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Chapter 6

Pig-to-Nonhuman Primate (NHP) Naked Islet Xenotransplantation

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Additional information is available at the end of the chapter

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Abstract

Islet transplantation is an established therapy for selected type 1 diabetes (T1D) patients with severe hypoglycemic unawareness and glycemic liability despite of insulin treatment. However, the donor organ is limited. Porcine islets are the best alternative source to overcome this limitation, and pig-to-nonhuman primate (NHP) naked islet xenotransplantation studies are being performed worldwide. Several studies including our own have presented successful proof-of-concept results based on immunosuppression regimen including the anti-CD154 monoclonal antibody. Particularly, long-term control of diabetes by adult porcine islet transplantation has been demonstrated in five consecutive monkeys, and the longest survival was ~1000 days after transplantation. Currently, pig-to-NHP islet xenotransplantation based on clinically applicable immunosuppression regimen is being pursued. In this chapter, we will describe all the procedures of pig-to-NHP naked islet xenotransplantation: (1) the porcine islet isolation from designated pathogen-free (DPF) miniature pigs, (2) diabetes induction in monkeys, (3) transplantation procedure via the portal vein, (4) immune monitoring comprising humoral and cellular immunity after porcine islet transplantation, and finally (5) liver biopsy and subsequent immunohistochemical procedure in detail.

Keywords: porcine islet, nonhuman primate, transplantation, immune monitoring, biopsy

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1. Introduction

Diabetes, a serious disease and a fast-growing worldwide epidemic, has culminated in that nearly 9% of global population is afflicted [1]. Most patients suffer from type 2 diabetes (T2D) in which genetic predisposition and harmful environmental exposure will lead to β-cell dysfunction and peripheral insulin resistance [2]. About 5–10% of the patients are afflicted with T1D where autoimmunity toward pancreatic β-cells induces β-cell death, and thus regular exogenous insulin treatment is absolutely required for the daily life of the patients [3]. In late 1960s, the procedure yielding large numbers of islets from the rodents was established using a commercial collagenase [4] and its ductal injection, and islet transplantation into the portal vein of diabetic animals could lead to recovery from experimental diabetes [5]. As islet isolation from large animals including the pigs and humans became possible [6, 7], several research groups including the Giessen group attempted clinical islet transplantation in the patients in 1980s–1990s [8]. Although early clinical experience had been unsuccessful with only some of the recipients being insulin-independent for a short while, other procedures such as organ preservation, islet isolation, and immunosuppressive regimen had been steadily improved [9, 10]. In 2000, Shapiro et al. reported a seminal paper in the *New England Journal of Medicine* and demonstrated that islet transplantation could lead to insulin-independence at least for a year in all seven type 1 diabetes (T1D) patients [11]. Since then, an international collaborative team reproduced this result from the clinical trials involving more patients from various ethnicities and continents [12]. Hering et al. very recently published an important result from a Phase 3 clinical trial, which had intended to test the efficacy and safety of the standardized human pancreatic islet product in the patients with impaired awareness of hypoglycemia (IAH) and severe hypoglycemic events (SHEs). The results showed that islet transplantation was effective for preventing hypoglycemic unawareness and providing sustained glycemic control (<5.6% of HbA1c) and thus should be considered for patients with T1D and IAH in whom other less-invasive treatments have been ineffective in preventing SHEs [13]. Although human allogeneic islet transplantation is now considered to be widely applicable to more T1D patients possibly under the coverage of health insurance reimbursement, the supply of donor organ is significantly limited, leading to only 0.1% of the patients gaining access to this promising therapy.

Porcine islets have long been considered to be the best alternative source for the human counterpart [14]. Pigs are easy to breed and have large litters at delivery and, most importantly, have high degree of physiological similarity to humans and a long history of use of porcine insulin for treating T1D patients. In addition, they can be bred in specific pathogen-free (SPF) and/or designated pathogen-free (DPF) status and thus healthier donor pancreata can be supplied unlimitedly, though relatively high cost is required for maintaining them in a clean facility which is regularly monitored for microbial pathogens. To achieve clinical xenotransplantation, several groups started pig-to-NHP islet xenotransplantation from the late 1990s [15]. However, in these early studies, porcine islets that had been transplanted into the portal vein or kidney subcapsule of the monkeys survived only

for several days to weeks despite of using strong immunosuppression regimen. Loss of the significant number of islets immediately after transplantation has been the most difficult obstacle to overcome. Goto et al. had focused on this phenomenon and tried to delineate the cellular and molecular mechanism underlying this early islet loss [16]. Indeed, they coined the term instant blood-mediated inflammatory reaction (IBMIR), and this problem still remains a significant barrier for long-term survival of porcine islet in the monkeys as well as in allogeneic and even in autologous islet transplantation settings [17, 18]. In 2006, two independent groups first succeeded in prolonging the survival of porcine islets (adult porcine islets and neonatal porcine islets) for longer than 3 months, using anti-CD154 monoclonal antibody-based immunosuppression [19, 20]. Since then, the survival duration of the porcine islet graft in the monkeys has been lingering around one year at most in a very limited number of the recipients, even though multiple genetically modified pigs were used [21]. In 2009, International Xenotransplantation Association (IXA) released a consensus statement on conditions for undertaking clinical trials of porcine islet products in T1D [22] and recently updated this document [23]. To justify clinical xenotransplantation, prerequisite results that should be obtained in preclinical NHP studies were to show the maintenance of fasting blood glucose levels (BGLs) of <150 mg/dL and nonfasting levels of <200 mg/dL in the absence of exogenous insulin or in the presence of greatly reduced insulin requirements in at least five out of eight consecutive NHPs (now four out of six consecutive NHPs in an updated version). Follow-up should be for a period of at least 6 months in all cases and ideally for 12 months in one or two successful cases [24, 25]. Recently, our group reported successful results where five consecutive diabetic monkeys achieved normoglycemia for at least 6 months after transplantation of adult porcine islets, with the longest survival day reaching to >603 days [26]. During a follow-up study, one monkey showed normoglycemia up to ~1000 days using a CD40-CD154 blockade such as anti-CD154 or anti-CD40 monoclonal antibody [27]. These two studies pose an important implication that porcine islet graft can survive in the liver, which is rather a harsh environment, for a significantly long duration. The caveat of these studies would be transplanting relatively high numbers of islets (100,000 IEQ/kg) to maintain normoglycemia for a long duration, which would reflect the species incompatibility between NHP and pig. The normal fasting blood glucose level (BGL) of a monkey has been known to be around 60 mg/dL, and the monkey requires higher amount of insulin for glycemic control than pigs and probably humans [28]. As rhesus monkeys have about 25-year lifespan, 1000 days could approximate to 3000 days (>8 years) in humans, suggesting that transplanted porcine islets can treat diabetic patients for a long time, given that immunosuppression should be well maintained with suitable drugs. Currently, our group is actively seeking to develop clinically applicable immunosuppressive regimen in the same pig-to-NHP islet xenotransplantation model.

