Enhanced Characterization of Beta Cell Mass in a Tg*(Pdx1-GFP)* Mouse Model of Beta Cell Destruction

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Abstract

Introduction

Measurement of pancreatic beta cell mass in animal models is a common assay in diabetes researches. Novel whole-organ clearance methods in conjunction with transgenic mouse models hold tremendous promise to improve methods for beta cell mass measurement. Here, we propose a refined method to estimate the beta cell mass using a new transgenic Tg(*Pdx1-GFP)* mouse model and a recently developed free-of-acrylamide clearing tissue (FACT) protocol.

Methods

First, we generated and evaluated a Tg(*Pdx1-GFP)* transgenic mouse model. By using the FACT protocol in this model, we could quantify the beta cell mass and alloxan-induced beta cell destruction in whole pancreas specimens.

Results

Tg(*Pdx1-GFP)* transgenic mice expressed green fluorescent protein (GFP) only in the beta cells of the pancreas and limited to the beta cells. This GFP expression enabled us to accurately measure beta cell loss in a beta cell destruction model. The results suggest that our proposed method can be used as a simple, rapid assay for beta cell mass measurement in studies of islet biology and diabetes.

Conclusion

The Tg(*Pdx1-GFP)* transgenic mouse in conjunction with the FACT protocol can enhance large-scale screening studies in the field of diabetes.

Introduction

Loss of pancreatic beta cell mass is a fundamental characteristic of type 1 diabetes and a prevalent symptom in advanced type 2 diabetes[1, 2]. Understanding the mechanisms underlying destruction and regeneration of the beta cell mass is a pivotal dogma in diabetes preclinical research[3]. Therefore, measurement of the beta cell mass is an important aspect of diabetes researches[2, 4].

Although many methods have been used to measure beta cell mass, most rely on time-consuming and laborious procedures or special imaging equipment. For instance, the traditional histological method for beta cell mass quantification entails sectioning, immunostaining, microscopic imaging, and quantitative analysis of approximately 1.5% of the pancreas volume[5]. Researchers have proposed automated beta cell mass measurement methods that use sophisticated microscopy and image analysis technologies[6]. Although being high-throughput and less laborious, fully automated methods are not convenient for many laboratories and may be prone to biased data from micrograph artifacts[7].

The use of transgenic models provides another approach for facilitating beta cell mass measurement. Different transgenic mouse models have been developed that express reporter genes such as green fluorescent protein (GFP) under the control of a beta cell specific promoter such as the insulin promoter[8]. These animal models eliminate the need for staining and hence provide a convenient mean for beta cell detection. However, to avoid massive histological sectioning, a complementary downstream detection method is required. For instance, positron emission tomography (PET) has been used in combination with a transgenic mouse that expresses a PET reporter gene, sr39tk, under the control of the mouse insulin 1 promoter (MIP)[9].

Downstream methods for the imaging of transparent and intact tissues are required to detect fluorescent proteins, specifically in whole-organ specimens. A recently developed free-of-acrylamide clearing tissue (FACT) protocol has shown promising results for large-scale imaging of a fluorescent protein reporter in neural tissues[10]. This protocol was successfully used for deep imaging of immunostained specimens of different mouse and avian organs, including the pancreas[11, 12]. Therefore, the FACT protocol holds tremendous promise to improve beta cell mass measurement in an intact pancreas.

Here, we report the generation of a Tg*(Pdx1-GFP)* transgenic mouse model that expresses GFP in its pancreatic islet beta cells. We investigated the effectiveness of the FACT protocol for quantification of the beta cell mass in whole pancreas specimens of this Tg*(Pdx1-GFP)* mouse model.

Materials And Methods

Tg*(Pdx1-GFP)* transgenic mice production and characterization

To generate the transgenic Tg(*Pdx1-GFP*) mouse, we used pEGFP-N1-Pdx1 plasmid (GenBank accession number: KU341334) which was reported previously [13]. This plasmid was generated by replacing CMV promoter in pEGFP-N1 with a 7115 bp fragment of genomic sequence upstream of the start codon of *Pdx1*. A 7962 bp fragment of pEGFP-N1-Pdx1 containing the *Pdx1*-upstream region, EGFP coding sequence and SV40 polyadenylation signal was excised with HindIII and MfeI (NEB corporation, Beverly, MA, USA) restriction enzymes and used for microinjection (Figure 1A).

