Research Article

Site-targeted non-viral gene delivery by direct DNA injection into the pancreatic parenchyma and subsequent in vivo electroporation in mice

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The pancreas is considered an important gene therapy target because the organ is the site of several high burden diseases, including diabetes mellitus, cystic fibrosis, and pancreatic cancer. We aimed to develop an efficient in vivo gene delivery system using non-viral DNA. Direct intra-parenchymal injection of a solution containing circular plasmid pmaxGFP DNA was performed on adult anesthetized ICR female mice. The injection site was sandwiched with a pair of tweezer-type electrode disks, and electroporated using a square-pulse generator. Green fluorescent protein (GFP) expression within the injected pancreatic portion was observed one day after gene delivery. GFP expression reduced to baseline within a week of transfection. Application of voltages over 40 V resulted in tissue damage during electroporation. We demonstrate that electroporation is effective for safe and efficient transfection of pancreatic cells. This novel gene delivery method to the pancreatic parenchyma may find application in gene therapy strategies for pancreatic diseases and in investigation of specific gene function in situ.

1   Introduction

Establishment of a robust in vivo gene delivery system that results in efficient transfection of pancreatic islet cells is one of the goals of gene therapy. Such a technolo-

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Abbreviations: GFP, green fluorescent protein; **IPPIGT,** intra-pancreatic parenchymal injection for gene transfer; **Pp,** poring pulse; **TB,** trypan blue; **Tp,** transfer pulse

gy would place cures for diabetes mellitus, cystic fibrosis, and pancreatic cancer within reach. To date, several in vivo approaches have been reported for the transfection of pancreatic cells, including a liposomal transfection system [1] a polyvinylpyrrolidone (PVP) system [2], adenovirus [3–9], adeno-associated virus (AAV) [10], and lentivirus [11] vectors. All of these reports describe the successful transfection of pancreatic cells in situ, but gene delivery efficiency and localization of gene products differs between reports. This may be partially due to the administration route of the DNA vector. For example, Wang et al. [10] injected adeno-associated virus DNA intraperitoneally, intravenously and intraductally to transfect pancreatic cells in adult mice. The intraductal route produced exogenous gene expression predominantly in

the peripheral zone of the islets, whereas the intravenous route (coupled with liver blockade) led to uniform gene transfer. The intraperitoneal route resulted in a combined intra-islet gene transfer pattern of the intraductal and intravenous routes.

In addition to those gene transfer routes, successful transfection of pancreatic acinar cells and islets has been repowered using intra-parenchymal injection of DNA [2, 12]. This approach is simpler than the other gene transfer routes because it does not require any microscopic apparatus or other surgery-related instrumentation. However, intra-parenchymal injection of DNA requires relatively large amounts of DNA-containing solution (100–120 µL) delivered with the aid of a 33- or 27-gauge needle. Using this approach, Yu et al. [2] demonstrated successful transfection of acinar and β-cells along with other tissues, such as the spleen and duodenum. The transfection of other tissues outside the pancreas might be due to the administration of large amounts of DNA-containing solution to the relatively small area of the pancreas. Consequently, DNA may have been transported through the blood stream or humoral fluids to other locations.

The exocrine pancreas has ducts that are arranged in clusters called acini, each of which has been clonally derived from juvenile stem cells [13]. Since these compartments are easily accessed and permit manipulation (i.e. injection of DNA by a micropipette) using a dissecting microscope, it is possible to transfect pancreatic cells efficiently by injected a small amount (2–4 µL) of DNA-containing solution intra-parenchymally and subsequently performing in vivo electroporation at the injected site. This procedure does not require as much DNA as other techniques [2, 12] and can prevent the possible spread of the introduced DNA outside the pancreas.

In this study, we determined the optimal delivery conditions for gene targeting to the mouse pancreatic parenchyma, and, using these customized conditions, we confirmed the utility of this technology for efficient transfection of pancreatic cells. We term this technology intra-pancreatic parenchymal injection for gene transfer (IPPIGT).

