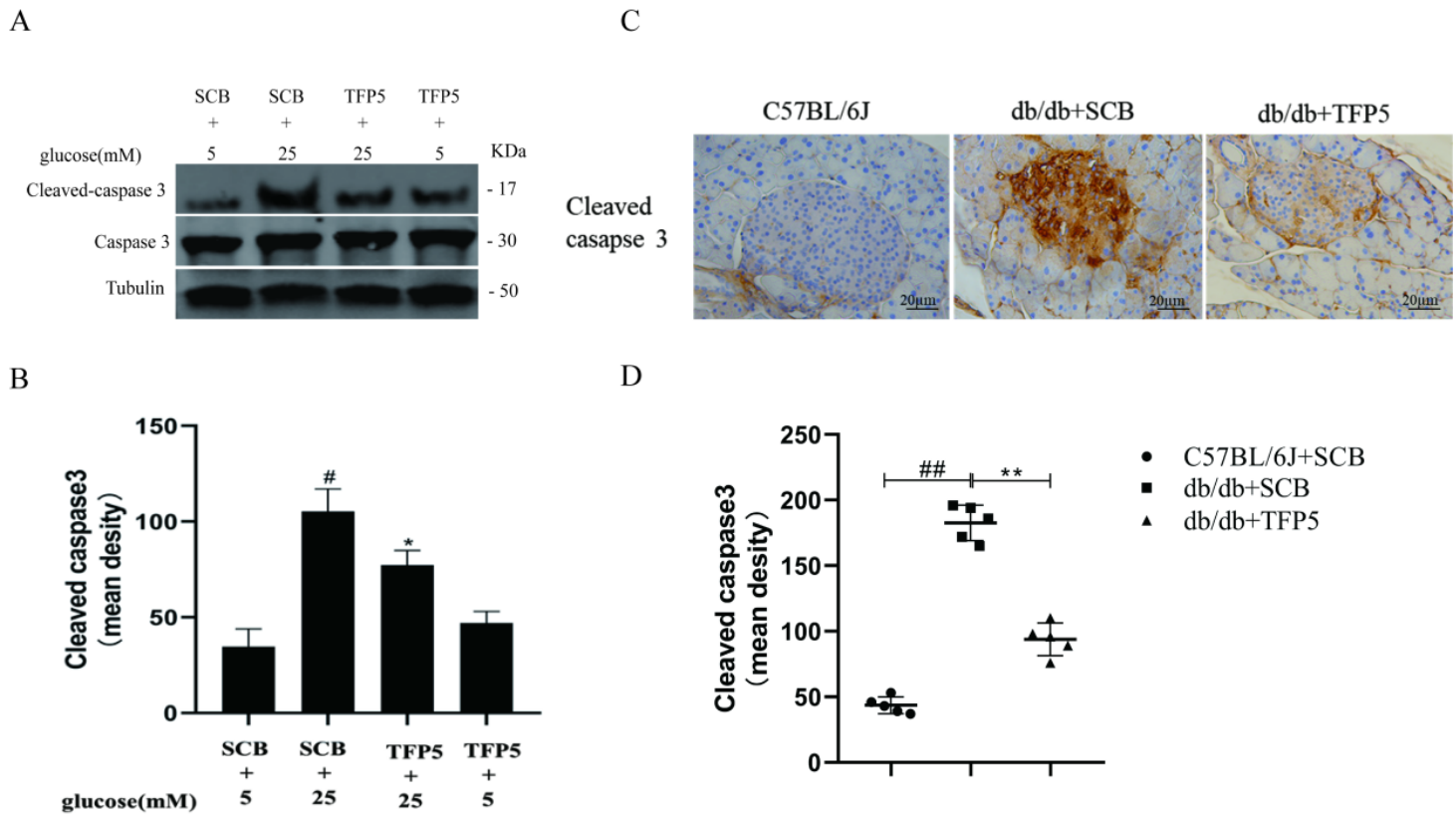
Figure 3




## Figure 3

**Figure 3. TFP5 restored insulin secretion in pancreatic β-cells.** MIN6 cells were exposed to TFP5 or SCB or infected with adenovirus-p5, as shown in Fig 2A. (A) Culture supernatants were collected and subjected to insulin measurement. ELISAs show the levels of insulin secretion in the supernatants. The data represent the mean ± SEM of four experiments (compared with the 5+SCB group, #$p < 0.05$; compared with the 25+SCB group, *$p < 0.05$) (B) Insulin gene expression in db/db mice and C57BL/6J mice as determined by Western blot analysis with anti-insulin antibody. (C) ELISA shows insulin secretion in islet culture supernatants for each group of mice (n=4). The data represent the mean ± SEM of four experiments (compared with the SCB+C57BL/6J group, #$p <0.05$; compared with the SCB+db/db group, *$p < 0.05$).