Transgenic mice were generated by pronuclear microinjection of fertilized eggs from the NMRI mouse strain (Pasteur Institute, Tehran, Iran) using a micromanipulator system installed on an IX71 inverted microscope (Olympus, Tokyo, Japan). The microscope was connected to an Eppendorf microinjector (FemtoJet, Eppendorf, Hamburg, Germany) and embryos were transferred to the pseudopregnant mice. A total of 400 eggs microinjected with the GFP transgene were transferred to pseudopregnant mice (Figure 1A). The founder mice were identified using a PCR assay on the tail DNA samples with primers 5ACAGCAGCAAGCAGGGATCAG3and 5CTTCAGGTCAGCTTGCCGTA3, which are complementary to specific region in the pdx1 promoter and EGFP sequence respectively (Figure 1B).

Pancreas dissection and tissue processing using the FACT and No-FACT protocols

All animal experiments were performed according to the Animal Research Ethics Guidelines at Royan Institute, which conforms to international guidelines. Tg*(Pdx1-GFP)* mice were used for breeding and the subsequent experiments. The mice were euthanized and the entire pancreases were removed. The pancreases were washed twice in phosphate-buffered saline (PBS) and the spleens and excess fat were removed. Next, the pancreas specimens were kept in PBS for 10 min before they were processed by either the FACT or No-FACT tissue processing methods. In order to prepare transparent tissue by the FACT protocol, each pancreas was fixed in 4% paraformaldehyde (PFA) in PBS solution and kept at 4 °C for three days. Subsequently, the tissues were treated with 8% sodium dodecyl sulfate (SDS) clearing solution at 37 °C for seven days. In order to obtain complete tissue transparency, the solutions were refreshed daily and transparency was checked visually[10]. After washing overnight by PBS with Tween detergent (PBST), each whole pancreas was mounted on a glass slide and covered with another slide, and then placed under a >13 kg weight to apply pressure overnight, followed by microscopic analysis the next day.

Tissue preparation according to the No-FACT method was performed by placing a heavy weight on top of the PBS-washed tissue, where it remained overnight.

Modeling beta cell destruction and diabetes induction

We obtained 10-week-old Tg*(Pdx1-GFP)* male mice (n=4) from the Royan Institute Animal Core Facility. The mice were fasted for 4 h before the challenge. alloxan (Sigma, 2244-11-3) was freshly dissolved in 1 mM HCl in 0.9% NaCl (45, 70, and 90 mg/kg body weight [bw]). The reagent was immediately injected via the dorsal tail veins to induce diabetes. The same volume of saline was given to the control group during the sham operation. Blood glucose measurement was performed 72h before and after fasting by obtaining a blood sample from the tail and assessing the glucose levels with an Accu-check Active blood glucose meter (Roche Diagnostics,
Basel, Switzerland). Glucose measurement was continued 48 h after the injection of alloxan or saline.

Measurement of beta cell area

The entire pancreases from 10-week-old mice (n=3) were obtained and processed by each of the two methods. The flattened tissues were analyzed by Olympus MVX10 (Olympus Corp, Tokyo, Japan) microscope that had a green fluorescent filter. In order to prepare a representative picture of the entire pancreas, approximately 60–80 images were captured manually of the tissues under a 20x objective and we created a tiled image for each mouse with ImageJ software 8 (NIH, Bethesda, MD, USA). After removing background using the beta cell mass area in each graph was calculated by automatic counting of the GFP signal. For calculation of beta cell destruction under each condition, we subtracted the remaining beta cell mass area from the total beta cell mass area in the corresponding control mice.

Statistical analysis

For each variable, at least three replicate experiments were conducted and 70–80 images were taken from each pancreas. Data were analyzed for statistical significance among the groups by using the independent t-test and Wilcoxon, using GraphPad Prism software (GraphPad software, CA, USA).

Results

Tg (Pdx1-GFP) **mice expressed GFP in their pancreatic islets**

The *Pdx1-GFP* fragment was excised from a *pEGFP-N1-Pdx1* plasmid and used as the microinjection material. Microinjected eggs with the GFP transgene were transferred to pseudopregnant mice and subsequent PCR analysis of DNA samples from 88 mice revealed the founder mouse (Fig. 1B). Microscopic observation of the pattern of GFP expression was limited specifically to the pancreatic islets and GFP signal was reliable and there was no background signal (Fig. 1C). Our observation was confirmed using antibody against GFP (Fig. 1D).

