Neonatal Pig as an Alternative Source of Islets for Transplantation

by

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Abstract

Background

Islet transplantation is an attractive alternative treatment for type 1 diabetes mellitus (T1DM). However, the further application of islet transplantation in the clinic is limited by the shortage of human organ donors and the immune-mediated rejection. Neonatal pig islets (NPI) are being considered as an alternative source of islets for transplantation. The potential human immune-mediated response to pig islet cells necessitates the development of new immunosuppressants. Genetically modified pigs (GMP) have been produced to alleviate the human organ shortage and rejection. However, the effects of genetic modifications on the function of NPI have not been carefully studied. Hence, a better understanding of NPI development may enable the generation of new GMP.

Objectives of this thesis project were: to investigate the immune response of peripheral blood mononuclear cells (PBMC) from individuals with T1DM to neonatal pig islet cells in *vitro*, and to explore the potential inhibitory effect of Suramin and Direct Red 80 (DR80) on the proliferation of PBMC after stimulation with neonatal pig islet cells; to investigate the immune response of individuals with or without T1DM to NPI in a humanized mouse model; to characterize and compare the islets from GalTKO, GalTKO/hCD46, GalTKO/hCD46/hCD39 and wild type neonatal pigs; to examine patterns of epithelial-cadherin (E-cadherin), neural-cadherin (N-cadherin) and vascular endothelial-cadherin (VE-cadherin) protein expression as islets develop in culture.

Methods

Proliferation curves of human PBMC from individuals with T1DM in response to pig cells were determined through one-way mixed lymphocyte reaction. The effect of Suramin and/or DR80 on this proliferation was traced with CFSE.

The rejection of NPI by human PBMC was tested in NOD.SCID gamma (NSG) mouse model. After achieving normoglycemic, mice were reconstituted with 15 million human PBMC from individuals with or without T1DM. Islet xenografts were harvested at 1-, 2- or 3-week post-reconstitution and analyzed for the presence of endocrine cells.

The difference in islet gene expression among GalTKO, GalTKO/hCD46, GalTKO/hCD46/hCD39 islets was evaluated by microarray analysis and function of islets were evaluated in an NSG mouse model.

Islets were isolated from 1-, 3-, 7- and 10-day-old neonatal pigs. Morphology of islets in culture was evaluated. In addition, islet samples were collected on day 0, 1, 3, 5, and 7 of culture to evaluate the expression of E-, N- and VE-cadherins by RT-PCR and western blot.

Results

Suramin inhibited NPI-induced proliferation of human PBMC with IC_{50}~62.5 to 125 μg/ml, and IC_{50}~125 to 250 μg/ml when induced by pig PBMC. Also, DR80 inhibited the proliferation of human PBMC stimulated with ConA, NPI cells or pig PBMC with IC_{50}~31.2 to 62.5 μg/ml.

Blood glucose levels were not increased in mice reconstituted with human PBMC, while hyperglycemia was observed in mice at 2 to 3 weeks post-reconstitution with NOD splenocytes. Immunohistochemistry revealed massive insulin-positive cells in islet grafts at 1-week post-reconstitution, which became sporadic and less at 2- and 3-weeks post-reconstitution. Macrophage, $CD4^+$ T cell, $CD8^+$ T cells were identified in grafts of reconstituted mice.

Microarray results showed that fewer genes were altered as more genetic modifications were introduced into the pigs. A delay in reversing hyperglycemia was observed when more genetic modifications were applied to islets. Islet xenografts of normoglycemic mice contained various

insulin and glucagon-positive cells, while less insulin- and glucagon-positive cells were identified in grafts of mice that remained hyperglycemic.

Islets from 1-, 3-, 7- and 10-day-old pigs matured and formed oval to round structure on day 3 of culture, while dramatical increase in E-cadherin, N-cadherin and VE-cadherin was observed on day 3 of culture, suggesting these cadherin molecules may play an important role in post-natal islet development.

Conclusions

These results demonstrate the inhibitory effect of DR80 and Suramin on the *in vitro* human immune response to pig cells. NPI rejection by human immune cells started at 2-3 weeks post-reconstitution and involved macrophage, $CD4^+$ and $CD8^+$ T cells. Islets from GMP have therapeutic effect on hyperglycemia. However, variations in the ability of these islet xenografts to reverse the diabetic state of recipient mice was observed and this may be due in part to the difference in patterns of their gene expression. E-cadherin, N-cadherin and VE-cadherin play important roles in the formation and maintenance of pig islet structure and may be important molecules to preserve when designing genetic engineered pigs.

Preface

This thesis is an original work by Wenlong Huang. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board: "AUP00000326, pancreatic islet cell transplantation and breeding colony" and "Pro00002005, mechanism of newborn pig islet xenograft rejection by type 1 diabetic human immune cells". We would also like to thank Ken and Denise Cantor, Ewa and John Burton, Colliers International Inc., and Martin Farrand for their generous donation. This work was supported by the Canadian Institutes of Health Research (CIHR, RES0011565) and Natural Sciences and Engineering Research Council of Canada (NSERC, RES0034259). Wenlong Huang is supported by a scholarship from the Li Ka Shing Foundation under the Sino-Canadian Program of the University of Alberta.

In Chapter 3, W.H. participated in the design of the study, performed the experiments, acquired and analyzed the data, and wrote the manuscript. G.R.R. designed and directed the study, and analyzed the data, and revised the manuscript. Both authors contributed to editing the manuscript and approved the final version.

In Chapter 4, W.H. participated in the design of the study, performed the experiments, acquired and analyzed the data, and wrote the manuscript. G.R.R. designed and directed the study, and analyzed the data, and revised the manuscript. Both authors contributed to editing the manuscript and approved the final version. I would like to thank Ping Wu for her assistance in helping collect samples and perform immunohistochemistry staining. Part of the data has been submitted to the 17th International Congress of Immunology (IUIS 2019 in Beijing, P.R. China) and 15th Congress of International Xenotransplantation Association (IXA 2019 in Munich, Germany).

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Dedication

This thesis is dedicated to my love Xiaojie and my adorable sweetheart Yishu, for the tremendous support and love that you have always given me.

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List of Abbreviations

ABC/HP	Avidin-biotin complex/horseradish peroxidase
ADCC	Antibody-dependent cell-mediated cytotoxicity
AHXR	Acute humoral xenograft rejection
Alpha-1,2-FT	Alpha-1,2-fucosyltransferase
Alpha-1,3-GalT	Alpha-1,3-galactosyltransferase
APC	Antigen presenting cells
BGL	Blood glucose level
CFSE	Carboxyfluorescein succinimidyl ester
CK-7	Cytokeratin-7
CMV	Cytomegalovirus
ConA	Concanavalin A
CPM	Count per minute
CTLA4-Ig	Cytotoxic T-lymphocyte-associated protein 4 immunoglobulin
DAB	Diaminobenzidine tetrahydrochloride
DMSO	Dimethyl Sulfoxide
DR80	Direct Red 80
EMT	Epithelial-mesenchymal transition
EPCR	Endothelial protein C receptor
E-cadherin	Epithelial cadherin
FBS	Fetal bovine serum
GAD65	Glutamate decarboxylase 65
GalTKO	Alpha-1,3-galactosyltransferase knockout
GalTKO/hCD46	Alpha-1,3-galactosyltransferase knockout/ hCD46
GalTKO/hCD46/hCD39	Alpha-1,3-galactosyltransferase knockout/ hCD46/hCD39
GMP	Genetically modified pigs
GnT-III	β-D-mannoside-1,4-Nacetylglucosaminytransferase
HAR	Hyperacute rejection
HBSS	Hank's balanced salt solution
hCD39	Human CD39

hCD46	Human CD46
hDAF	Human decay accelerating factor
HLA	Human leukocyte antigen
hTM	Human thrombomodulin
IBMIR	Instant blood-mediated inflammatory reaction
IC50	50% inhibition concentration
IPGTT	Intraperitoneal glucose tolerance test
mAb	Monoclonal antibody
MCP	Membrane cofactor protein
MLR	Mixed lymphocyte reaction
NK	Natural killer cells
NOD	Non-obese diabetic
NPI	Neonatal pig islets
NSG	NOD scid gamma
NT1D	Non-type 1 diabetes
N-cadherin	Neural cadherin
PAK	Pancreas-after-kidney transplant
PBMC	Peripheral blood mononuclear cells
PERV	Porcine endogenous retrovirus
PI	Proliferation index
pPBMC	Pig peripheral blood mononuclear cells
PTA	Pancreas transplant alone
SCNT	Somatic cell nuclear transfer
SD	Standard deviation
SEM	Standard deviation of mean
SMGT	Sperm-mediated gene transfer
SPK	Simultaneous pancreas-kidney transplant
SPLK	Simultaneous deceased donor pancreas and liver donor kidney
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
TCR	T cell receptor