2 Materials and methods

2.1 Mice

Female ICR mice, 8–10 wks of age (Kyudo Co., Ltd., Tosu, Saga, Japan), were used for IPPIGT. The mice were maintained on a 12 h light/dark cycle (lights on from 07:00 to 19:00), and were provided with food and water *ad libitum*. The experiments described were performed according to the "Guide for the Care and Use of Laboratory Animals" of the National Academy of Sciences, USA and were approved by the "Animal Care and Experimentation

Committee" of Kagoshima University (Sakuragaoka Campus). The experiments describing in vivo transfection of mouse pancreas by IPPIGT were accompanied by surgery (exposure of spleen/pancreas) and operation/manipulation (DNA injection toward pancreatic parenchyma and in vivo electroporation).

2.2 Plasmid DNA

The plasmid used in this study was green fluorescent protein (GFP)-expressing pmaxGFP (Lonza GmbH, Wuppertal, Germany). For IPPIGT, this plasmid was dissolved in phosphate-buffered saline (PBS) + 0.1% (v/v) trypan blue (TB) (Trypan Blue Stain 0.4% (Invitrogen Carlsbad, CA, USA) used to enable identification of the injected site) at a final concentration of 0.5 µg/µL.
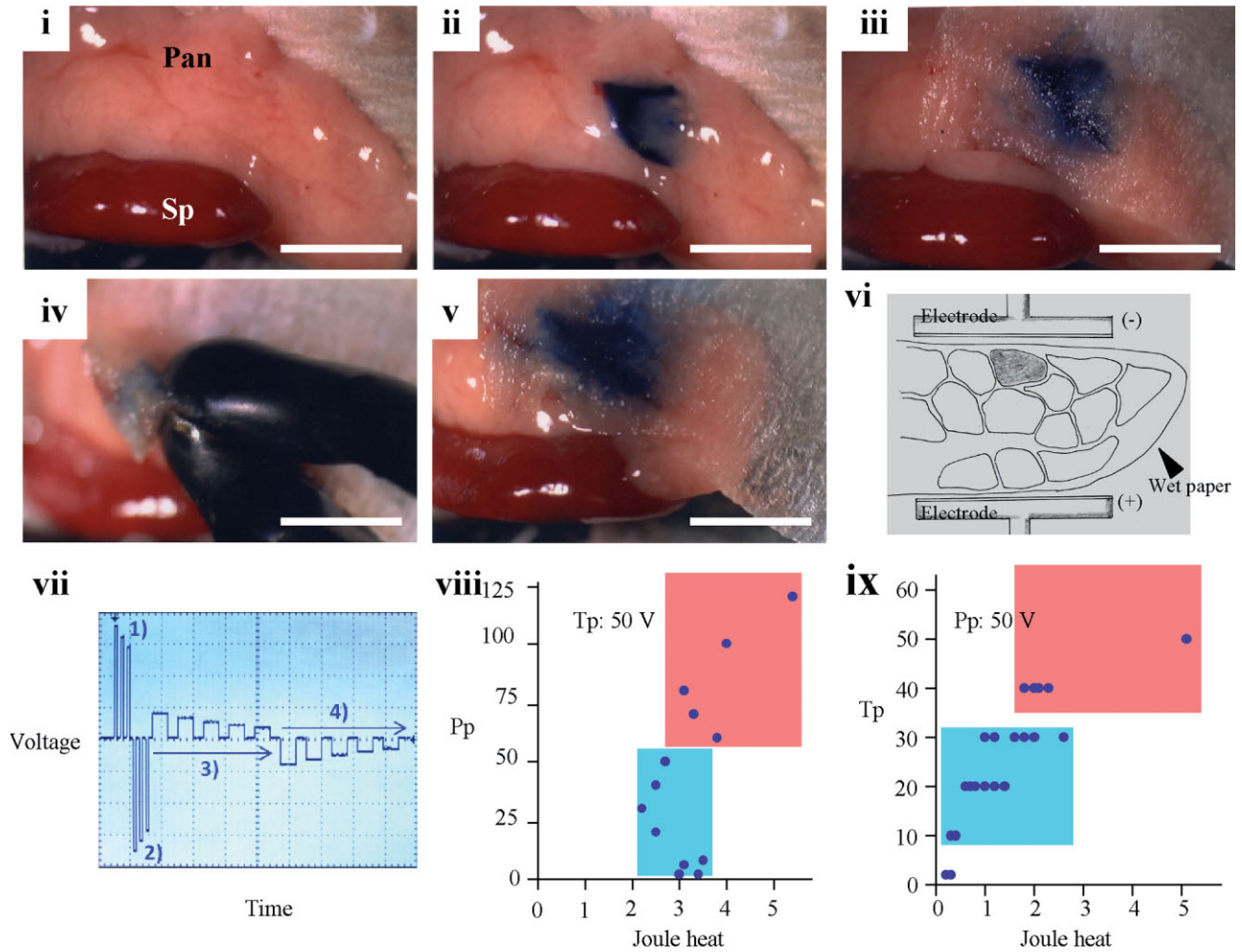
2.3 IPPIGT