The FACT protocol improved beta cell microscopy and mass quantification

We compared tissue sections prepared by the FACT and No-FACT protocols to determine the best method for obtaining clear pancreatic tissue while maintaining a fluorescent signal (Fig. 2A). Our data suggested that although pancreatic tissue embedded between chamber slides under a heavy weight in the No-FACT method showed clear tissue (Fig. 2B), step-wise clearance of pancreatic tissue by using 11 days of the FACT method improved transparency of the pancreatic tissue (Fig. 2B). This transparency resulted in increased intensity and lower background noises in FACT-cleared sections compared to the No-FACT approach (Fig. 2B), which helped to bring the islets into better focus. The binary pictures obtained by ImageJ enabled us to detect islets of more diverse sizes by FACT-cleared sections. This was particularly noted with the smaller-sized islets (Fig. 2B). Furthermore, we observed higher beta cell mass areas in FACT-cleared sections (2928869 ± 120215 AU) compared to No-FACT cleared sections (1292372 ± 325632 AU) by using compiled fluorescent images of each pancreas (Figs. 2C and 2D). Additionally, the total number of detected islets with this method was significantly higher than the other clearance method (155.7 and 109 respectively) (Fig. 2E).

The FACT protocol improved analysis of the alloxan-induced beta cell destruction model in Tg (Pdx1-GFP) mice

In order to prepare a diabetic model of beta cell destruction, we injected 45, 70, and 90 mg/kg.bw of alloxan into 3 different groups of Tg*(Pdx1-GFP)* mice. The control group received saline injections. Fasting blood glucose levels were monitored 72 h before the alloxan or saline injections and 48 h later (Fig. 3A). We observed an increase in blood glucose levels in accordance with the concentration of alloxan in all of the experimental groups (Figs. 3B and 3G). in A45 group blood glucose level increased from 105.3 ± 39.02 to 345.0 ± 120.2. Moreover, blood glucose level increased from 117.66 ± 9.29 in A70 and 101.0 ± 9.64 in A90 to 600.00 48h post-injection. The mice were euthanized 48 h post-injection and their pancreases were harvested and processed according to either the FACT or No-FACT methods. We observed that the total beta cell mass area gradually decreased in accordance with the alloxan concentration. In both processing methods, the 70 and 90 mg/kg.bw injections of alloxan resulted in significant spikes in glucose levels and entire destruction of the islets (Figs. 3G and 3H). The trend was the same in both experimental groups that were processed by the FACT or No-FACT clearance approaches. We chose the experimental group that received the 45 mg alloxan (A45) for further analyses. Tissue specimens from this group were assessed via the FACT and No-FACT protocols.

Next, we sought to assess the differences in beta cell destruction after 48 h between the FACT and No-FACT methods. We measured the total numbers of islets and the beta cell mass area in the experimental group injected with 45 mg alloxan (A45) and was processed by the FACT or No-FACT methods. Our data demonstrated that although the numbers of islets detected by the FACT method was higher in the control group (Fig. 3C), there was a more substantial decrease in the number of islets in FACT-cleared sections of A45 group compared with the No-FACT-cleared sections (Fig. 3D). A comparison of beta cell area showed more than 75% beta cell destruction in the A45 group processed by the FACT method and approximately 50% destruction was observed in the A45 group that was processed by the No-FACT method. The same trend was obtained for total islet numbers in the A45 group processed by these two methods (Figs. 3D and 3E).

The results of our observation were supported by fluorescent microscopy observations. We suggest that the FACT-cleared pancreas resulted in an easier and more precise calculation of islet numbers and mass areas. In contrast, shadows in the No-FACT method increased errors in counting the islets (Fig. 3F). Taken together, although the tissue was better compression-rolled over the slide in the No-FACT method, the FACT approach appeared to be a better platform for beta cell destruction studies.

Discussion

In this study, we propose a simple, rapid method that uses a Tg*(Pdx1-GFP)* transgenic mouse and FACT protocol to estimate the beta cell mass. Our results indicate that this method can detect the loss of beta cell mass in an alloxan-induced beta cell destruction model. A comparison of the FACT protocol with the conventional protocol shows that the FACT protocol can improve tissue clearance, and result in more accurate quantification during the image processing step.