TEM	Transmission electron microscopy
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TRAIL	TNF alpha-related apoptosis inducing ligand
VE-cadherin	Vascular epithelial cadherin
WT	Wild type

Chapter 1 GENERAL INTRODUCTION

1.1 TYPE 1 DIABETES MELLITUS

The term "diabetes" was first used by Aretaeus of Cappadocia in the 2nd century A.D. to describe the polyuric state of patients[1]. However, the observation of this polyuric condition dated back to 300-1500 BC in Ebers papyrus in Egypt[1]. The term "mellitus" was introduced by John Rollo in 1809 to describe the sweetness in the urine, which was first noted by Hindu physicians Charak and Sushrut around 450 BC[1]. Diabetes mellitus, colloquially referred to as diabetes, is a group of metabolic disorders which are characterized by high blood glucose levels. These disorders include immune mediated or idiopathic type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes. T1DM, previously known as insulin-dependent or childhood-onset diabetes or juvenile diabetes, is a type of autoimmune disease which results in the absolute deficiency of insulin[2]. T2DM, previously referred to as non-insulin-dependent diabetes mellitus or adult-onset diabetes, is characterized by insulin resistance and/or a lack of insulin due to excessive body weight and insufficient exercise. Gestational diabetes refers to high blood glucose level in pregnant women without a previous diagnosis of diabetes. T1DM is the central focus of this thesis.

T1DM accounts for 5-10% of all diabetic cases[3], or 11-22 million worldwide (WHO data). Though the total number globally is not known, it is reported that approximately 80,000 children develop T1DM each year[4].

1.1.1 Etiology of T1DM

The cause of T1DM remains unknown. A multifactorial theory is accepted in the field as the cause of T1DM: genetic susceptibility, environmental factors, and diabetogenic triggers including

viral infection, chemicals or allergens[5]. The genetic contribution to T1DM was demonstrated in a study by Redondo et. al, in which they showed a concordance in monozygotic twins over lifetime[6]. Multiple genes related to T1DM have been identified [7], such as *PTPN22, TLR7, CTLA4, GSDMB, STAT4, IL7R, C1QTNF6, CD55, CTSH, ERBB3, HLA-DQA1, HLA-DQB1, HLA-DRB1, HLA-DPB1* and *INS*. Multiple studies have demonstrated that the expression of these genes is influenced by environmental factors[8, 9]. The importance of environmental factors in the expression of genes has been demonstrated in monozygotic twins. Despite similar genetics, twins exposed to different environmental factors were found to have a difference in mortality in terms of T1DM development. Infection is an important environmental factor in the development of T1DM as demonstrated by the molecular mimicry or modification of beta cell antigens[10], as some bacteria or viruses express molecules that have similar structure with molecules in beta cells.

1.1.2 Pathophysiology of T1DM

Several models have been proposed to explain pathophysiological events during the onset of T1DM. Among them is the linear beta cell mass decline hypothesis proposed by Eisenbarth in 1986[11]. In this model, individuals with genetic susceptibility encounter environmental triggers, followed by the production of autoantibodies and insulitis, leading to the continuous linear loss of beta cell mass. Hence individuals develop hyperglycemia with the onset of T1DM and eventually C-peptide levels (connecting peptide, which is released with the synthesis of insulin) are depleted as the beta cell mass is damaged. Though Eisenbarth's hypothesis is widely accepted, it does not explain the variability in the time for the onset of T1DM. Later, Chatenoud and Bluestone developed several scenarios to explain the variation and these include: i) primary chronic progression that is concordant with Eisenbarth's model; ii) benign fluctuation of the beta cell mass;

iii) a fluctuation model that depends on the interaction between genetic factors and environmental triggers; and iv) secondary chronic progression model[12]. Von Herrath *et al.* regard T1DM as a relapsing-remitting disease that depends on the balance of beta cell damage by the dysregulation of effector T cells and regulatory T cells, as well as the restoration of beta cells by proliferation[13]. Current views on the pathophysiology of the onset of T1DM is as follows[14]: individual with genetic variations such as in i) HLA; ii) insulin; iii) IL-2Ra; iv) CTLA4; v) PTPN22; vi) IFIH are triggered by an environmental event like viral/bacterial infection, nutritional factor or exposure to certain chemicals, creating a proinflammatory state in the individual with down regulation of regulatory T cell function. Meanwhile in the pancreas, IFN-α (interferon alpha) and MHC-I (major histocompatibility molecule, type I) molecules are up-regulated in beta cells, which attracts killing by $CD8^+$ T cells. Damaged cells release beta cell specific antigens, which will be picked up by the antigen-presenting cells (APC) in the adjacent pancreatic lymph nodes. The $CD4^+$ T helper cells activated by the APC thus promote the transformation of naïve B cells into insulin autoantibody secreting plasma cells and the proliferation of CD8+ T cells, thus creating a second killing wave of beta cell mass by the release of perforin, IFN-γ and TNF-α (tumor necrosis factor alpha). The newly released antigens from beta cells are picked up by B cells and other APCs. Then more $CD4^+$ T helper cells are activated, followed by more B cell and $CD8^+$ T cell proliferation. More autoantibodies against insulin, Glutamic Acid Decarboxylase (GAD65), Insulinoma Associated 2 (I-A2), Zinc Transporter 8 (XnT8) and killing by CD8+ T cells lead to the onset of T1DM when 70-90% of beta cell mass are destroyed[14, 15].