In this chapter, we will describe the detailed procedures of islet isolation from adult SNU miniature pigs, diabetes induction and islet transplantation, immune monitoring after transplantation, and finally biopsy and subsequent immunohistochemical analyses. Because other related topics including encapsulated pig islets, islet sources, immunosuppression regimen,

and overall results from several pig-to-NHP islet xenotransplantation have been elegantly described elsewhere [15, 29–32], here we will focus on the above themes while briefly reviewing past and current status of each area.

2. Porcine islet isolation DPF SNU miniature pigs

2.1. History and characteristics of SNU miniature pigs

Islets for transplantation can be obtained from adult, neonatal, fetal, and embryonic pigs. There is still a debate on the best source of the porcine islets, but at least adult, neonatal, and embryonic pancreata have been shown to be efficient for controlling hyperglycemia in higher mammals including NHPs [19, 20, 33]. Because advantages and disadvantages of using each islet source have been repeatedly discussed elsewhere [30, 34], here we focus on adult porcine islets from DPF miniature pigs. Our group had obtained an SPF miniature pig strain from the University of Chicago in 2004 and have been breeding and maintaining a closed herd in a SPF barrier facility. About 41 viral, 35 bacterial, 2 fungal pathogens, and 25 parasites were screened and confirmed negative in microbial examinations that have been performed on a regular basis (at least once two years), implying that this closed herd is in DPF status [35]. Also, all SNU miniature pigs have been tested for the presence of porcine endogenous retrovirus (PERV) via reverse transcription-polymerase chain reaction (RT-PCR). The results showed that PERV A, B, and C genotypes were present in the genome. However, reverse transcription activity of PERV assessed by *in vitro* reverse transcriptase assay in >60 of monkeys that underwent porcine islet transplantation was not observed. This observation is supported by the gene sequence data of PERV in SNU miniature pig, showing that most of the PERV genes were integrated into the chromosome as defective forms such as deletion, insertion, or inversion (data not shown).

2.2. Islet yield from SNU miniature pigs

In 2007, we have published the first results of islet isolation from SNU miniature pigs (at that time, this pig was named Chicago Medical School [CMS]). In that report, we compared the islet yield from 9 adult SNU pigs (>12 months old), 6 young SNU pigs (6–7 months old), 4 other adult miniature Prestige World Genetics (PWG) pigs (>12 months old), and 13 adult market pigs, and found significantly higher yield of islets from adult SNU pigs than the other three groups: The yield was ~9600 islet equivalent per 1 g pancreas (IEQ/g), which marked the highest value that had ever been reported worldwide [36]. Moreover, we published the results based on 68 successful cases of isolation attempts and found several parameters that predicted for higher yield of islet isolation in 2009: old age of >2 years, male preference, pregnancy experience in female, and good distension of pancreas by collagenase injection [37]. Since then, we have preferred to use adult SNU pigs older than 2 years and standardized all isolation procedures from pancreas procurement to islet purification (**Figure 1**). The results of islet isolation remained stable during the past 5 years, and the yield was ~6000 IEQ/g pancreas and total ~300,000 IEQ/isolation attempt.