We used a 7962 bp fragment of pEGFP-N1-Pdx1 spanning from the *Pdx1*-upstream region and EGFP coding sequence to SV40 polyadenylation signal to derive GFP expression in the mouse beta cells. Previously, researchers have used a mouse model that expressed GFP under the control of the *Pdx1* promoter and a human growth hormone (hGH) minigene GFP[14]. However, the hGH minigene commonly used to increase transgene expression in transgenic mice has been reported to impair pancreatic islet function through the activation of STAT5 signaling[15]. In our model, we achieved an elevated, readily detectable level of GFP expression without the use of the hGH minigene. Our results showed that GFP expression was confined to the pancreatic islets. We did not detect any GFP expression in the surrounding exocrine tissues. Furthermore, we did not detect any GFP expression in the high dose (70 and

90 mg/kg.bw) alloxan-treated mice. Since the toxic effects of alloxan are highly restricted to Glut2-expressing beta cells absence of GFP expression after alloxan treatment strongly suggests that GFP is specifically expressed in beta cells[16–18].

Researchers have suggested that the FACT method preserves protein structure, including fluorescent proteins through PFA crosslinking, while providing tissue clearance by SDS-mediated lipid removal[19]. This suggestion supports the results of our study where the FACT method provided superior tissue clearance compared to the conventional No-FACT protocol. The latter resulted in detection of higher islet numbers and area in healthy mice.

Our observation also suggests that erroneous beta cell detection in the No-FACT group is due to the bright background fluorescence and lower contrast between dimmer islets and their surrounding area. This supports a previous report of decreased background fluorescence in the FACT protocol compared to similar protocols[10].

In this study, we used an alloxan-induced model of beta cell destruction to investigate the potential use of our model for diabetes research. We chose different doses of alloxan, which enabled us to detect the loss of beta cell mass by quantification of GFP expressing beta cells in the FACT-processed specimens. Previously, researchers have used various dosages of alloxan (90–200 mg/kg.bw) to obtain complete, irreversible destruction of beta cell masses[20]. We detected complete loss of beta cell mass using 70 and 90 mg/kg.bw doses of alloxan in the current study. However, when we used a suboptimal dose of alloxan (45 mg/kg.bw), we observed different ratios of beta cell destruction in the No-FACT and FACT groups. This was mostly attributed to the different detection power of these methods in the baseline healthy samples.

Conclusions

Collectively, our study presents a simple, effective method for measuring beta cell mass that uses the *Tg(Pdx1-GFP)* mouse model combined with the straightforward FACT protocol. This method does not require a massive sectioning procedure or specialized microscope equipment, and it can be used for large-scale screening studies.

Declarations

Acknowledgment

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Competing interests

The authors declare no conflict of interest.

Authors contribution

FK, BAA, and SP performed the experiments, analyzed the data, and wrote the manuscript. AF designed the experiments, AT analyzed the data, MB designed the experiments, KK, and MF performed the experiments, YT provisioned of study materials and equipment, designed the experiments, wrote and finalized the manuscript, and supervised.

Study Highlights

What is the current knowledge?

- Most methods to measure beta cell mass rely on time-consuming and laborious procedures or special imaging equipment.
- The use of transgenic models provides appropriate approach for facilitating beta cell mass measurement

What is new here?

- We represented a simple, effective method for measuring beta cell mass using the *Tg(Pdx1-GFP)* mouse model combined with the straightforward FACT protocol.
- Our method does not require a massive sectioning procedure or specialized microscope equipment, and suitable for large-scale screening studies.