Mice were anesthetized by pentobarbital, and then a small dorsal incision was made on the left side from which both spleen and pancreas were removed (Fig. 1A-i). Two µL of solution containing plasmid DNA and TB was injected into one compartment of the pancreatic parenchyma by a glass pipette with an attached mouthpiece (Fig. 1A-ii).) The pipette (G-1; outer diameter 1 mm; Narishige, Tokyo, Japan) was created using a pipette puller (PD-5; Narishige). The tip was cut by forceps to make the inner diameter 40–60 µm. After injection, the

Figure 1. Optimization of direct gene transfer toward pancreatic parenchyma. **(A)** IPPIGT procedure. i: Spleen (Sp) and pancreas (Pan) were exposed after dorsal incision of skin and muscle wall under anesthesia. ii: Pancreatic parenchyma after injection with a small volume of solution (2 µL) containing plasmid DNA and TB. iii: Pancreatic parenchyma covered with wet paper after DNA injection. iv: In vivo electroporation (*n* = 40). v: Pancreatic parenchyma after in vivo electroporation. Scale bars = 3 mm. vi: Schematic presentation of IPPIGT. The DNA introduced within a pancreatic compartment is delivered to pancreatic cells, including acinar cells and islets, under a strong electric current. vii: Pulses generated from the square-pulse generator NEPA21 (cited from www.nepagene.jp/E/Ecatalogue/Enepa21.htm). Upon electroporation, Pp, shown as (1) and (2) are first generated, which induce formation of fine pores at the cell membrane to facilitate DNA uptake. Secondly, Tp, shown as (3) and (4), are generated, which induce efficient DNA transfer through a cell membrane. Pulses (2) and (4) were polarity-reversed. viii: Relationship between Joule heat and applied Pp voltage with Tp voltage held constant at 50 V (*n* = 13). ix: Relationship between the Joule heat and applied Tp voltage with Pp voltage held constant at 50 V (*n* = 21). **(B)** GFP fluorescence in the pancreas 1 day after IPPIGT. i and ii: Pancreatic parenchyma electroporated at Pp voltage of 125 V and Tp voltage of 50 V. Tissue solidification along with whitening (enclosed by dotted lines in a and c). iii and iv: Magnified views of i and ii, respectively. Arrows indicate the presence of TB, showing the sites where DNA was injected. i and iii: Photographs taken under white light; ii: Photograph taken under ultraviolet light; iv: Image taken under white light merged with image taken under ultraviolet light. Scale bars = 1 mm. v and vi: Hematoxylin-eosin-stained sections of the pancreas shown in **B**i–iv. Scale bars = 100 µm.
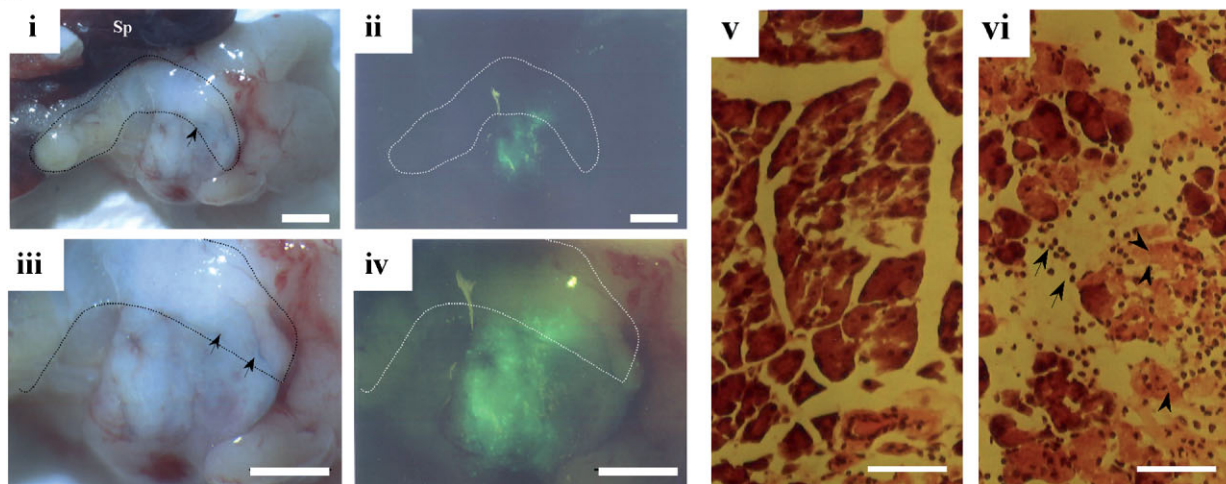
injected site was covered with a small piece of wet paper (Kimwipe; Jujo-Kimberly Co. Ltd., Tokyo, Japan) (Fig. 1A-iii) that prevented possible damage resulting