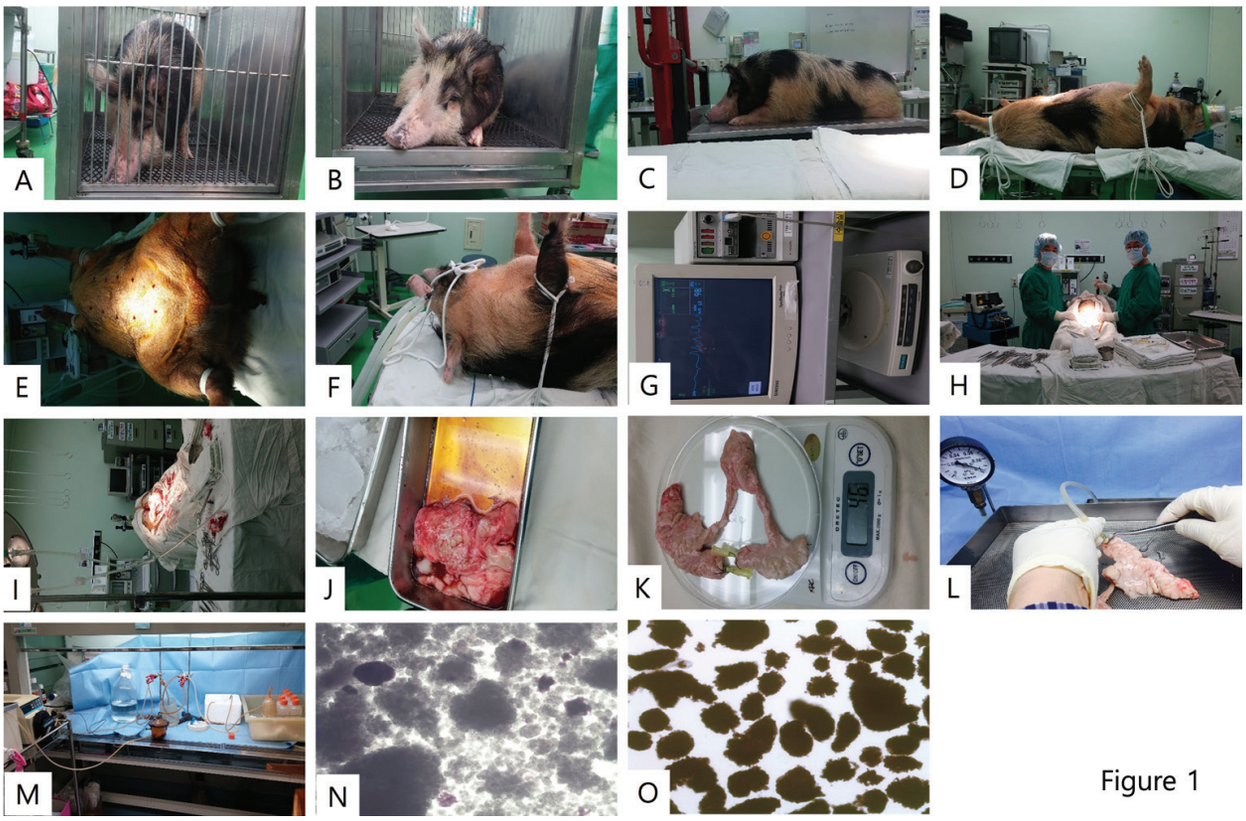

Figure 1. Whole procedure of islet isolation from a DPF SNU miniature pig. (A) A pig transferred to an operating room in a cage. (B) The pig was anesthetized with ketamine and xylazine. (C–F) The pig was moved to an operating table using a lift, intubated, and kept under anesthesia under isoflurane gas. G; Oxygen saturation was monitored. (H–I) Surgery was started under aseptic condition. (J–K) The pancreas was removed, trimmed, and weighed. (L) Collagenase was infused into the pancreatic duct in a cold infusion chamber while monitoring the pressure. (M) The pancreas was incubated in a Ricordi chamber for 20–25 min. (N) The tissue digest was examined under a microscope before the purification step using a COBE2991 processor. (O) Final islet preparation that has been stained with dithizone after purification.

2.3. Quality control of isolated islets from SNU miniature pigs

In order to gain consistent glycemic control after transplantation of porcine islets in NHPs, quality control of isolated islets is important. For our pig-to-NHP islet xenotransplantation experiments, we performed three independent assays that included (1) islet cell viability test using β-cell specific fluorescent dye Fluozin-3, mitochondrial activity indicator Tetramethylrhodamine, Ethyl Ester (TMRE), and a fluorescence-activated cell sorting (FACS) machine, (2) glucose-stimulated insulin secretion (GSIS), and (3) nondiabetic obese severe combined immunodeficiency (NOD/SCID) mouse bioassay where four streptozotocin (STZ)-induced diabetic mice were transplanted with 2500 IEQ of porcine islets under the kidney subcapsule, and their BGL were monitored 2–3 times per week for at least 2 months (**Figure 2**). Our recent study showed that the isolated porcine islets were >90% pure, contained >80% healthy β-cells, and had >60% diabetes correction capacity, each demonstrated by dithizone staining, FACS analysis, and NOD/SCID bioassay, respectively [26]. Although the fold increase of insulin upon glucose stimulation of porcine islets overall reached >1, the results from GSIS assay were highly variable and did not reflect the potency of the isolated pig islets, unlike those from other species (data not shown).

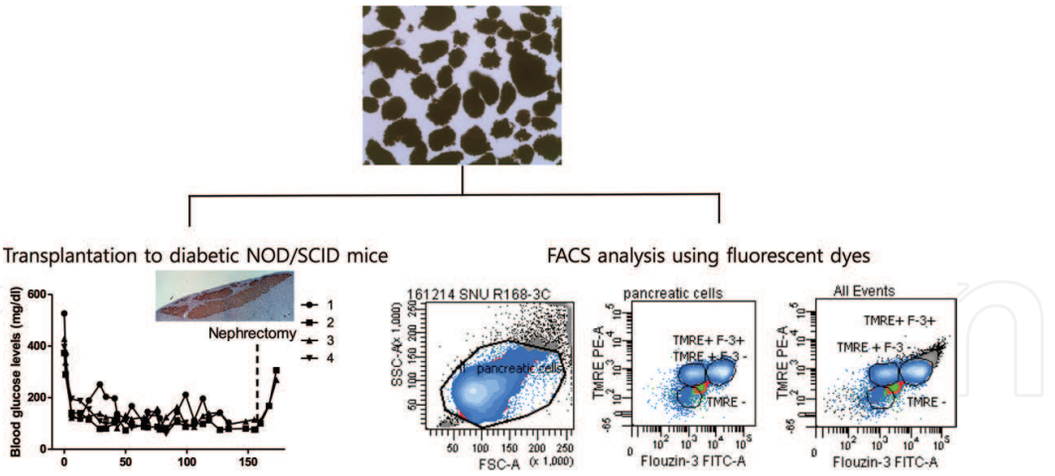

Figure 2. Quality control of the isolated porcine islets. A portion of the isolated porcine islets was tested for their potency using two independent assays as follows: *NOD/SCID bioassay; four mice were rendered diabetic by injecting STZ (200 mg/kg) and BGLs were followed after porcine islet transplantation (marginal mass; 2500 IEQ) into subcapsular region of the kidney. After nephrectomy, hyperglycemia was confirmed, and the harvested kidney was immunostained with anti-insulin antibody. FACS index; porcine islets were dissociated into single cells and were stained with Fluozin-3 and TMRE for measuring β-cells and mitochondrial potential, respectively. Then, the stained cells were analyzed by FACS. FACS index was calculated by Fluozin-3 positive (β-cells) × TMRE positive cell fraction.

3. Diabetes induction in monkeys and transplantation procedure via the portal vein

3.1. Diabetes induction in monkeys

There are several methods to induce diabetes mellitus (DM) in the monkeys such as total pancreatectomy [38], partial pancreatectomy (75% resection of the pancreas) followed by low-dose STZ (15 mg/kg) injection [39], and high-dose STZ (80–150 mg/kg) injection [40]. Pros and cons of each DM induction method are summarized in **Table 1**. STZ is selectively uptaken by the glucose transporter 2 (GLUT2), and induce cell death by massive DNA alkylation [41]. Because GLUT2 is mainly expressed in the pancreatic β-cell, hepatocytes, and basolateral membrane of small intestine and renal tubular cells [42], those organs can be damaged by STZ injection. To prevent systemic side effect of STZ, one group suggested that STZ should be injected into celiac artery and branches supplying blood to the pancreas after temporary embolization of the hepatic and gastric arteries [43]. However, the equipment such as C-arm or fluoroscopy and higher degree of technical skill for arterial catheterization is required to use this method. Zhu et al. reported an in-depth review article for DM induction in NHPs for islet transplantation [44]. Recently, our group published the procedures of STZ-induced DM induction and subsequent DM management before and after islet transplantation in rhesus monkeys [45].