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Figures

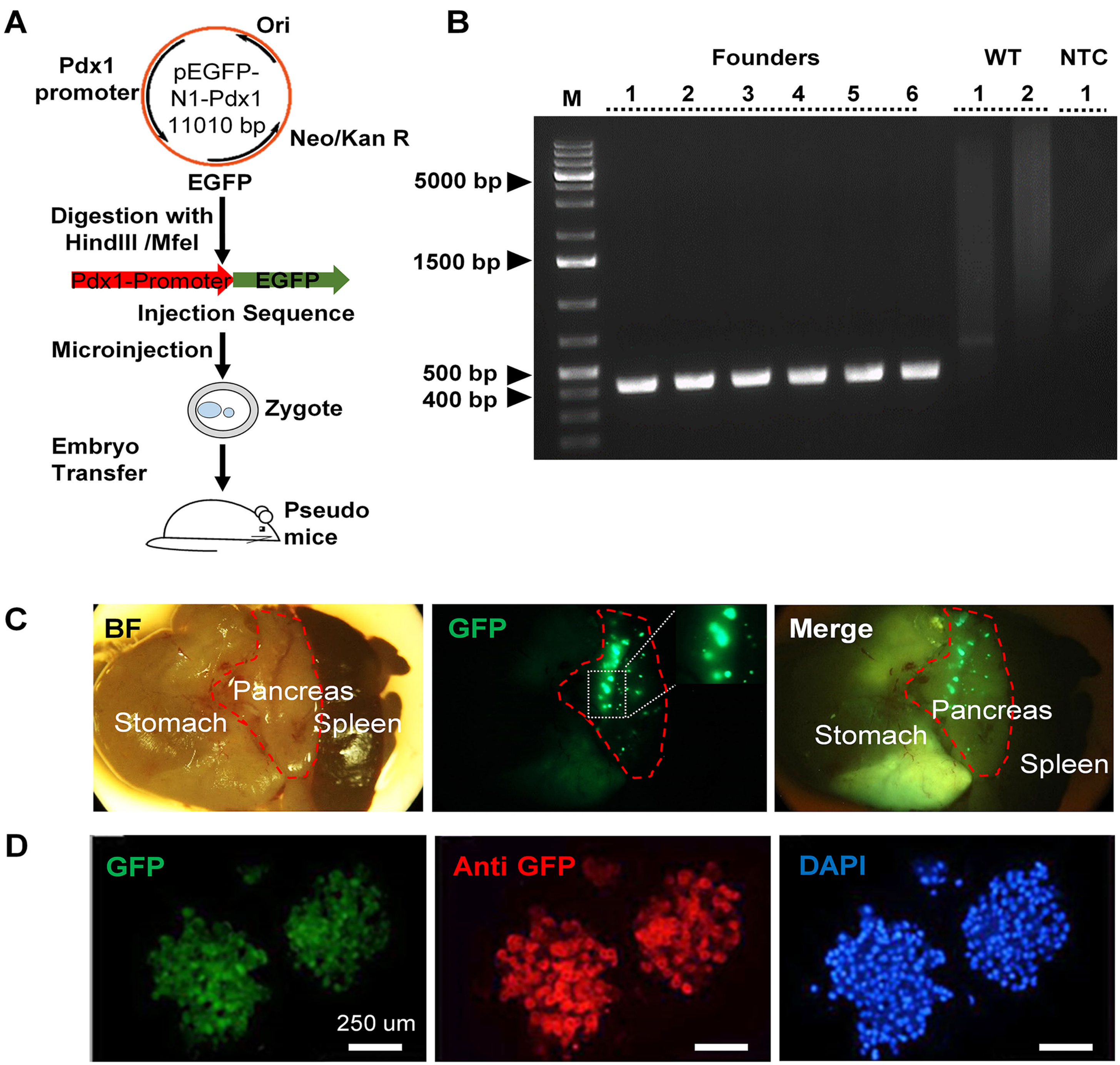

Figure 1

Production of transgenic mice and pancreatic expression of green fluorescent protein (GFP). A) Schematic view of transgenic mice production using pronuclear microinjection of a 7962 bp fragment that contains a Pdx1 promoter,GFP coding region, and SV40 poly-adenylation signal. B) Founder mice were identified by PCR products from tail DNA samples. C) Representative pictures of GFP expression localized to the pancreas and compared to the spleen and stomach. Dashed lines in each micrograph show the pancreas boundaries. D) Representative micrographs of GFP and anti-GFP staining show co-localization of GFP signals to the pancreas. The green and blue signals represent GFP and nuclear staining, respectively. Red signals represent anti-GFP staining. Scale bars: 250µm. WT: Wild type, NTC: Non-template control group, BF: Bright field.