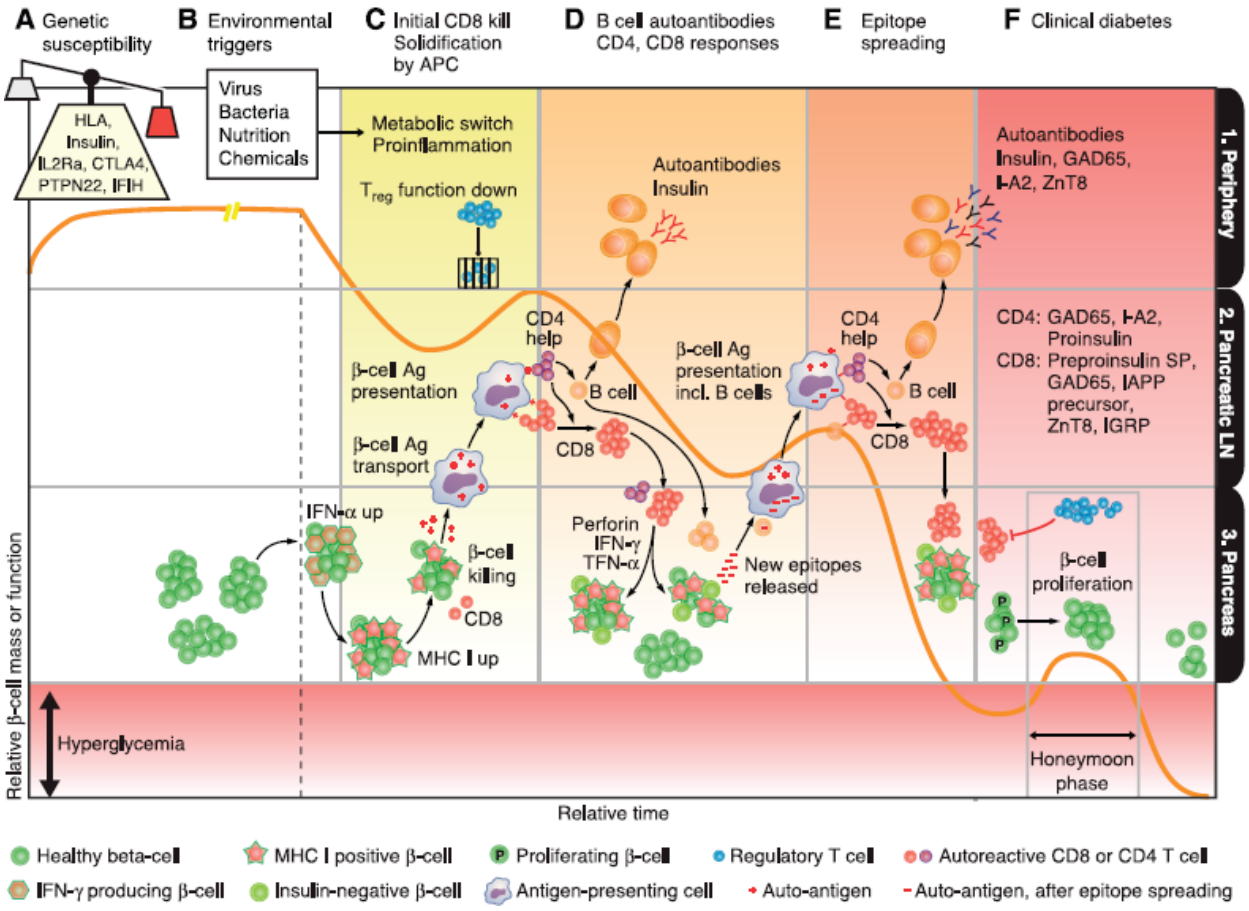

Figure 1-1. How T1DM might arise[14]. (*Van Belle, Tom L., Ken T. Coppieters, and Matthias G. von Herrath. "Type 1 diabetes: etiology, immunology, and therapeutic strategies." Physiological reviews 91.1 (2011): 79-118.*)

1.1.3 Clinical Manifestations and Diagnosis of T1DM

Typical symptoms of T1DM include:

- Polyuria – excessive urine
- Polydipsia – thirsty
- Polyphagia - excessive hunger
- Unexplained weight loss

Other symptoms may include: fatigue, nausea, blurred vison.

According to the American Diabetes Association[16], the diagnostic criteria include:

- Hemoglobin A1C (Hgb A1C) ≥6.5%, or
- A fasting plasma glucose level ≥126 mg/dL (7.0 mmol/L), or
- A 2-hour plasma glucose level ≥200 mg/dL (11.0 mmol/L) during a 75-gram oral glucose tolerance test, or
- A random plasma glucose level ≥200 mg/dL (11.0 mmol/L) with typical symptoms of hyperglycemia or hyperglyemic crisis

1.1.4 Secondary Complications of T1DM

Without proper treatment, secondary complications of T1DM may occur, which can be classified into acute and long-term complications. Acute complications include diabetic ketoacidosis (DKA) and hypoglycaemia, and long-term complications include cardiovascular disease, diabetic kidney disease, retinopathy, and diabetic neuropathy[17].

DKA as a classical acute complication, is manifested by hyperglycaemia and metabolic acidosis. DKA is commonly caused by insufficiency of insulin (e.g., patient forgets to inject insulin) or by stress such as an infection or acute myocardial infarction. When in stress, hormones such as glucagon, cortisol, and catecholamines promote ketogenesis. The accumulation of ketone body (acetoacetic acid, acetone, and beta-hydroxybutyric acid) and blood glucose leads to osmotic diuresis and therefore cause dehydration[18].

Hypoglycaemia is a main complication of insulin therapy, especially when there is an overdose injection. Clinically significant hypoglycaemia is defined as blood glucose level <3.0 mmol/L, while severe hypoglycaemia is any low blood glucose level that causes cognitive impairment in

patients[17].

Cardiovascular disease is the leading cause of death in diabetic individuals. Macrovascular disease such as atherosclerosis may affect arteries of the heart, brain, low extremity and kidney, causing coronary heart disease, cerebral ischemia, low extremity gangrene and nephropathy, respectively. There is evidence showing that intensive glycemic control decreases the incidence of macrovascular disease in diabetic patients[19].

Diabetic kidney disease is the most common cause of end-stage kidney disease. A cohort study shows the cumulative risk of ESRD was 2.2% and 7.0%, at 20, 30 years respectively, after the diagnosis of the disease[20]. Diabetic kidney disease is characterized with glomerular mesangial sclerosis, which results in proteinuria and progressive decline in glomerular filtration.

Retinopathy as the typical microvascular complications, may cause blindness in diabetic individuals. High level of glycosylated hemoglobin (HbA1c) is related to a higher incidence of retinopathy in diabetic patients. The incidence is 1 in 100 person-years for patients with a mean HbA1c value of 5.5%, whereas 9.5 in 100 person-years for patients with a mean HbA1c value of 10.5%[21]. Retinopathy is asymptomatic at early stages, hence, screening is critical especially in patients with > 5 years of diabetes onset[17].

Up to 50% of diabetic individuals develop neuropathy[22]. Diabetic neuropathy includes peripheral distal symmetrical polyneuropathy, predominantly sensory, autonomic neuropathy, proximal painful motor neuropathy and cranial mononeuropathy (ie, cranial nerve III, IV, or VI). The distal symmetrical polyneuropathy is most common[23]. Hyperglycaemia is the greatest risk factor for diabetic neuropathy, and strict glycemic control can prevent the onset and delay the progression of diabetic neuropathy[17].

All the complications described above impair the quality of life in diabetic individuals. Some

of these complications are lethal, especially when without proper treatments. Next we will discuss the treatments for T1DM.

1.1.5 Treatments for T1DM

The treatments for T1DM include lifestyle control, insulin therapy, pancreas transplantation or islet transplantation. Lifestyle control involves diet control and exercise. However, there is no evidence that T1DM can be treated solely by diet and exercise alone. In combination with other anti-diabetic drugs, injections of insulin *via* subcutaneous route or the use of insulin pump is critical in the treatment of T1DM. The life-long course of insulin administration, the side effects of insulin therapy which include low blood glucose level, high potassium level as well as skin reactions at the site of injection make this treatment unattractive, and therefore, the need for more effective treatments for T1DM like beta cell replacement in the form of pancreas or islet transplantation are being considered. Though a successful pancreas transplantation can restore glucose regulation to some extent, the intake of immunosuppressive medicine can do more harm to patients than continued insulin therapy[24]. In addition, the surgical procedures for pancreas transplantation are complicated and surgery can result in high morbidity and mortality. An alternative treatment is to replace the damaged beta cells with islets. The surgical procedures involved in islet transplantation are not as complicated as in pancreas transplantation. This is performed by injecting islets into the portal vein of diabetic recipients. However, there is great shortage in human donors[25]. Pancreas transplantation or islet transplantation would not be widely applied in the clinic until the alternative source of organ donor are available.

With the above understanding, the current treatment of T1DM focuses on insulin replacement therapy by insulin injection. However, insulin therapy itself does not stop the progression of

autoimmune disease, which would also damage the new islets transplanted into the recipients. Hence. another direction in treatment of T1DM is to protect the remaining insulin-secreting β cells by eliminating the pathogenic cells or increasing regulatory T cells. Here we focus on immuno-modulatory therapy in slowing down the progression of T1DM onset. The first trial in this field was reported by Stiller C et al. in 1984, in which they used cyclosporine as immunosuppression for the treatment of new onset T1DM. Since then, multiple therapies either targeting specific antigens or broad-based immunomodulation have been developed to restore self tolerance or preserve the remaining insulin-secreting β cells.

1.2 IMMUNOTHERAPY FOR T1DM

1.2.1 Antigen-specific Immunomodulatory Therapies

Since T1DM is a type of autoimmune disease, researchers in the field have identified several potential antigens specific for T1DM. As summarized by Tom L Van Belle et al., molecules include several of the autoantigens in individuals with T1DM as follows: (pro/pre)-insulin, GAD65, IA-2, ZnT8, PDX1, DiaPep277[14].

1.2.1.1 Insulin

Insulin and proinsulin/pre-insulin are considered as primary antigens present in patients with T1DM. In a preclinical study by Nakayama M et al., the results showed a primary role for an insulin epitope in the development of T1DM[26]. Similar findings were also demonstrated in a non-obese diabetic (NOD) mouse model, which develops diabetes after 12 weeks old when autoreactive T cells infiltrate islets and destroy insulin secreting beta cells. In NOD mice, the administration of

insulin, either orally[27] or intranasally[28], could prevent the development of the disease. However, when similar experiments were done in individuals with recent onset T1DM, the parenteral or oral administration of insulin did not slow down the progression of the autoimmune disease[29-31]. However, another trial on high risk people demonstrated that nasal or parenteral insulin did not prevent T1DM[32]. In a recent trial by Fourlanos et al. intranasal insulin administration showed some decrease in the antibody response, but it did not prevent the continuous destruction of insulin-secreting β cells in patients with early onset T1DM[31]. The discrepancy of results between animal and human trials indicated a more complicated mechanism in the progression of the disease. Recently, a trial by Achenbach P et al. tried to determine the route, dose and timing of administration of insulin on children at high genetic risk and modulated the nature onset of T1DM[33]. With this trial being finished, role of insulin in T1DM development would be revealed. Other than insulin, other molecules such as GAD65 may also play an important role in the onset of T1DM.