3.2. Induction of DM using high dose of STZ injection

A central venous catheter (5Fr. Dual-Lumen PICC; Bard Access Systems, Salt Lake City, UT, USA) was inserted into the right internal jugular vein in monkeys under general anesthesia. Monkeys

	Total pancreatectomy (TP)	Partial pancreatectomy (PP) + Low dose STZ injection	High-dose STZ injection
Pros	Usage for auto- or alloislet source [81] Unfailing method of DM induction	Usage for alloislet source Less invasive compared to TP Less systemic toxicity compared to high-dose STZ injection	Less invasive More convenient Low possibility of regeneration of β-cell [82]
Cons	Invasive Requires a precise surgical technique Loss of exocrine function (sometimes requirement of oral administration of pancreatin and other islet hormones) High surgical mortality	Increased possibility of regeneration of β-cell More invasive compared to high-dose STZ injection More systemic toxicity compared to TP	Instability of STZ in solution Liver and kidney toxicity Nausea and vomiting

Table 1. Pros and cons of each diabetes mellitus induction method.

were fasted overnight and were prehydrated with normal saline (NS; 0.9% NaCl, 5 mL/kg/hr intravenously [i.v.]) via a tether system for 12 h before STZ (Sigma–Aldrich, St Louis, MO, USA) administration to reduce adverse nephrotoxic effects. A high dose of STZ (110 mg/kg) was diluted with 10 mL of normal saline and given i.v. within 10 min at 4 pm to prevent hypoglycemia at 9 am the next day. Because maximum nadir of hypoglycemia usually occurs about 17 h after STZ injection, 5% dextrose solution was infused at 1 h after STZ injection to prevent hypoglycemia and nephrotoxicity. Intravenous glucose tolerance test (IVGTT) and arginine stimulation test (AST) were conducted within 1–2 weeks after STZ injection. For IVGTT, a bolus of glucose solution (0.5 g/kg) was administered into the right saphenous vein. Two mL of blood was collected from the left saphenous vein at baseline, immediately before injection of glucose, 2, 5, 15, 30, 60, 90, and 120 min after injection of glucose to measure blood glucose and C-peptide. BGL were measured using a small electrode-type blood glucose meter (Accu-Chek™, Roche Diagnostics, Seoul, Korea). For AST, 70 mg/kg of arginine (Sigma–Aldrich, St Louis, MO, USA) was administered into the right saphenous vein. Two mL of blood was collected from the left saphenous vein at baseline, 0, 1, 2, 3, 4, 5, and 10 min after administration of arginine to measure C-peptide. Complete DM was confirmed by persistent hyperglycemia and <1 ng/mL of fasting C-peptide levels and absence of C-peptide responses in IVGTT and AST.

3.3. Exogenous insulin treatment procedure in diabetic monkeys

Because the monkeys require high doses of insulin to sustain normoglycemia and are easily succumbed to metabolic deteriorations such as ketone body formation if they are not adequately treated, insulin treatment is very important to keep animals healthy after STZ injection. Animals were fed on commercially available certified primate biscuit diet (2050C, Harlan, Indianapolis, IN, USA). Calorie intake was maintained within 70–130 Kcal/kg/day which were divided equally at 9 am and 4 pm. After confirming complete DM induction, BGL was checked at least two times per day. Desired target value of the fasting BGL was approximately 80–150 mg/dL in the diabetic monkeys. To do so, each meal was not fed until the fasting BGL was measured at 9 am and 4 pm. Intermediate-acting form of insulin (NPH; Novolin N, Green Cross Corp., Yongin, Korea) and long-acting form of insulin (glargine; Lantus, Sanofi-Aventis Korea, Seoul, Korea) were injected subcutaneously after feeding at 9 am and 4 pm, respectively. Because insulin glargine and NPH that had been injected at different time points can influence BGL at the time

of subsequent measurement, the algorithm of insulin dose adjustments that have been modified from the method used in human clinic [46] was used to maintain target fasting BGL (**Table 2**).

Fasting plasma glucose (mg/dL)	Insulin dosing
<40	No insulin injection
<60	Reduce by 1–2 U
60–80	Reduce by 0.5 U
80–150	No change
151–250	Increase by 0.5 U
251–350	Increase by 1 U
>350	Increase by 1.5 U

Table 2. Insulin dose adjustments according to the fasting blood glucose levels [45].

3.4. Transplantation procedure via the portal vein

There are two popular methods to transplant the islets via the portal vein: one is to infuse the islets through a jejunal vein after laparotomy [26]; and the other is to use percutaneous transhepatic portal catheterization guided by ultrasound technology [47]. The latter is less invasive than the former, but special equipment—such as ultrasound and C-arm—and the technique for ultrasound-guided percutaneous transhepatic portal vein catheterization are indispensable. Because of this limitation, most research groups prefer to use the former method in NHP study. In our study, the monkeys were fasted for 12 h and a laparotomy was performed. The jejunal arch was exposed, and a 22-gauge catheter was inserted through the jejunal vein and advanced near the portal vein. The porcine islets were infused under gravity pressure for 8–12 min (**Figure 3**). The vessel was ligated with a 5–0 prolene suture, and the tether system was applied for continuous fluid therapy and infusion of low-dose glucose, if necessary.

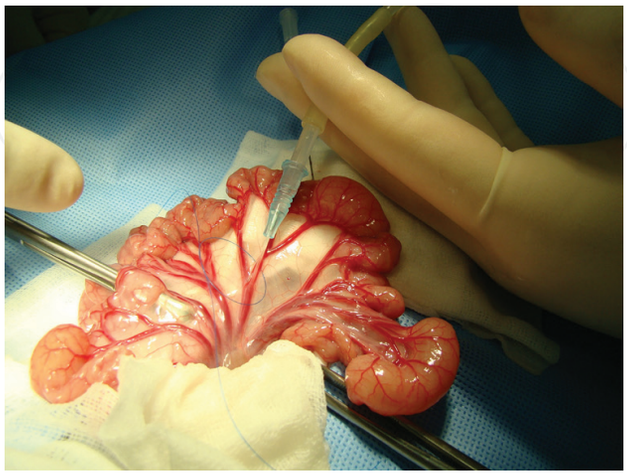

Figure 3. Islet transplantation into the portal vein through a jejunal vein. Porcine islets were resuspended in tissue culture media and infused via a jejunal vein of the diabetic rhesus monkey under anesthesia.