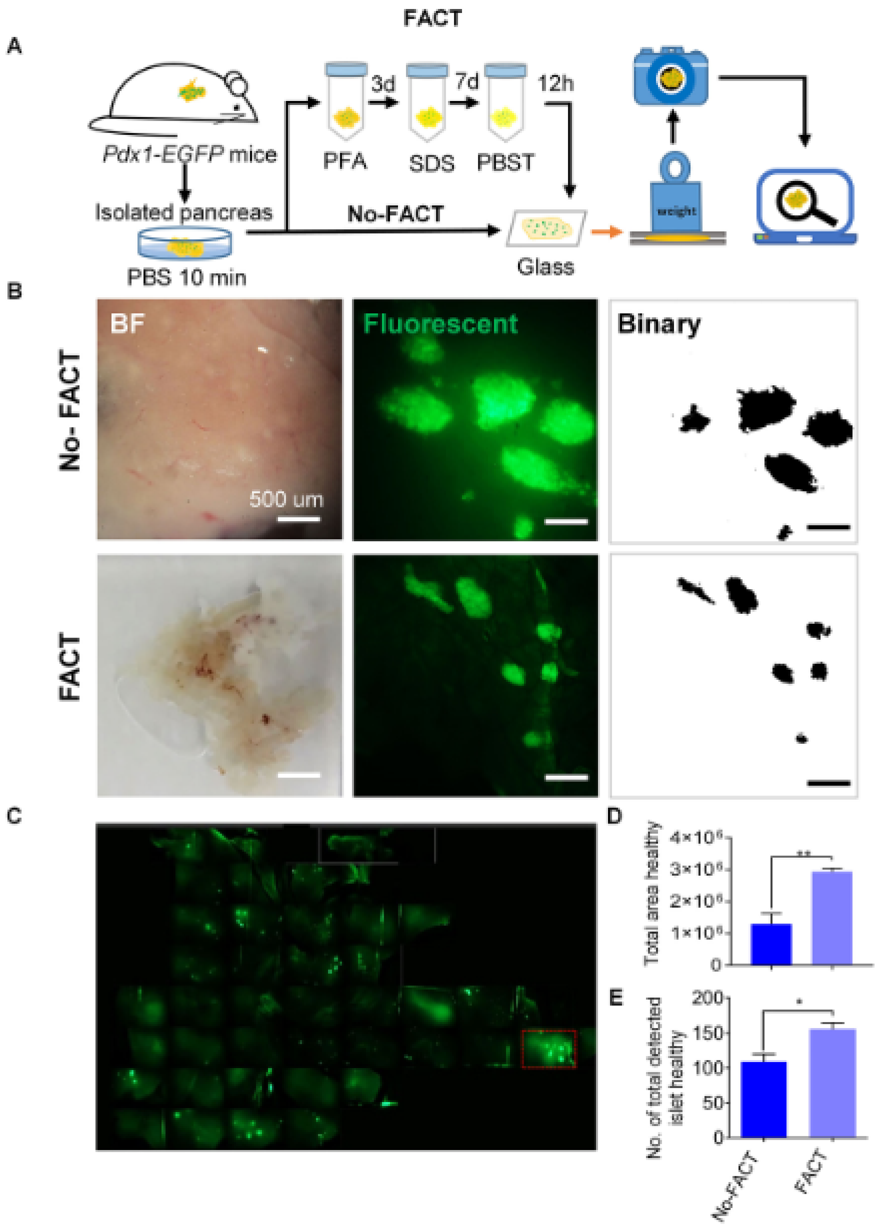

Figure 2

Pancreatic tissue processing and beta cell mass measurement. A) The entire pancreas was removed from each Tg(Pdx1-GFP) mouse and washed with phosphate-buffered saline (PBS), followed by either the step-wise clearance free-of-acrylamide clearing tissue (FACT) method or the No-FACT approach. The whole pancreas was then placed between glass slides and heavy pressure was applied overnight. The flattened tissues were characterized by microscopic analysis. B) Transparency of the pancreas tissue in the No-FACT and FACT approaches indicated that the better cleared tissues were seen in the FACT approach. Fluorescent microscopy observation showed that the FACT-cleared tissues had less background noise and enhanced intensity. Binary pictures from ImageJ software showed increased numbers of islets detected in the FACT-cleared tissues. C) Representative picture of the entire pancreas built by captured images of a whole flattened pancreas that was used to obtain the total area (D) and number of islets (E) in healthy mice. The results showed increased values in the FACT-cleared tissues. Scale bars: 500 µm. Data are presented as mean±SEM (n=4 mice/group). *P<0.05 and **P<0.01.