1.2.1.2 GAD65

GAD65 is a glutamate decarboxylase isoform expressed in β cells. Although it was first identified by Baekkeskov S et al. more than 20 years ago[34], the mechanism of its involvement in the development of T1DM remains unknown. In NOD mouse model, treatments targeting GAD65 before the onset of the autoimmune disease prevented the development of T1DM[35]. Different route of GAD65 administration alone or with adjuvant showed similar effects. In a human trial by Agardh CD et al., GAD65 immunomodulation in adult-onset autoimmune diabetes showed some exciting results in modulating the course of disease[36]. However, another trial by Wherrett DK et al. demonstrated that GAD65 immunization did not protect β cell destruction[37]. The literature continues to demonstrate conflicting results between animal studies and human trials,

thus reinforcing the notion that the mechanisms must be further elucidated. Further experiments focusing on difference in dose, timing, route of administration, and species will reveal the underlying mechanisms.

1.2.1.3 DiaPep277

DiaPep277 is a peptide translated from the sequence of human Heat Shock Protein 60 (HSP60). Early studies indicated DiaPep277 as an autoantigen recognized by immune cells, but later scientists found its function in regulating the inflammatory immune response, as shown by Jiang H et al[38]. Two randomized, double-blind, phase II trials in 2001[39] and 2007[40] showed immunomodulatory effects in preserving β cell function and improvement in C-peptide response with DiaPep277 treatments. A phase III trial reported similar effect on C-peptide levels and a decrease in hemoglobin A1c level (≤7%) in some of the patients involved. However, not all studies investigating DiaPep277 showed positive results. Two randomized, double-blinded, control studies in children showed no effect in preserving β cell functions[41, 42]. Hence the effect of DiaPep277 in T1DM treatment remains controversial.

Other antigen-specific immunomodulatory therapies involve the autoantigens such as IA-2, ZnT8 and PDX1[14]. All the antigen-specific approaches described above target the autoantigen found in the progression of T1DM. The underlying mechanisms may be the induction of regulatory T cells or the production of regulatory cytokines, which protects beta cells from destruction. In summary, most of these strategies showed exciting results in animal models, but variable efficacy in human trials. The inconsistency may result from differences in the route of administration, doses, and timing of treatment.

1.2.2 Non-antigen-specific Immunomodulatory Therapies

1.2.2.1 Anti-CD3 mAb

CD3 (cluster of differentiation 3) is a protein complex that consists four distinct chains (a CD3γ chain, a CD3δ chain and two CD3ε chains) on T cell surface, which, together with T cell receptor and ζ-chain, constitute the TCR complex. Anti-CD3 therapy has been used for the treatment of allograft rejection in patients after organ transplantation. Teplizumab and otelixizumab, two representative FcR non-binding anti-CD3 mAbs, have been used in T1DM studies. The mechanism below involves the induction of self-tolerance or a shift to regulatory T cells[43, 44]. Several clinical trials in T1DM cases showed a protective effect in preserving β cell function from 1 to 5 years[45, 46]. However, following discontinuation of the medication the disease progression occurs. Recently, a phase III clinical trials using different dose of Teplizumab revealed an inconsistency in C-peptide response compared to a previous study[47]. In summary, anti-CD3 strategy seems to be protective but in a temporary manner.

1.2.2.2 Anti-CD20 mAb

B-lymphocyte antigen CD20 is a glycosylated phosphoprotein expressed on the surface of B cells. B cells, as antigen presenting cells, can recognize and produce autoantibodies against the autoantigens in β cells. As mentioned before, β cells are damaged by autoreactive T cells in the progression of T1DM and the disease is independent of B cells in late stage of disease onset. However, the administration of anti-CD20 mAb to deplete B cells can prevent or reverse the progression of T1DM in NOD mouse model[48]. In a randomized control study the humanized monoclonal antibody against human CD20 called Rituximab showed a protective effect in preserving β cell function[49]. Significant difference in regulating levels of glycosylated hemoglobin and insulin were observed for 1 year after treatment, after which the protective effect

disappeared following termination of the treatment[49]. These results show a potential function of B cells in the early stage of the disease. However, the requirement of B cells for the progression of T1DM in the early stage became controversial when a case report showed that a patient with X-linked agammaglobulinemia (no B cells development) developed T1DM[50]. This contradiction makes anti-CD20 mAb controversial.

1.2.2.3 CTLA4-Ig

The activation of naïve T cells requires 3 signals. These are the binding of TCR complex and antigen presented on MHC molecule, a co-stimulation CD28 signaling by binding to CD80 or CD86 on antigen presenting cells (APCs), and cytokines released by APCs[51]. Cytotoxic T-lymphocyte-associated protein 4 Ig (CTLA4-Ig, Abatacept) is a soluble form of immunoglobulin that binds to CD80 and CD86 and blocks the co-stimulation signal and T cell activation. However, studies using human CTLA4-Ig in NOD mouse model showed no protective effect in preserving β cell function[52] and the murine CTLA4-Ig even deteriorated the disease[53]. Nonetheless, another study by Londrigan SL et al. indicated that the transduction of CTLA4-Ig provided *in situ* protection against islet allograft rejection[54]. In a double-blinded randomized control trial by Orban T et al., co-stimulation blockade in patients with recent-onset T1DM showed an increase of 59% in C-peptide response and decreased level of glycosylated hemoglobin when treated with abatacept compared to placebo after 2 years of treatment[55]. However, the protective effect of abatacept on preserving β cell function was not persistent as it diminished after discontinuing treatment[55]. This suggests the need for long-term use of abatacept. Multicenter clinical trials are needed to provide more evidence on the application of abatacept to prevent T1DM.

1.2.2.4 Rapamycin/IL-2

There is a shift of T cell balance in autoimmune disease from regulatory T cells to effector T cells. Rapamycin inhibits effector T cell proliferation through inhibiting mTOR signaling[56]. Evidence in NOD mouse model showed that IL-2 administration can increase regulatory T cell populations and prevent the disease[57]. These results indicate that we can combine rapamycin and IL-2 to favor a regulatory T cell response in the prevention or treatment of T1DM. A pilot study using the combination of rapamycin and IL-2 was performed in T1DM patients with residual insulin production[58]. The treatment showed an increase in $Foxp3^+$ and $Helios^+$ regulatory T cell populations but a decrease in C-peptide response[58]. This unexpected result suggests that the dose and timing of IL-2 needs to be refined in order to promote an increase in regulatory T cell populations relative to other cells.

1.2.3 Future Direction of Immunotherapy for T1DM

Our understanding in the prevention and treatment of T1DM has significantly improved since the first trial using cyclosporine in the 1980s. Strategies targeting specific autoantigens as well as various immune cells involved in the development of the disease have been applied in animal studies and clinical trials. Although these studies showed some exciting results in preserving the remaining β cells and slowing down the progression of T1DM, problems remain to be solved. There is great discrepancy in the results between different studies and some have even showed contradictory results. Furthermore, most studies focus on pre-existing cases of T1DM, but few have investigated pre-clinical stages of the disease. For clinical trials in humans, monoclonal antibodies targeting effector T cells, B cells or regulatory T cells showed positive effects but in a temporary manner as the protective effects disappear after discontinuing medical therapy.

For antigen specific immunomodulatory therapy, the dose, route and timing of administration

need to be standardized or optimized in susceptible individuals. For example, Culina et al. tried to apply immunomodulatory strategies in order to induce insulin immune tolerance in early stages of intra-uterine fetal development[59], when T cell education begins. They vaccinated the fetus with pro-insulin in the uterus on day 16 of embryonic stage in a $CD8^+$ T cell murine model. The results showed a 3-fold decrease in terms of morbidity and less cytotoxicity of T cells[59]. This study emphasized the importance of early intervention for the prevention of disease. On the other hand, for the immunomodulatory therapies that have positive effects in animal models but not in clinical trial, it is important to compare the difference in different models and elucidate the underlying mechanism to finally translate into human trials. In addition, the study of autoantigens or autoantibodies may help in finding a strategy that can predict the onset of T1DM.