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4. Immune monitoring after porcine islet transplantation

4.1. Monitoring of humoral immune responses

Porcine islet transplantation in NHP has been known to elicit humoral responses against porcine antigens, including Galα1,3Gal (Gal), and non-Gal antigens [48]. Gal is a carbohydrate antigen, which is expressed universally in most species including bacteria and fungi, but not in humans and old world NHPs. Anti-Gal is the most abundant form of natural antibody in humans (mostly found as IgM, IgG, and IgA isotypes to a lesser extent). CD40 signaling on B cells, which is acquired through the interaction with CD154 expressed on T cells, is critical for the survival and proliferation of B cells, antibody production, isotype switching, germinal center formation, memory generation, and production of numerous cytokines [49]. Antibodies targeting CD40-CD154 costimulation pathway have been shown to efficiently suppress humoral responses including anti-Gal and anti–non-Gal antibodies in the recipients [19, 20]. Indeed, immunosuppression regimen including anti-CD154 antibody suppressed the induction of anti–non-Gal and anti-Gal antibodies and prolonged islet graft survival for up to >603 days in NHP recipients [26]. In contrast, similar immunosuppression regimen including anti-CD40 antibody, instead of anti-CD154, suppressed xenoreactive IgG responses after islet transplantation as well, but could not sustain the graft function for a prolonged period [50]. Therefore, suppression of humoral responses against xenoantigens seems to be essential, but not quite enough to sustain graft survival in porcine islet transplantation. In our pig-to-NHP islet xenotransplantation model, monitoring of humoral responses after islet transplantation in the recipients is performed as follows: (1) weekly measurement of anti-Gal IgG and IgM using an in-house enzyme-linked immunosorbent assay (ELISA), (2) measurement of anti-donor pig peripheral blood mononuclear cells (PBMC) IgG and IgM using an in-house flow cytometry assay at every 2–3 months interval with the serum samples that had been collected weekly and stored in aliquots, and (3) measurement of anti-porcine endothelial cells (PEC) IgG every 2–3 months by flow cytometry using weekly collected and stored serum aliquots. As a control, the levels of IgG binding to galactosyl transferase knockout (GTKO) PECs (kindly provided by Dr. Shuji Miyagawa in Osaka University, Japan) were measured in parallel.

The levels of anti-Gal IgG and IgM antibodies are measured by ELISA as follows [50]: each well was coated with 100 μL of Galα1,3Gal β1-4GlcNAc-human albumin (5 μg/mL; GlycoTech, Gaithersburg, MD, USA) and then blocked with 1% human albumin (Green Cross Corp., Yongin, Korea) diluted in phosphate-buffered saline (PBS). Monkey plasma (100 μL) diluted 1:50 (for anti-Gal IgG) or 1:100 (for anti-Gal IgM) in 0.1% human albumin-supplemented PBS was added into each well in duplicate and incubated at 37°C for 30 min. Then, the signal was detected with the peroxidase-conjugated anti-human IgG or anti-human IgM (Sigma-Aldrich, St. Louis, MO, USA) and subsequent color development. Serial dilutions of the selected lot of monkey plasma (as a calibrator) were tested in parallel. A mean absorbance of the sample was compared with those of the calibrator and each antibody level of the sample was calculated from the calibration curve. High-level and low-level control plasma samples were simultaneously tested in each run to validate the performance of assays.

The detection of xenoreactive antibodies binding to porcine cells was performed by flow cytometry [51]. Single-cell suspensions (10^5/tube) of cultured PEC cells or PBMC obtained from the donor pigs were mixed with 50 μL of the plasma diluted 1:10 in PBS containing 1% human albumin and 30 mM EDTA, and incubated at 4°C for 30 min. The plasma was then incubated with FITC-conjugated $F(ab)_2$ fragments of a rabbit immunoglobulin specific for human IgG or human IgM (DAKO, Glostrup, Denmark) and measured using a FACSCalibur (BD Biosciences, San Jose, CA, USA) flow cytometer. The extent of antibody binding was expressed as the mean fluorescence intensity (MFI): MFI of the sample subtracted by the MFI of the negative control (porcine plasma). To reduce inter-variation, cohort samples obtained every week were assayed in duplicate in a single run, and the number of samples in each assay did not exceed 20. High-level and low-level control plasma samples were simultaneously tested in each run to validate the performance of assays.

4.2. Monitoring of cell-mediated immune responses

Since the current clinical islet transplantation procedure has been performed in the liver through the portal vein, IBMIR mediated by diverse nonimmunological and immunological factors is known to contribute to early islet loss [52]. Although the exact mechanisms underlying IBMIR need to be elucidated further in pig-to-NHP islet transplantation, activation of coagulation cascades together with platelet activation, tissue factor release, and thrombin release is observed during IBMIR, and the extent may be stronger than that observed in allogeneic islet transplantation [53]. Strong complement activation has been observed during IBMIR [54]. In particular, activation of alternative complement pathway was profound in pig-to-NHP islet transplantation [55]. Following immediate responses by soluble inflammatory mediators, infiltration of islet grafts by large numbers of activated $CD11b^+$ neutrophils and macrophage was observed [56]. In turn, the degranulation of cytotoxic granules and release of inflammatory cytokines, such as TNF-α and IL-6 from neutrophils and macrophages, induce the apoptosis of islets. In this sense, infiltrating innate immune cells may strengthen the subsequent adaptive immune responses from T and B cells. Among the diverse immune responses against porcine islets, T cell response has been the most critical barrier against long-term graft survival [57, 58]. Indeed, many T cell-targeting immunosuppressants have been developed to control T cell–mediated immune responses against porcine islets. Particularly, costimulation blockade such as CD40-CD154 and B7-CD28 interactions have been proven to be highly effective for prolonged graft survival [19, 20, 59].

To establish an optimal immunosuppressive regimen and to individualize the immunosuppressive therapy, the existence of reliable and predictable immunological tools for monitoring immunological status after clinical porcine islet transplantation is necessary. Yet, there are only a few reports on predictive immune parameters that can estimate the fate of the graft in pig-to-NHP islet transplantation model. Therefore, we will describe our own experience for finding the appropriate monitoring methods to oversee the immunological events happening during graft rejection. In addition, the role of *de novo* induced-immunosuppressive $CD8^+$ T cells will be discussed for the potential markers for predicting graft rejection.