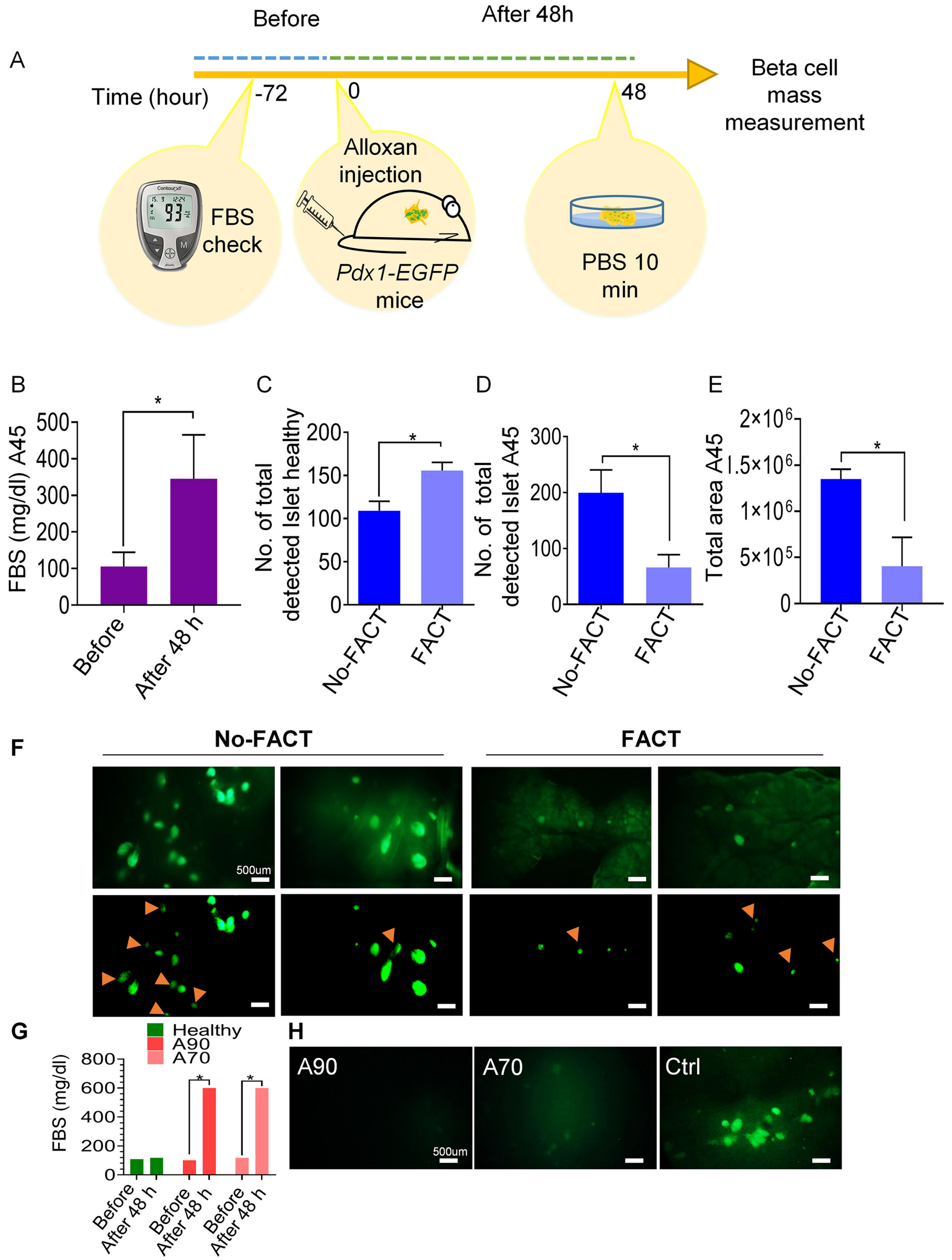

Figure 3

Preparation and characterization of a beta cell destruction model. A) Schematic view of beta cell loss from alloxan-induced beta cell destruction. B) FBS measurement (mg/dl) before and after injection of 45 mg/kg body weight (bw) alloxan. C-D) Total numbers of islets detected in healthy mice that showed more islets in the control group cleared with the free-of-acrylamide clearing tissue (FACT) method. While No-FACT method showed more number of islets in the A45 group. E) Total area of islets according to the FACT and No-FACT methods in the A45 group. F) Representative graphs confirm enhanced, more precise detection of islets in FACT-cleared tissues. G) FBS (mg/dl) before and after injection of alloxan (70 and 90 mg/kg.bw) and the saline-injected control group. H) Representative micrographs of healthy, A70, and A90 mice showed complete destruction of islets in the A70 and A90 groups 48 h post-injection. Data are presented as mean±SEM (n=4 mice/group). *P<0.05. FBS: Fasting blood sugar; A45: 45 mg/kg alloxan group; A70: 70 mg/kg.bw alloxan group; A90: 90 mg/kg.bw alloxan group

Supplementary Files

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