Another direction of future study is the combination of the strategies mentioned above. As in many clinical trials, the administration of monoclonal antibodies only provided a temporary protective effect and sometimes have caused dysregulation of other immune cells. Hence, for the prevention of T1DM, a combination of different immunomodulation should include an agent that can reduce or diminish the autoantibodies, a monoclonal antibody that targets the autoreactive effectors, a monoclonal antibody that could enhance regulatory T cells and other agents that could help in preserving β cell mass and function[60]. The optimization and standardization of the dose, timing, and route of administration should be also considered when designing strategies for combination therapy.

1.3 INSULIN REPLACEMENT THERAPY FOR T1DM

Insulin is a polypeptide consisting of two chains, the A-chain and B-chain, which are comprised of 21 amino acids (with a disulphide bond between A6 and A11) and 30 amino acids

(linked by two disulphide bonds between A7-B7 and A20-B19), respectively. Insulin replacement therapy, also known as intensive insulin therapy or basal-bolus therapy, aims to optimize blood glucose control through defined insulin delivery that mimics physiological secretion[61, 62]. Here, the history of insulin discovery and development and the types of insulin clinically available will be discussed.

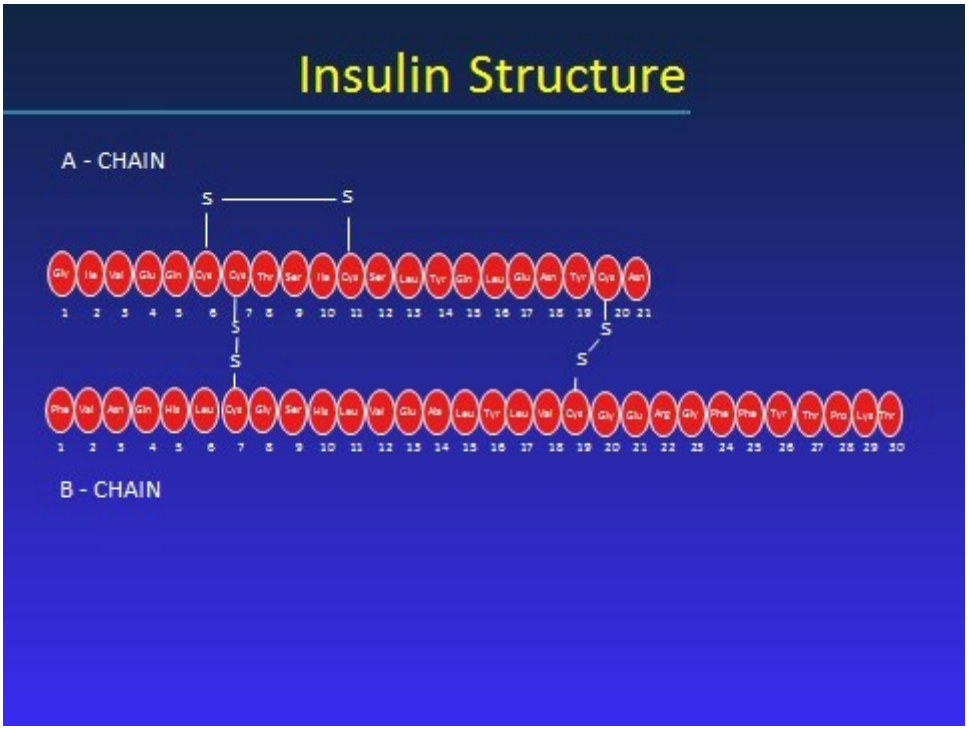

Figure 1-2. Insulin Structure (*Donner, Thomas. "Insulin–pharmacology, therapeutic regimens and principles of intensive insulin therapy." Endotext [Internet]. MDText. com, Inc., 2015*.)

1.3.1 History of Insulin Discovery

Pancreatic islets, also named islets of Langerhans, were first described by Paul Langerhans in 1869[63]. In the 1890s, Von Mering and Minkowski first reported the development of diabetes

after the removal of the pancreas in a dog[64]. Later, Schafer introduced the term “insuline” for the secretion from pancreatic islets[65]. In 1920, Barron noticed that diabetes could be induced by the destruction of islets in the pancreas[65]. In 1921, Banting, Best, Collip and MacCleod reported the anti-diabetic effect of an extract from dog pancreas[66]. The successful use of the extract in patients with diabetes in 1923 stimulated research in the field, and thereafter, animal derived insulin sources have been used in the clinic[65].

1.3.2 Types of Insulin

Insulin sources include pig, cow, human and insulin analogs. Pig insulin and cow insulin are similar in structure compared to human insulin, with differences in one amino acid and three amino acids, respectively. Due to the slight differences in structure, pig and cow insulin are more immunogenic than exogenous human insulin. Pig insulin and cow insulin are no longer available in the US after the introduction of recombinant DNA technology to produce human insulin in 1980s[67]. In this technology, the proinsulin gene is inserted into the genome of either *Saccharomyces cerevisiae* or a non-pathogenic strain of *Escherichia coli*, followed by the isolation and purification of insulin from these bacteria. Later, researchers in the field discovered that the amino acids in B26 to B30 are not essential in the binding of insulin to the insulin receptor, rather, the alternation of these amino acids can dramatically change the efficacy of insulin. Thereafter, various insulin analogs have been generated.

In terms of the efficacy of insulin (time needed for insulin to function), types of insulin include rapid-acting, short-acting, intermediate-acting, long-acting and mixed insulin. Table 1-1 lists the different types of commercially available insulin and their pharmacodynamics. Rapid-acting insulin includes Insulin Aspart (aspartate added to B28), Insulin Glulisine (Glutamate added to

Proline and to B29, lysine added to B3), Insulin Lispro (lysine added to B28) and Technosphere Insulin (a type of inhalable insulin in powder). Rapid-acting insulin usually works within 15 minutes and peaks in 1 hour. Regular Human Insulin is regarded as short-acting insulin, which works in about 1 hour and peaks at 2-4 hours. Intermediate-acting insulin usually refers to neutral protamine Hagedorn (NPH), in which regular human insulin is mixed with protamine to delay the absorption, as such, NPH works at 1-2 hours and peaks at 4-10 hours after subcutaneous injection. Protamine is also mixed with rapid-acting insulin to make insulin mixtures, which have prolonged onset/peak/duration time. Long-acting insulin usually has late onset time and longer duration (more than 24 hours), whereas providing a baseline dose of insulin. Commercially available long-acting insulin includes Insulin Glargine (2 arginines added to B30 and asparagine substituted to glycine in A21), Insulin Detemir (B30 deleted and 14-C fatty acid added), Insulin Degludec (B30 deleted and 16-C fatty acid added).

Table 1-1. Commercially Available Insulin and Their Pharmacodynamics*

Category/Name	Brand Name	Onset (hr.)	Peak (hr.)	Duration (hr.)
Rapid-acting				
Insulin Aspart	Novolog	Within 15 min	1-3	3-5
Insulin Glulisine	Apidra	15-30 min	0.5-1	4
Insulin Lispro	Humalog	Within 15 min	~1	3-5
Technosphere Insulin	Afreeza	Within 5 min	15 min	3
Short-acting				
Regular Human Insulin	Humulin R, Novolin R	~1	2-4	5-8
Intermediate-acting				
NPH Human	Humulin N, Novolin N	1-2	4-10	14+
Insulin Mixtures				
Protamine/Lispro (50%/50%)	Humalog Mix 50/50	0.25-0.5	0.5-3	14-24
Protamine/Lispro (75%/25%)	Humalog Mix 75/25	0.25-5	0.5-2.5	14-24
Protamine/Aspart (70%/30%)	Novolog Mix 70/30	0.1-0.2	1-4	18-24
Long-acting				
Insulin Glargine	Lantus, Basaglar, Toujeo	1.5	Flat	24
Insulin Detemir	Levemir	3-4	6-8	20-24
Insulin Degludec	Tresiba	1	9	42

*Table modified from Thomas Donner and Sudipa Sarkar's book chapter[62].