from heat generated by application of electrical current, and the site was immediately sandwiched with a pair of tweezer-type electrodes whose tips consisted of 3-mm-

diameter disks (model CUY650P3; Nepa Gene Co., Ltd., Ichikawa, Chiba, Japan) (Fig. 1A-iv).) In the case described, the orientation of current was set as constant, as schematically shown in Fig. 1A-vi.

Next, in vivo electroporation was carried out using the square-pulse generator NEPA21 (Nepa Gene Co., Ltd. Chiba, Japan), which can generate two types of pulses: poring pulse (Pp) and transfer pulse (Tp) (Fig. 1A-vii). The Pp, corresponding to (1–2) in Fig. 1A-vii, produces fine pores on the cell membrane, through which exogenous DNA can be transferred. The Tp, corresponding to (3–4) in Fig. 1A-vii, drives DNA into the cell interior. In pulses (2) and (4), it should be noted that the pulses are polarity-reversed, which aids gene delivery. Joule heat, an important indicator of tissue damage, can be measured with the square-pulse machine. For in vivo electroporation, the following conditions were used: 4 Pp (2.5 ms wavelength/50 ms duration) and 8 Tp (50 ms wavelength/50 ms duration).

To explore optimal gene transfer conditions, we focused our study on the effects of variable voltages of Pp and Tp on gene transfer efficiency. Other parameters (such as the number of pulses, wavelength, and pulse durations) were unaltered during the experimental series. For example, for testing variable voltages of Pp, four pulses with varied voltages of Pp (2 – 125 V) at 2.5 ms wavelength and 50 ms pulse duration followed by eight pulses at 50 V Tp, 50 ms wavelength and 50 ms pulse duration were used (group A; Fig. 1A-viii). For testing variable voltages of Tp, four pulses at 50 V Pp, 2.5 ms wavelength and 50 ms pulse duration followed by eight pulses with varied voltages of Tp (2 – 60 V), 50 ms wavelength and 50 ms pulse duration were used (group B; Fig. 1A-ix). Three or four sites on pancreatic parenchyma per mouse were injected with 2 μL of pmaxGFP + TB and then electroporated. Four mice were used for each group.

After IPPIGT, there was no appreciable diffusion of the introduced DNA from the injection site (Fig. 1A-v). Finally, the electroporated pancreas was returned to its original position, and the abdominal wound was closed.

2.4 Fluorescence microscopy and immunohistochemistry

At the indicated days after in vivo gene delivery, mice were sacrificed by cervical dislocation. The electroporated portion of the pancreas, which was easily identified by the presence of TB, was directly inspected for fluorescence under an Olympus SZX12 fluorescence stereomicroscope (Tokyo, Japan). In some cases, the brain, lung, liver, spleen, stomach, kidney and intestine were concomitantly dissected and inspected for fluorescence. Microphotographs were obtained using a digital camera (FUJIX HC-300/OL; Fuji Film, Tokyo, Japan) and were printed using a digital color printer (CP700DSA; Mitsubishi, Tokyo, Japan).