Enzyme-linked immunosorbent spot (ELISPOT) assay is based on the detection of a cytokine (e.g., IFN-γ, IL-4, and IL-2) produced by single cells after stimulation with cognate antigens

[60, 61]. The secreted cytokine is detected by specific monoclonal antibodies and revealed by the generation of discrete spots, reflecting the number of cytokine-secreting cells. It has been widely used in measuring antigen-specific responses in the context of vaccine development for infectious diseases [62], cancer [63], and autoimmunity [64]. In the transplantation field, it has been also used to examine the presence of donor-specific T cells in the patients. For example, following human kidney transplantation, it has been proved useful to screen the patients at high risk for acute or chronic graft rejection [65]. Also, an increased number of IFN-γ-secreting donor-specific cells were detected by ELISPOT in the patients who experienced an acute rejection [66]. Standardization and cross-validation of alloreactive IFN-γ ELISPOT assay were reported in clinical allotransplantation [67, 68]. However, ELISPOT assay is yet to be determined for pig-to-NHP islet xenotransplantation model. Recently, our group reported the results of the retrospective IFN-γ ELISPOT assay using a time-series of PBMC samples from the monkeys with long-term surviving islet grafts (**Figure 4**) [69].

Accumulating evidence indicates that immunosuppression after T cell depletion affects $CD8^+$ T cell homeostasis in the periphery, resulting in the loss of CD28 expression on some subset of T cells. Interestingly, repopulated $CD8^+CD28^-$ T cells have been shown to have immunosuppressive activity and be closely related to the graft survival in some allogeneic transplantation [70, 71]. In our pig-to-NHP islet xenotransplantation model, absolute number of $CD8^+CD28^-$ T cell population significantly increased during homeostatic reconstitution of T cell subpopulation [26] in which the monkeys were treated with ATG and immunosuppressive agents such as

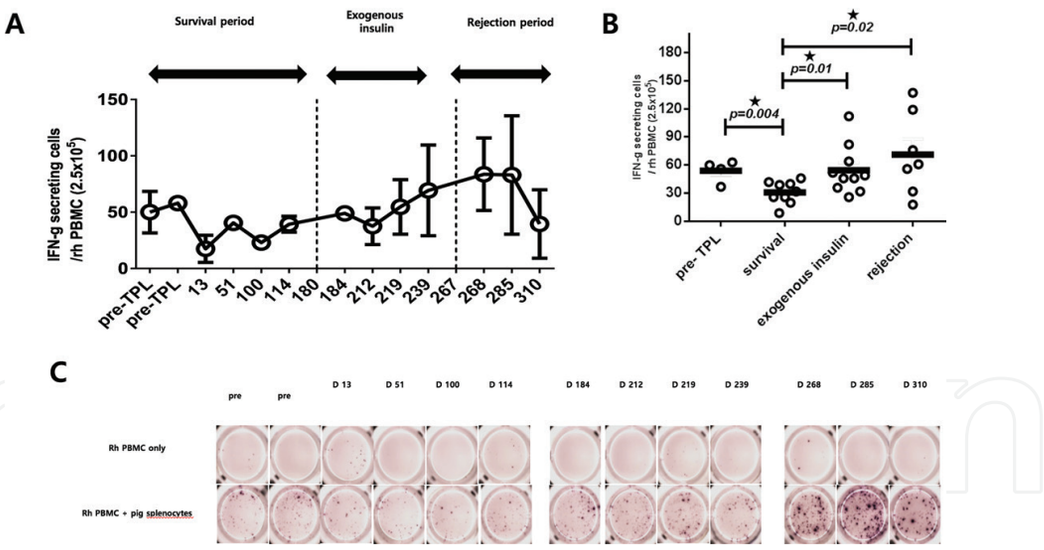

Figure 4. One representative IFN-γ ELISPOT result. (A) STZ-induced diabetic monkey (R015) was transplanted with porcine islets (100,000 IEQ/kg) through a portal vein under immunosuppressive regimen comprised of anti-thymoglobulin, sirolimus, anti-CD40 antibody (2C10R4), and tacrolimus. Fasting BGL and porcine and monkey C-peptide were measured. Grey line: fasting BGL, red bar (bar during normoglycemic period): porcine C-peptide, filled inverted triangle (▼): monkey C-peptide, pink bar (bar during hyperglyemic period): exogenous insulin. The values above red bar indicate porcine C-peptide, (B) After porcine islet transplantation, PBMCs from the recipient monkey were sampled at different time-points and stored. Stored PBMCs (2.5×10^5) were cocultured with 5.0×10^5 splenocytes for 40 h and the number of IFN-g producing spots was measured. The number of IFN-γ secreting spots was enumerated and compared according to the status of graft functioning. (C) Raw data showing the images of IFN-γ secreting cells as visualized by chromogen development.

rapamycin or methyl-prednisone. These resurged $CD8^+CD28^-$ T cells were immunosuppressive to $CD4^+$ T cell activation and proliferation *in vitro*, suggesting that these cells are regulatory subsets. Importantly, blood glucose levels indicative of function of the transplanted islet were closely associated with the ratio of $CD8^+CD28^-$ T to $CD4^+$ T cells, and the transient hyperglycemia or terminal graft loss was observed after $CD8^+CD28^-$ T/$CD4^+$ T cell ratio dropped below 2.0 approximately. In this regard, monitoring immunosuppressive $CD8^+CD28^-$ T cell population together with $CD4^+$ T cells will be helpful for predicting graft function in some allogeneic or xenogeneic transplantation. However, it is highly likely that reconstituted $CD8^+CD28^-$ T cells are heterogeneous in nature and are mixed together with regulatory $CD8^+$ T cells and cytotoxic $CD8^+$ T cells. This fact may hinder broad application of CD28 as a regulatory $CD8^+$ T cell marker. Further study for the identification of surface or lineage markers which could differentiate regulatory CD8+ T cell subset among $CD8^+CD28^-$ T cells is warranted.