1.4 PANCREAS TRANSPLANTATION

1.4.1 History of Pancreas Transplantation

The purpose of pancreas transplantation is to restore euglycemia, alleviate the secondary complication of T1DM. The surgical skills for pancreas transplantation have been well established in the past 50 years. The first attempt was by Kelly and Lillehei e*t al.* at the University of Minnesota in Minneapolis in 1966, in which they transplanted a duct-ligated segmental pancreas graft simultaneously with a kidney graft into a 28-year-old female with T1DM[68]. However, the grafts failed due to pancreatitis, which was likely caused by ligation of the pancreatic duct. Later, Lillehei and others performed a series of whole pancreas transplantation (up to 25 pancreas transplants by 1970) in which exocrine secretion was drained by ligation, cutaneous duodenostomy or enteric drainage[69, 70]. Only one of these transplants functioned more than 1 year. Pancreatic exocrine drainage into the bladder through the ureter was first reported by Gliedman at Montefiore Hospital and Medical Centre in New York in 1971[71]. They demonstrated variable graft survival due to leakage of exocrine secretion. The foundation of the International Pancreas Transplantation Registry (IPTR) in the early 1980's, as well as the Spitzingsee Meeting advanced the field of pancreas transplantation. In the late 1980's pancreatic exocrine drainage into bladder via ureteric anastomosis became the most popular surgical technique for pancreas transplantation. To avoid complications of bladder drainage such as urinary tract infection, cystitis, urethritis, reflux pancreatitis, hematuria, metabolic acidosis and dehydration, Tom *et al*. from the University of Cincinnati first reported a successful pancreas transplantation with enteric drainage[72]. Thereafter, the surgical techniques for pancreas transplantation have been well developed with either bladder or enteric drainage.

1.4.2 Development of Immunosuppressive Drugs

In addition to the advancement in surgical skills, the progress in immunosuppression has also contributed to the success of pancreas transplantation. Among them is the first use of cyclosporin A (calcineurin inhibitor) to prevent rejection in pancreas transplant recipients in 1979, reported by Calne and associates[73]. Due to the nephrotoxicity of large dose cyclosporin A, steroid was first introduced into anti-rejection therapy regimens by Starzl *et al*. to reduce the dose of cyclosporin A[74]. Later, the synergistic effect of azathioprine was tested by Squifflet's group, thus further reducing the dose of cyclosporin A[75, 76]. Thereafter, the triple combination of cyclosporin A, steroid and azathioprine had been the anti-rejection treatment regimen for pancreas transplantation. In the late 1980's and early 1990's, another type of calcineurin inhibitor, tacrolimus (also named fujimycin, or FK-506) was used to replace cyclosporin A and resulted in less toxicity. Sollinger and associates from the University of Wisconsin started to use mycophenolate mofetil rather than azathioprine in the anti-rejection recipe[77]. Later, the use of anti-T cell therapy i.e. anti-thymocyte globulin, was found to excitingly increase graft survival[78]. Thus, a quadruple recipe of induction by anti-T cell preparation and maintenance with tacrolimus, mycophenolate mofetil and steroid, has been widely used in the field. After the year 2000, treatment regimens without steroid have been developed by various groups. Looking through the history of immunosuppressive drug development, the search for specific anti-rejection agents with less toxicity but more efficacy has never stopped and remains a hot topic in the field of transplantation.

1.4.3 Current Pancreas Transplantation

With the progress and advancement in surgical skills and anti-rejection strategies above, pancreas transplantation has become the gold standard for the treatment of T1DM with kidney

failure. Currently in the clinic, four major types of pancreas transplantation are available: simultaneous pancreas-kidney transplant (SPK), pancreas-after-kidney transplant (PAK), pancreas transplant alone (PTA), and simultaneous deceased donor pancreas and live donor kidney (SPLK). To date, over 40,000 pancreases have been transplanted worldwide. Patients with SPK have a 1-year-survival rate of 96% and a 5-year-survival rate of 83%. In terms of pancreatic graft survival (insulin independence, defined by fasting blood glucose levels ≤1 mmol/l and 2-hour postprandial levels ≤10 mmol/l without exogenous insulin), SPK, PAK and PTA have 86%, 80% and 78%, respectively, at 1 year after transplantation. Despite the success of pancreas transplantation in curing end-stage T1DM, complications such as cardiac morbidity, post-operative infection and thrombosis may occur post-transplantation. In addition, the transplantation per se may be a huge trauma that many patients cannot bear, and the health of the patients may be compromised while waiting for a suitable donor organ. Therefore, scientists in the field turned to islet transplantation rather than transplanting the whole pancreas.

1.5 Islet Transplantation

1.5.1 History of Islet Transplantation

The development of modern clinical islet transplantation relies on the advancement in techniques of islet isolation and knowledge of transplantation. The idea of transplanting pancreatic tissue was inspired from Von Mering and Minkowski's findings in 1889 that the removal of pancreas led to the failure of maintaining normal blood glucose level in canine[79]. Later, transplantation of pancreatic fragments was performed in dog or sheep, which had various outcomes[80-82]. The idea of separating endocrine and exocrine pancreas was developed. However, the practice of transplantation in individuals was not performed until Hellerstroem

introduced a method for the microdissection of intact pancreatic islets under the microscope in 1964[83]. One year later, Moskalewski used collagenase to dissociate guinea pig pancreas, which improved islet yield[84]. In 1967, Lacy and Kostianovsky developed protocols to isolate rat islets by flushing pancreas with saline before collagenase dissociation followed by islet handpicking[85]. To purify the islet preparation from exocrine cells, density gradient purification with ficoll was proved to be efficient[86, 87]. While Younoszai *et al*. first reported islet transplantation in 1970[88], Ballinger and Lacy showed the reversal of chemically induced diabetes in rats following intraperitoneal autologous islet transplantation in 1972[89], and Reckard *et al*. demonstrated success in reversing diabetes in a chemically induced model in 1973[90]. Later, the concept of intrahepatic islet embolization was put forward in 1973[91]. Rajotte et al. developed protocols for cryopreservation of islets, which showed reversal of diabetes in animals with surgical or chemically induced diabetes in 1983[92]. Though results were promising in rodent models, when the same techniques were applied to large animals and human beings, new problems emerged. The more compact and fibrous pancreatic tissue, especially in human beings, are more resistant to digestion, leading to poor yield and viability in islets[93]. Methods based on intraductal injection of collagenase were developed and proved to be efficient in islet isolation from large animals and human[94, 95]. An "automatic method" using the Ricordi® Chamber (BioRep, Miami, FL, USA) was introduced in human islet isolation in 1988. Using this automatic method, consistent islet yield was obtained[96]. The advancement in isolation technology progresses islet transplantation in the clinic.

1.5.2 Clinical Islet Transplantation

With the progress in islet isolation, transplantation of autologous pancreatic fragments in

humans was performed by Najarian *et al.* in 1977, with the administration of azathioprine and corticosteroids[97]. The first cases of successful allogeneic pancreatic fragment transplantation was reported in patients with T1DM by Largiader *et al.* in 1980, with more than 200,000 human islets of mean diameter 150 μm[98]. Warnock *et al*. in 1989 reported the first series of allogeneic simultaneous islet and kidney transplantation in two T1DM individuals, with continued function of pancreatic islets[99]. Two patients (35 years old male, T1DM for 17 years with retinopathy, neuropathy and end-stage nephropathy; 26 years old male, T1DM for 20 years with end-stage nephropathy) received 260, 845 and 261,370 islets with a standard diameter of 150 μm. The raised C-peptide level indicated continued function of the transplant islets in patients[99]. Two years later, a researcher in the same group reported the first series of insulin independence and/or continued graft function following transplantation of freshly isolated or cryopreserved allogenic islets[100]. Though these advancements were promising, the International Islet Registry Report in 1999 revealed that only 9% of the 267 islet transplant recipients were insulin independent for more than 1 year[25]. In 2000, the Edmonton Group reported a series of 7 patients receiving allogeneic islet transplantation alone achieved 100% insulin independence[101]. Highlights of the Edmonton Protocols includes the use of combined islets from at least two donors and a steroid-free immunosuppressant regimen with daclizumab, which is an anti-interleukin-2 receptor antagonist antibody[101]. In 2006, a multicentre international trial reported 58% insulin independence at 1 year following Edmonton Protocol[102]. In the Eighth Annual Report in 2014 by Collaborative Islet Transplant Registry, more than 1,500 patients have received islet transplantation in 40 international centres[103]. An insulin independent rate of more than 50% at 5 years has been achieved in these patients, which is close to the 5 years success rate of whole-pancreas-alone transplantation[104]. In 2015, a phase III multicentre trial of the NIH CIT Consortium

demonstrated the safety and effectiveness of islet transplantation as a treatment for selected patients with hypoglycaemic unawareness, severe hypoglycaemic episodes and glycaemic lability[105].