Some of the samples were fixed in 4% paraformaldehyde in PBS at 4°C for 2 days, dehydrated by immersion in 0.25% sucrose in PBS at 4°C for 2 days, and then dehydrated in 0.4% sucrose in PBS at 4°C for 4 days. These samples were then embedded in O.C.T. compound (Tissue-Tek® [no. 4583]; Miles Scientific, Naperville, IL) for cryostat sectioning. The sections were then embedded in a solution containing glycerol and 600 nM 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes Inc. Eugene, OR, USA) for 5 min at room temperature, then inspected for fluorescence using an Olympus BX60 microscope and photographed using a Mitsubishi digital color printer. In some cases, the sections were reacted overnight with the primary C-peptide antibody (1:100, #4593, Cell Signaling Technology, Inc., Danvers, MA) at 4°C, and then with the second Alexa Fluor 647-conjugated anti-rabbit IgG Fab 2 antibody (1:100, #4414S, Molecular Probes Inc.).

3 Results

3.1 Determination of optimal IPPIGT

To optimize the conditions for IPPIGT in the mouse pancreas, we explored the effect of voltage applied during Pp and Tp delivery regimes, as outlined in section 2.3.

At 1 day after gene delivery, pancreases were dissected and immediately inspected for GFP fluorescence under ultraviolet illumination. In group A, each electroporation resulted in expression of bright fluorescence (representative image shown in Fig. 1B). Although there was no noticeable difference in the strength of fluorescence between samples (data not shown), voltages > 60 V applied during the Pp waveform produced deleterious effects on pancreatic tissue. Consequently, tissue solidification, along with whitening, was observed within the electroporated tissue portion (enclosed by dotted lines in

Figure 2. (**A**) IPPIGT performed using increasing Tp voltages during in vivo electroporation. After injection with pmaxGFP + TB, the injected portion was immediately electroporated under the conditions in which the pulses, wavelength, and duration of Tp were constant (eight pulses of 50 ms wavelength and 50 ms duration,), and Tp voltages were changed from 2 (i, ii) to 10 (iii, iv), 20 (v, vi), 30 (vii, viii), and 40 V (ix, x). An inset in viii is a magnified view of a quadrant. Arrowheads indicate possible fluorescent islets. Arrows (in i, iii, v, vii, and ix) indicate the presence of TB, showing the site where DNA was injected. i, iii, v, vii and ix: Photographs taken under white light; ii, iv, vi, viii, and x: Photographs taken under ultraviolet light. Scale bars = 1 mm. (**B**) Cryostat sections of pancreas 1 day after IPPIGT. i–iii: Observation for fluorescence under a fluorescence microscope. iv and v: Immunostaining with C-peptide antibody. i: Photograph taken under white light; ii–v: Photographs taken under ultraviolet light. Scale bars = 100 μm. (**C**) Fluorescence observed in pancreas 3 (i, ii), 7 (iii, iv), and 14 (v, vi) days after IPPIGT. i, iii, and v: Photographs taken under white light; ii and vi: Photographs taken under ultraviolet light; iv: Images taken under white light and ultraviolet light merged. Scale bars = 1 mm. Each experiment was performed twice.