5. Liver biopsy and subsequent immunohistochemical procedure

In organ transplantation, tissue biopsy is the standard way to evaluate graft dysfunction or rejection. Recent advances in the development and expansion of antibody application have generated more sophisticated and powerful diagnostic methods based on tissue biopsy. Since the 1990s, diagnosis and determination of graft rejection through a biopsy have been a routine clinical practice in human kidney, heart, and lung transplantation. In kidney transplantation, standardization of criteria for renal allograft rejection has been published [72]. There were many reports on biopsy-based diagnostic methods for other allograft [73, 74] as well as xenograft rejection [75]. The infiltration of immune cells including T cells, B cells, macrophages, and the deposition of antibody/complement to the graft causing graft dysfunction and rejection can be revealed by histological analysis through biopsies. Thus, the histology-based results were usually semiquantitatively analyzed and correlated to the degree of cell infiltration and antibody/complement deposition [74, 76]. For example, C4d deposition was correlated with the presence of donor-specific anti-human leukocyte antigen (HLA) antibodies [77–79] and was deemed a specific marker for acute humoral rejection [76]. Also, $CD68^+$ and $CD3^+$ cell infiltration in the grafts was highly correlated with the extent of cellular rejection in heart transplantation [74]. However, in islet transplantation, posttransplantation monitoring tools to examine the graft site are limited, because the porcine islets are engrafted throughout the liver in a scattered pattern. Immune-monitoring with peripheral blood after transplantation such as measuring absolute counts of T cells, B cells, neutrophils, and NK cells is simple and less invasive, but it is difficult to predict whether the islets in situ are attacked by immune cells and antibodies in the liver via observing these immune parameters. Particularly, after ATG injection, immune cells such as T cells, B cells, and NK cells are depleted and detected in very low numbers in the peripheral blood. In contrast, a large number of immune cells were observed in the biopsied liver samples, and in some cases, overt graft rejection ensued. Therefore, the histological examination of biopsied liver sample would have higher predictive value in determining the status of immune response against islet graft.

In our pig-to-NHP islet xenotransplantation study, scheduled or event biopsy was performed as needed (**Figure 5**). Ordinarily, biopsies of the distal portion of the liver were performed. Under general anesthesia, the monkey was placed in the supine position. The abdominal wall was incised from the xyphoid process to the umbilicus. The margin of the central lobe of the liver was gently grasped and excised about 10 mm distal to the margin (wedge biopsy; 1 × 1 cm). Hemorrhage from the biopsy site was controlled with electrocautery and absorbable hemostat (SURGICEL®, Ethicon Inc.). Routine abdominal wall closure was then performed [26]. Finally, biopsied sample was washed three times with PBS and transferred to 4% paraformaldehyde in PBS for fixation.

Troxell and Lanciault [73] reported practical applications of immunohistochemistry for human organ transplantation, and elegantly described all the details of antibody selection for immune cells and blood coagulation factors in human tissues. However, when these antibodies are used in NHP experiments, antigen-antibody reaction did not work as expected in many cases. Thus, many researchers have tried to find the antibodies that are suitable for NHP tissues. In 2009, Kap et al. reported an important paper entitled "A monoclonal antibody selection for immunohistochemical examination of lymphoid tissues from non-human primates [80]." In this study, they have tested over 100 antibodies against 69 antigens expressed in tissues from the great apes, old world monkeys, and new world monkeys. This report was of great help in selecting antibodies for use in pig-to-NHP islet xenotransplantation. Antibodies used to determine the distribution of B cell, T cell, and macrophages and deposition of complement, antibody, and α-Gal are listed in **Table 3**.

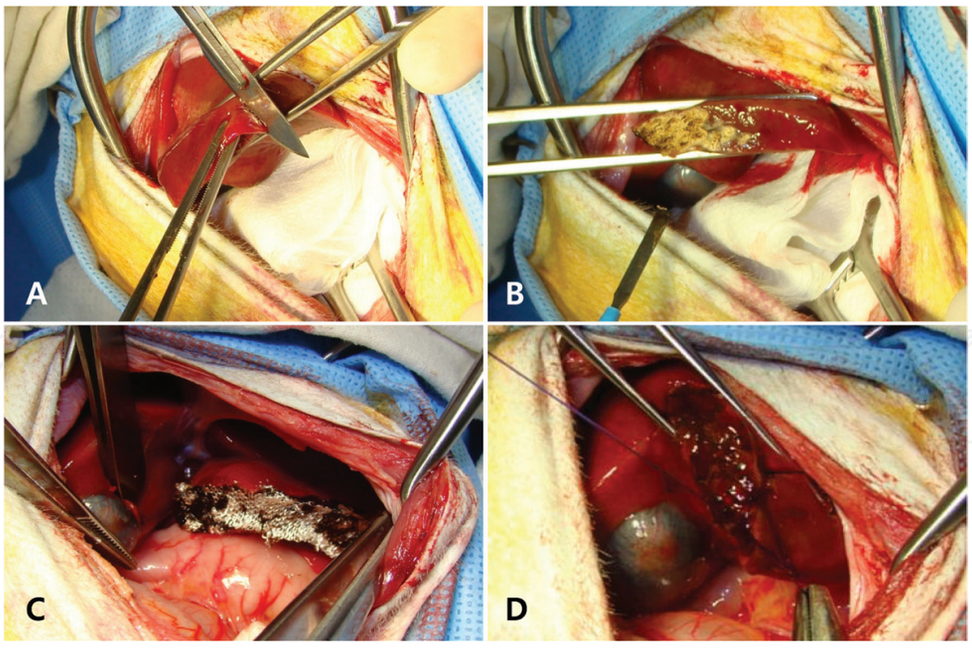

Figure 5. Procedure of liver biopsy in pig-to-NHP islet xenotransplantation. (A) Marginal liver of left central lobe (approximately 1 × 2 cm) is resected. (B) After the resection, the resected surface of liver is electro-cauterized for bleeding control. (C) After the electro-cauterization, Surgicel® is applied on it. (D) When anti-coagulant or anti-platelet agents are administered in the recipients, overlapping guillotine suture method is used to control bleeding because those procedures are not enough for bleeding control.