Currently, clinical islet transplantation is through the portal vein. Briefly, islets are isolated and purified using the Automated Method described previously, followed by 24 to 72 hours of culture to enable quality assessment of islet preparation[105]. At least 5,000 IEQ (islet equivalent, a parameter for standardizing number and size of islets) per kilogram body weight of the recipient are infused into the portal vein through a minimal invasive percutaneous transhepatic approach under interventional radiology[103]. The islet transplants are settled in sinusoids of the liver where they function. This provides a physiological response to glucose fluctuation, which is more effective than exogenous insulin injection. Also, the minimally invasive approach makes islet transplantation stand out from pancreas transplantation. With the advancement in transplantation techniques as well as the development of safer immunosuppressants, the demand for clinical islet transplantation will expand. However, this expansion will be limited by the shortage of human organ donors. Hence, it is necessary to find alternative sources of islets.

1.6 ISLET XENOTRANSPLANTATION

1.6.1 Advantages of Pigs as Donors for Islet Xenotransplantation

Pigs are being considered as optional organ donors for xenotransplantation. The size, anatomy and physiology of pig organs are similar to human organs. In addition, pigs have a short gestation period and high reproduction rate allowing an adequate supply of pig donors to become available in a short time. Furthermore, the expense to produce pigs is relatively low and they can be manufactured in high hygienic standards to reduce pathogens. Finally, methods of genetic modification to overcome the immune mediated rejection have been developed in pigs[106].

1.6.2 Immunological Obstacles for Pig Islet Xenotransplantation

In addition to the shortage of human organs, another limitation for the wide application of islet transplantation is the immune mediated rejection of the islet transplant. The immune mechanisms against pig islet transplant include instant blood-mediated inflammatory reaction (IBMIR), hyperacute rejection (HAR), acute humoral xenograft rejection (AHXR), immune cell-mediated rejection and chronic rejection. The correction of functional and physiological incompatibility between the porcine donors and human recipients are also prerequisites for successful xenotransplantation. The presence of porcine endogenous retroviruses (PERV) is also considered as another obstacle for further application of islet xenotransplantation. Here, genetic modification to overcome the immune-mediated rejection and PERV infection will be discussed.

1.6.2.1 Instant Blood-mediated Inflammatory Reaction

Though pre-clinical trials have shown encouraging results in islet xenotransplantation, early islet destruction by innate immune mechanisms remains to be solved, among which is the instant blood-mediated inflammatory reaction (IBMIR) encountered after the transplantation of islets into the portal vein[107-109]. The exact mechanisms for IBMIR is not fully understood, but it is known that IBMIR is characterized by activation of complement, platelets, and the coagulation pathway[110]. Scholars found that the tissue factor (TF) expressed on the islets is a key molecule in initiating the response. Moberg et al used monoclonal antibodies to inhibit the tissue factors and showed promising results[111].

1.6.2.2 Hyperacute Rejection

Hyperacute rejection (HAR) is induced by the pre-formed natural antibodies in the host against

carbohydrate epitopes, such as the epitope that is synthesized by alpha-1,3-galactosyltransferase (alpha-1,3-GalT, encoded by GGTA1 gene). Alpha-1,3-GalT transfers a galactose molecule to terminal N-acetyllactosamine (N-lac)[112]. The natural antibodies against alpha-1,3-Gal epitopes exist in Old World primates including human but not in pigs. After pig-to-primate xenotransplantation, those antibodies bind to the epitopes on the endothelium, deposit on the endothelium of vascularized xenografts, activate the complement system and finally activate the coagulation cascade[113]. The activation of the complement system leads to immediate graft destruction demonstrated by widespread hemorrhage, edema, thrombosis and a relative lack of cellular infiltration[114]. Thus, different strategies can be used to reduce HAR, including the knockout of alpha-1,3-galactosyltransferase encoded gene, transgenic expression of human alpha-1,2-fucosyltransferase to compete with the glycosylation, or expression of human complement regulatory proteins such as hCD46, hCD55, hCD59 and/or combination of these proteins[115, 116].

1.6.2.3 Acute Humoral Xenograft Rejection

Acute humoral xenograft rejection (AHXR) begins with the activation of endothelial cells, then disruption of the anti-coagulation property of the endothelium and finally results in thrombotic microangiopathy and disseminated intravascular coagulopathy (as shown in Figure 1-3). The expression of human CD39 (ecto-ADPase), endothelial protein C receptor (EPCR), heme oxygenase 1, thrombomodulin and tissue factor pathway inhibitor (TFPI) have been applied to overcome AHXR and show some exciting results[117]. All these strategies will be discussed further in the following section of genetically modified pigs.

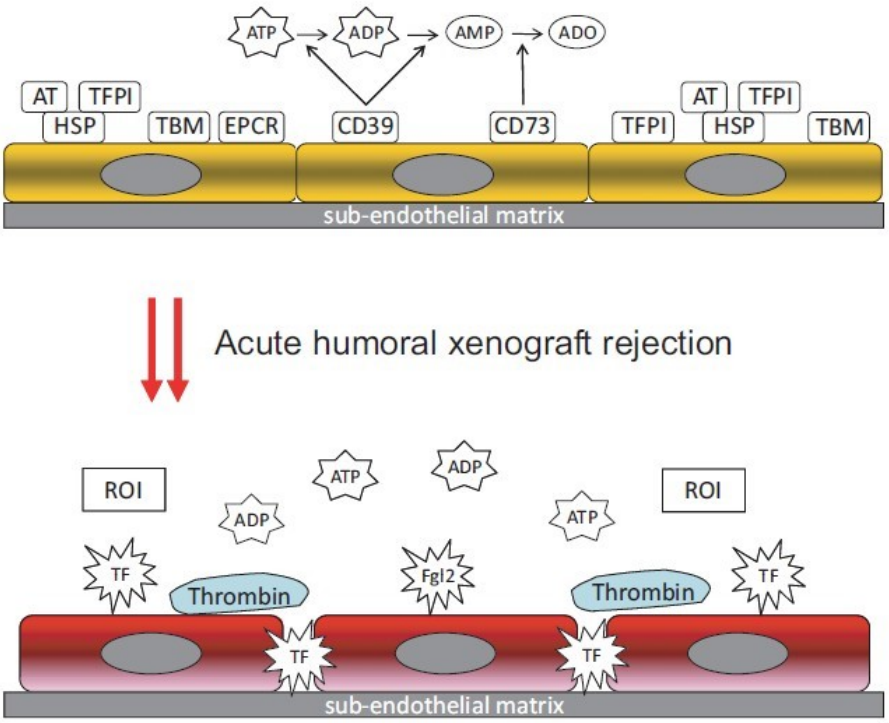

Figure 1-3. Prothrombotic changes associated with acute humoral xenograft rejection, characterized by increased vascular exposure of the immune coagulant fgl2/fibroleukin, tissue factor (TF), thrombinproduction and release of natural anti-coagulants viz. tissue factor pathway inhibitor (TFPI), the protein C pathway with thrombomodulin (TBM), antithrombin (AT), and endothelial protein C receptor (EPCR). CD40L-dependent thrombin generation and release of reactive oxygen intermediates (ROI) are also noted. Loss of endothelial CD39 and CD73 bioactivity leads to increased levels of extracellular adenosine triphosphate (ATP) and adenosine diphosphate (ADP), with the latter acting as a major agonist for platelet aggregation. (*Schmelzle M, Cowan P J, Robson S C. Which anti-platelet therapies might be beneficial in xenotransplantation[J]. Xenotransplantation, 2011, 18(2): 79-87.*)

1.6.2.4 Immune Cell-mediated Rejection

In addition to HAR and AHXR, immune cell-mediated rejection is also involved in pig-to-primate xenotransplantation. Innate immune cells such as natural killer cells (NK), polymorphonuclear neutrophils (PMNs) and monocytes/ macrophages are considered as obstacles

to the successful pig-to-primate xenotransplantation (as shown in Figure 1-4). NK cells can infiltrate pig organs perfused with human blood ex vivo[118]. *In vitro* studies have shown that NK cells can lyse the islet cells directly or in the presence of human serum by antibody-dependent cell-mediated cytotoxicity (ADCC)[119]. The autoreactivity of NK cell is inhibited by the expression of MHC class I ligands of inhibitory receptor CD94/NKG2A, which specifically binds to HLA-E and is expressed in a majority of activated NK cells[120]. Garcia et al. found that HLA-E is not polymorphic and induces allogenic T cell response to a lower degree, which is different from classical MHC molecules[121]. Evidence has been found that the expression of HLA-E can provide partial protection against human NK cell cytotoxicity, which will be discussed in the following sections[122].