Fig. 1B-i, ii). Histological examination revealed that the damaged portion exhibited necrosis (arrowheads in Fig. 1B-vi) with massive infiltration of macrophages (arrows in Fig. 1B-vi), contrasting with the neighboring normal-looking pancreas (Fig. 1B-v). Joule heat frequently exceeded 3 J when electroporation was performed using
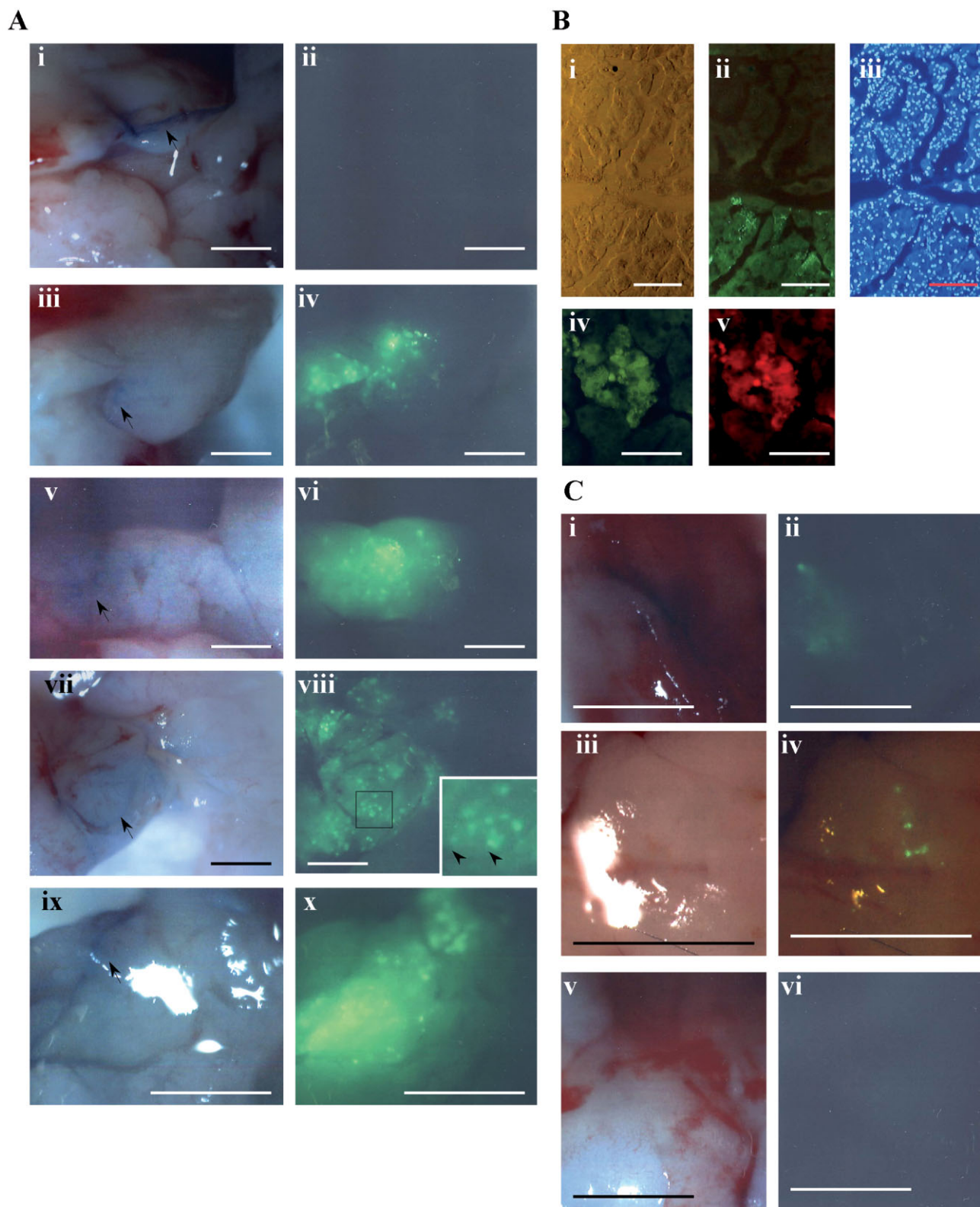
Pp voltage > 60 V (indicated by the red zone in Fig. 1A-viii). In contrast, electroporation with Pp voltage < 50 V resulted in no appreciable damage to the tissue, which appears to be consistent with lower Joule heat measurements (below 3 J) when Pp voltage < 50 V (indicated by blue zone in Fig. 1A-viii). We observed that within group B there was a correlation between the voltage applied during the Tp waveform regime and the intensity of fluorescence with the electroporated tissue (Fig. 2A). At 2 V Tp, no noticeable fluorescence was seen (Fig. 2A-i, ii), but using Tp voltage between10 and 30 V, moderate fluorescence was observed (Fig. 2A-iii–viii). Tp voltage > 40 V yielded bright fluorescence (Fig. 2A-ix, x), but also caused serious damage to the tissues as previously described. A correlation was apparent between Joule heat and the Tp voltage applied, similar to that observed when voltage was varied in Pp regime (Fig. 1A-ix). Increased Tp voltage was associated with increased Joule heat. We considered that an applied Tp voltage of 10 – 30 V was less harmful to pancreatic tissue (shown by blue zone in Fig. 1A-ix); therefore, in the subsequent experiment, we used Pp and Tp voltages of 50 and 30 V, respectively, for IPPIGT.

Injection of plasmid DNA without electroporation resulted in no expression of GFP fluorescence (data not shown). When direct inspection for fluorescence was performed using major organs (including brain, lung, liver, spleen, stomach, kidney and intestine) 1 day after IPPIGT, there was no detectable fluorescence in those samples (data not shown).