Antibody	Description	Company	Host	Clonality	Clone
Anti-αGal	Galactose-α-1,3-galactose	Vector Laboratories			Fluorescein labeled *Griffonia Simplicifolia* Lectin I (GSL I) isolectin B4
Insulin	*Pancreatic β-cell marker*	DAKO	Guinea Pig	Polyclonal	
Insulin	*Pancreatic β-cell marker*	Abcam	Guinea Pig	Polyclonal	
Glucagon	*Pancreatic alpha cell marker*	Santacruz	Rabbit	Polyclonal	
CD3	*T cell coreceptor*	DAKO	Rabbit	Polyclonal	UCHT1
CD3	*T cell coreceptor*	Santacruz	Mouse	Monoclonal	A1
CD4	*Glycoprotein found on the surface of immune cells*	Santacruz	Mouse	Monoclonal	1F6
CD4	*Glycoprotein found on the surface of immune cells*	Abcam	Mouse	Monoclonal	1F6
CD8	*Transmembrane glycoprotein that serves as a coreceptor for the T cell receptor*	Abcam	Rabbit	Polyclonal	
CD20	*Activated-glycosylated phosphoprotein expressed on the surface of all B cells*	Thermo	Mouse	Monoclonal	L26
CD20cy	*Activated-glycosylated phosphoprotein expressed on the surface of all B cells*	DAKO	Mouse	Monoclonal	L26
CD68	*Glycoprotein which binds to low density lipoprotein. expressed on monocytes/macrophages*	Thermo	Mouse	Monoclonal	KP1
FOXP3	*Specific marker of natural T regulatory cells*	Abcam	Mouse	Monoclonal	236A/E7
CD31	*Platelet endothelial cell adhesion molecule (PECAM-1)*	Santacruz	Rabbit	Polyclonal	H300
CD31	*Platelet endothelial cell adhesion molecule (PECAM-1)*	DAKO	Mouse	Monoclonal	JC70A
C4d	*Complement system activation marker*	LSBio	Mouse	Monoclonal	
Fibrinogen	*Glycoprotein that helps in the formation of blood clots.*	Abcam	Rabbit	Polyclonal	

Table 3. Antibody specification of immunohistochemistry for pig-to-NHP islet xenotransplantation.

In our study, triple immunohistochemical staining was routinely performed because it has an advantage: capability of examining multiple cell types in the same tissue section. For example, T cells and B cells or T cells and macrophages can be observed simultaneously in the islet graft site. As it is not easy to identify the islet if the islet has been destroyed or only a few β-cells remain, insulin staining should always be performed at the same time (**Figure 6**). Biopsy samples from

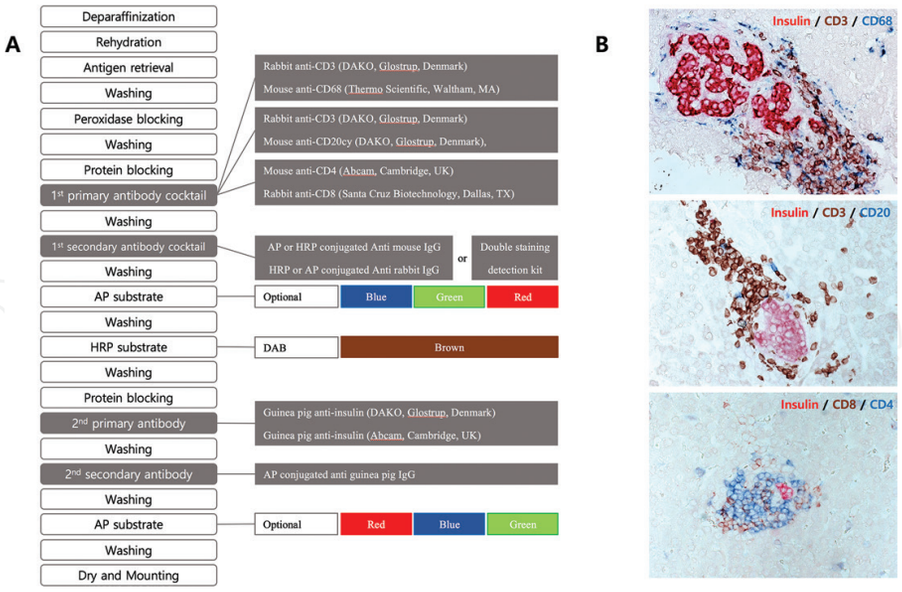

Figure 6. Flow chart for anti-insulin antibody-based triple immunohistochemical staining. (A) Whole procedure of triple staining is depicted. (B) One representative result of anti-insulin antibody-based triple staining. These micrographs show insulin positive b-cells (red) and other immune cells that are either infiltrated or peripherally located (blue or brown).

the liver were fixed in 4% paraformaldehyde in PBS and embedded according to the conventional paraffin-embedding protocol. Paraffin-embedded tissues were sectioned at 4-μm thickness using a microtome. The sections were incubated with primary antibody cocktail designed for each combined targets. The sections were subsequently incubated with secondary antibody cocktail of anti-rabbit/HRP + anti-mouse/AP polymers. For color development, the slides were incubated with blue chromogen (Thermo Scientific, Waltham, MA, USA) for AP and DAB chromogen (DAKO, Glostrup, Denmark) for HRP. After the slides were treated with protein block solution (Thermo Scientific, Waltham, MA, USA), they were incubated with guinea pig anti-insulin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), AP-conjugated goat anti-guinea pig secondary antibody (Abcam, Cambridge, UK) and then incubated with red chromogen substrate (Zytomed Systems, Berlin, Germany). After the staining procedure, all slides were dried at 60°C and mounted with aqueous mounting medium (Thermo Scientific, Waltham, MA, USA). The stained sample was examined by Carl Zeiss Axio Imager A1 microscope, and the images were taken with a micrograph with AxioVision software (Carl Zeiss AG, Oberkochen, Germany).

6. Conclusion

Recent advances in preclinical studies in pig-to-NHP naked islet xenotransplantation have granted a huge momentum in the endeavor for clinical adoption of porcine islets to overcome donor organ shortage in islet transplantation as a cure for T1D. Safety concerns related to porcine islet transplantation have also been significantly lessened by the fact that no infection had been detected in several clinical studies using encapsulated neonatal porcine islets and porcine islets cotransplanted with Sertoli cells. Importantly, clinically applicable

immunosuppressive regimen without anti-CD154 antibody is now being developed. Our detailed procedures of porcine islet isolation from DPF miniature pigs, islet transplantation, immune monitoring with peripheral blood after transplantation, and biopsy and subsequent immunohistochemistry described in this chapter will help other scientists to expedite clinical realization of naked porcine islet transplantation using clinically acceptable immunosuppression in the near future. Also, a quantum leap in advances on gene editing technique that will generate multiple genetically modified pigs or possibly PERV-free pigs within a few months, and on generation of interspecies chimera that will provide human organs in the pigs will heighten the potential of xenotransplantation.

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