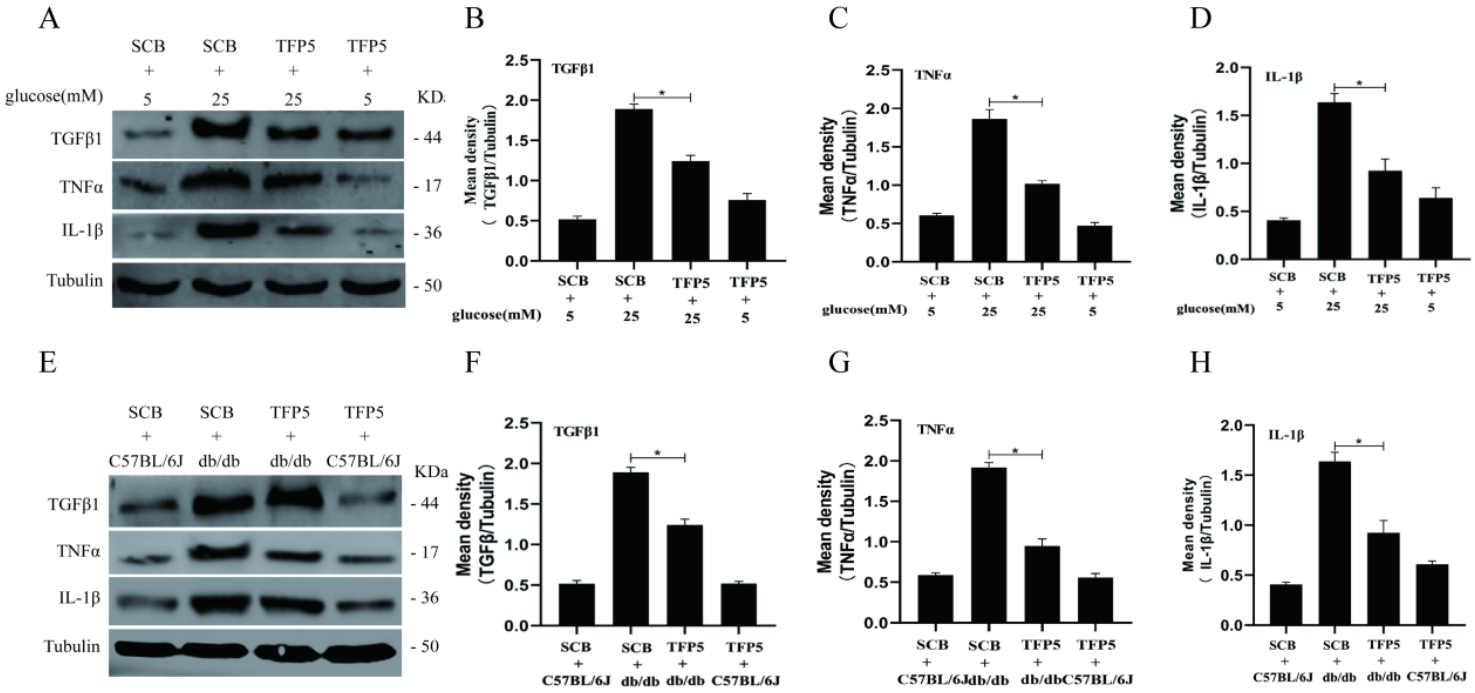
Figure 4




## Figure 4

**Figure 4. TFP5 protects pancreatic β-cells from high glucose-induced apoptosis.** High glucose-induced apoptosis of MIN6 cells. MIN6 cells were treated with TFP5 or SCB. After 24 h, the cells were starved overnight with glucose-free medium and then treated with 5 mM (control) or 25 mM glucose (high glucose) for 24 h. (A) Expression levels of cleaved caspase-3 and total caspase-3 in MIN6 cells by Western blot analysis. (B) The quantified ratio of cleaved caspase-3 in the these treated MIN6 cells. The data represent mean ± SEM of four experiments (compared with the 5+SCB group, #$p$<0.05; compared with the 25+SCB group, *$p$ < 0.05). (C) IHC assay of cleaved caspase-3 in mice models, (bars = 20μm). (D) Density quantification analysis of the assay results shown in (C). The data represent the mean ± SEM of four experiments (compared with the C57BL/6J+SCB group, #$p$<0.05; compared with the db/db+SCB group, *$p$ < 0.05).

Figure 5




## Figure 5

**Figure 5. TFP5 protected pancreatic β-cells by reducing inflammation.** MIN6 cells were treated with TFP5 or SCB. After 24 h, the cells were starved overnight with glucose-free medium and then treated with 5 mM (control) or 25 mM glucose (high glucose) for 24 h. (A) Expression levels of TGF-β1, TNF-α, and IL-1β in MIN6 cells by Western blot analysis. (B, C, and D) The quantified ratio of TGF-β1, TNF-α, and IL-1β in the these treated MIN6 cells. The data represent mean ± SEM of four experiments (compared with the 25+SCB group, *$p < 0.05$). C57BL/6J and db/db mice were exposed to the same levels of TFP5 peptide or SCB. Then, purified pancreatic islets were collected and used for western blot analysis. (E) Western blot analysis results show the expression levels of the inflammatory cytokines TGF-β1, TNF-α, and IL-1β in pancreatic islets (n=3). (F, G, and H) Mean density analysis of inflammatory biomarkers in tissues shown in (E) (compared with the SCB+db/db group, *$p < 0.05$).