3.2 Histological examination of reporter gene expression after IPPIGT

To examine localization of GFP expression in pancreases transfected with pmaxGFP, cryostat sections were prepared from pancreas tissue harvested 1 day after gene delivery and GFP fluorescence within the sectioned tissue observed. Strong fluorescence was observed in acinar cells within some clusters (as shown by the lower cluster in Fig. 2B-ii). However, neighboring clustering did not fluoresce (as seen in the upper cluster in Fig. 2B-ii), suggesting that the injected DNA did not spread beyond the boundary of each cluster. Structures similar to islets exhibited fluorescence when inspected under a dissecting fluorescence microscope (indicated by arrowheads in Fig. 2A-viii). This was confirmed by immunostaining of cryostat sections using C-peptide antibody, since the antibody-reactive cells also exhibited GFP-derived green fluorescence (Fig. 2B-iv, v).

3.3 Reporter gene expression is transient

To determine the duration of expression of the reporter gene, GFP expression in transfected pancreases was monitored at various time points after IPPIGT. At 3, 7, and 14 days after IPPIGT, pancreases were dissected and immediately inspected for GFP fluorescence. At 3 days after IPPIGT, low levels of GFP expression were observed (Fig. 2C-i, ii), but a rapid decline in fluorescence was noted 7 and 14 days after IPPIGT compared to the day 1 measurements (Fig. 2C-iii–vi).

4 Discussion

In vivo electroporation has been employed to transfect a range of of cells, including skin epidermal cells [14, 15], oviductal epithelium [16], efferens ductal epithelium [17], bladder epithelium [18], ovarian cells (immature oocytes and interstitial cells) [19], hepatocytes [20] and renal epithelium [21, 22]. An exceptional feature of this system is the ability to transfect cells located far from the surface of an organ or tissue, since electrical currents allow physical transfer of negatively charged DNA. This approach is also applicable to in vitro gene transfer in tissues or organs isolated. For example, Lefebvre et al. [23] demonstrated that cells located in the inner region of islets could be successfully transfected. This unique feature is in contrast with those in other transfection systems, which depend on cell contact with DNA brought via the blood stream or humoral fluids. Therefore, as suggested by Wang et al. [10], intraperitoneal, intravenous and intraductal delivery of viral vectors can reach islets and result in pancreatic cell transfection. However, localization of virus DNA occurred mainly at the cell surface of islets rather than in the inner regions. Notably, our present results indicate successful transfection of cells throughout islet aggregates (arrowheads in the inset of Fig. 2A-viii; Fig. 2B-iv, v). This implies that this in vivo electroporation-based gene delivery system would be useful for transfecting β-cells in situ.

We observed gene expression in acinar cells 1 day after IPPIGT (Fig. 2B-i–iii). These observations demonstrate that the exocrine acinar cells are highly susceptible to IPPIGT-based gene delivery. This highly efficient gene transfer to exocrine acinar cells might be applicable for the gene therapy of pancreatitis, pancreatic malignancy and exocrine enzyme insufficiency, for example.

Shinoda et al. [12] and Yu et al. [2] reported in vivo gene delivery to pancreatic parenchyma, similar to our present IPPIGT. However, there are some critical differences between the previous systems and ours. For example, other reports involved the injection of large amounts (100–120 μL) of DNA solution using a 33- or 27-gauge needle. In contrast, we used a small amount of solution (2 μL), which was introduced using a glass micropipette. Injection of large amounts of solution often allows widespread vector distribution to the other non-pancreatic organs, which might have been facilitated by the blood stream and/or humoral fluids. In fact, Yu et al. [2] demonstrated transgene expression in the spleen and duodenum apart

from the pancreas. Other researchers may have intended to transfect a large number of acinar cells and β-cells, cure streptozotocin-induced apoptosis in islets or investigate the localization pattern of several types of glucose transporters; in these cases the overflow of gene expression to other tissues would be inconsequential. We intended to transfect pancreatic cells in a very limited area at the acini. The major advantages of the IPPIGT protocol we describe here are: (i) the low quantity of DNA required for successful transfection; and (ii) reduced distribution of DNA to non-pancreas organs and tissues. Compared to preexisting gene delivery routes, such as intravenous, intraperitoneal, and intraductal vector administration, IPPIGT allows us to examine gene function in the pancreas on a finer, more localized, scale.

5 References

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