Figure6

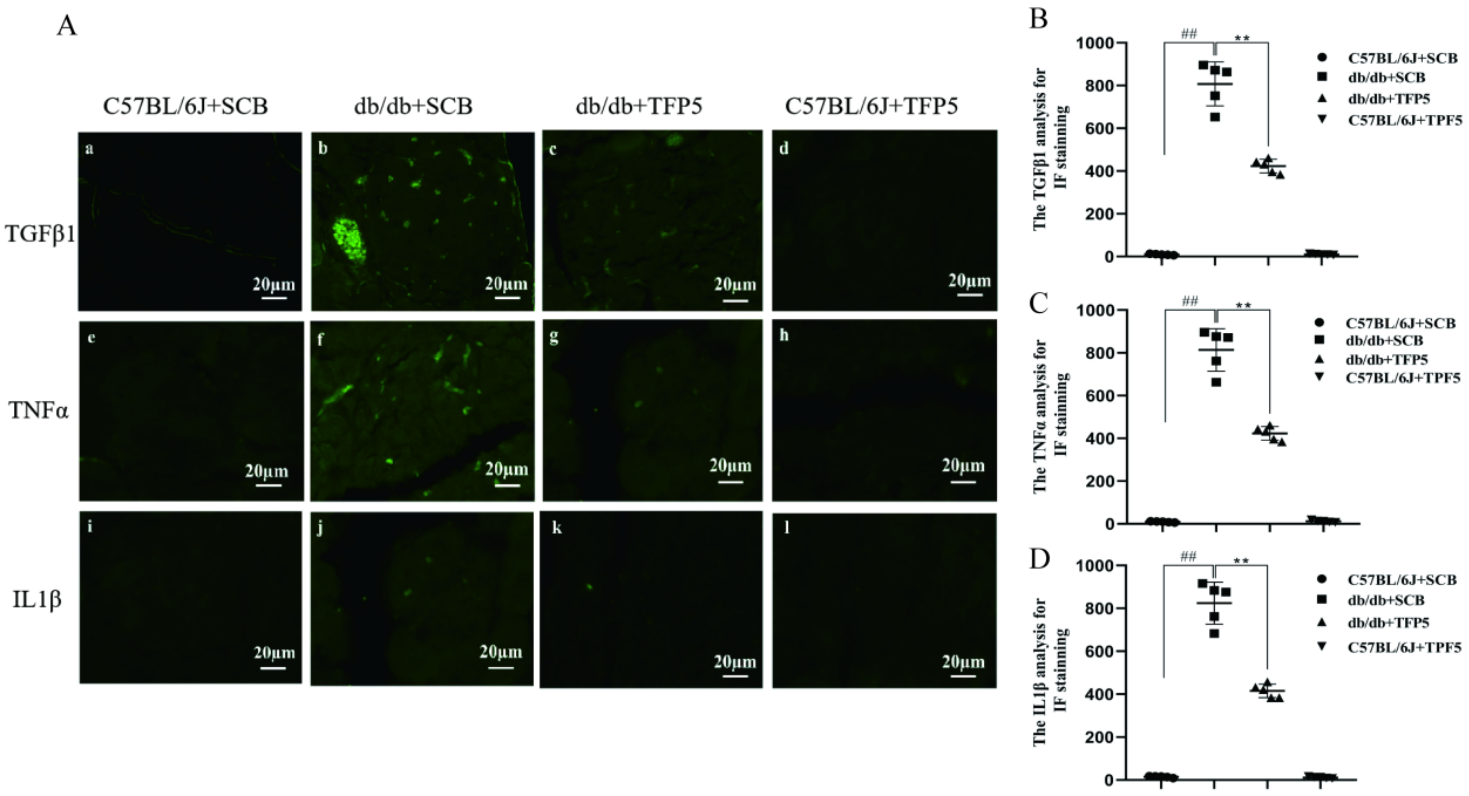



**Figure 6**

**Figure 6. The inhibition of inflammation by TFP5 in pancreatic islets.** C57BL/6J and db/db mice were exposed to the same levels of TFP5 peptide or SCB. Then, pancreatic tissue slides were created for immunofluorescence staining. (A) Inflammatory cytokines TGF-β1, TNF-α, and IL-1β in pancreatic tissue were subjected to immunofluorescence staining, and their expression was analyzed (bars=20μm). (B-D) Semiquantitative analysis of the expression of inflammatory biomarkers subjected to immunofluorescence staining. Five representative sections on each slide were imaged, and the intensity of the fluorescence signals was measured by Image J software (compared with the SCB+C57BL/6J group, #$p$<0.05; compared with the SCB+db/db group, *$p$ < 0.05).

# Supplementary Files

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