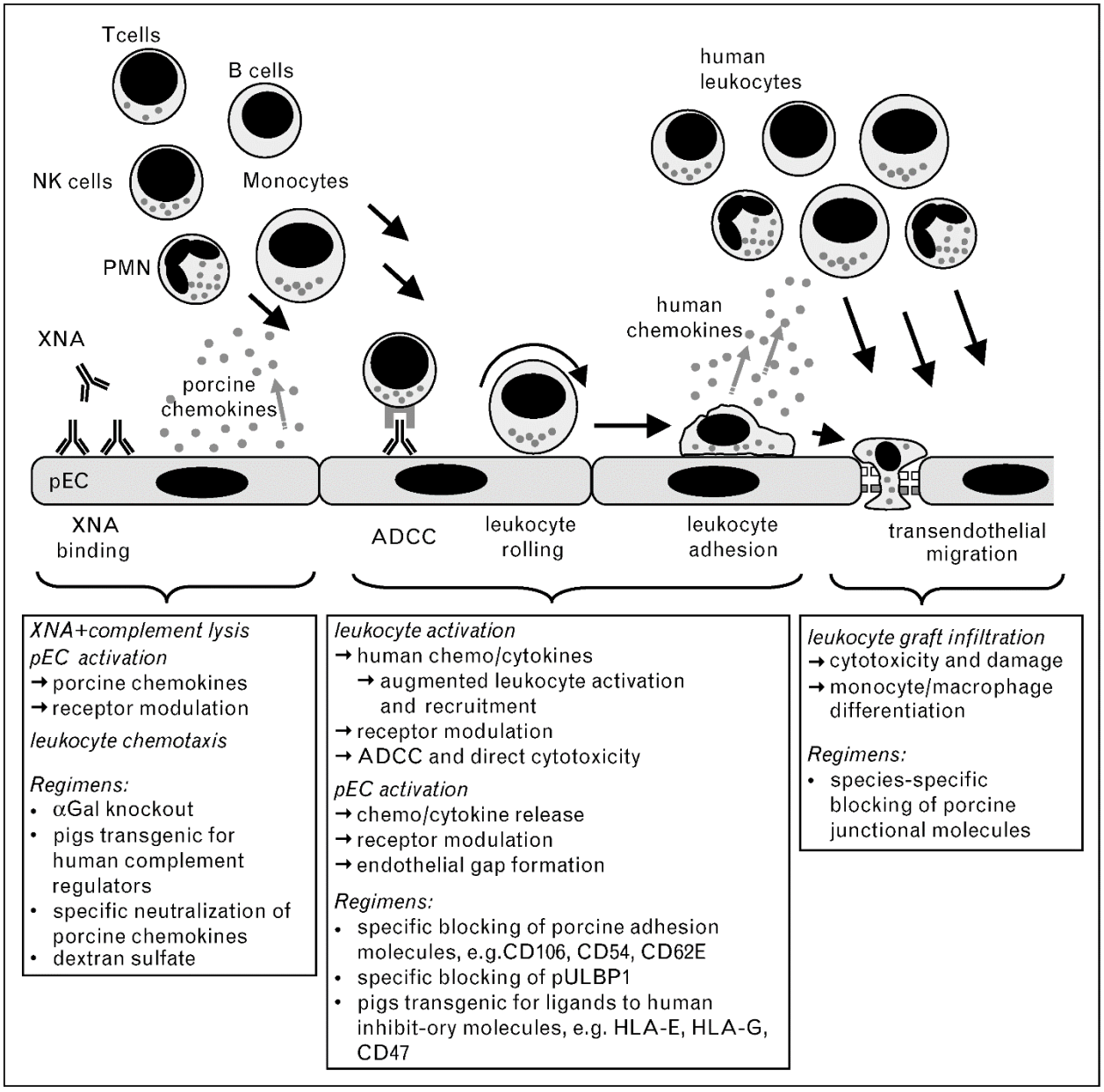

Figure 1-4. An overview of the initial interactions between human innate immune cells and the porcine endothelium, as well as putative regimens to inhibit such interactions (*Schneider MK, Seebach JD. 2008. Current cellular innate immune hurdles in pig-to-primate xenotransplantation. Curr Opin Organ Transplant 13:171–177*). NK cells, natural killer cells; PMN, polymorphonuclear neutrophil; XNA, xenogeneic natural antibody; pEC, porcine endothelial cell; ADCC, antibody dependent cell cytotoxicity.

T cells act in either an indirect or direct pathway to the xenograft. In the indirect pathway, the T cells are activated through the recognition of the major histocompatibility complex (human leukocyte antigens, HLA) on host APCs that present peptides from the porcine xenograft to host CD4+ T cells. In the direct pathway, host CD4+ T cells recognize the porcine leukocyte antigens presented on donor antigen-presenting cells (as shown in Figure 1-5)[123]. In a clinical trial, the intensity and mechanisms of cell-mediated rejection of pig islet cells were investigated in 49 T1DM and 34 healthy subjects. A dominant CD4 class II-restricted Th2 response in an indirect recognition pathway and a CD8 T cell response resulting from direct recognition were involved in the xenoreaction to pig islet cells[124].

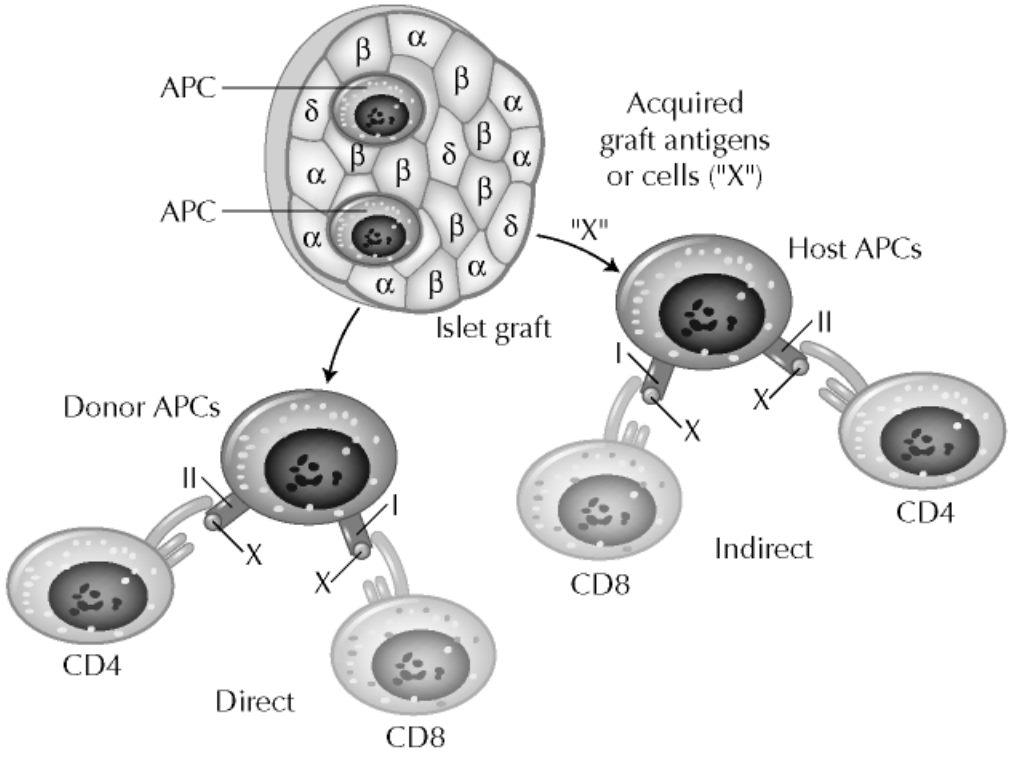

Figure 1-5. "Direct" (donor major histocompatibility complex *[*MHC*]*-restricted) and "indirect" (host MHC-restricted) pathways of graft antigen presentation. (*Rayat G, Gill RG. Pancreatic islet xenotransplantation: barriers and prospects. Curr Diab Rep 2003: 3: 336-343*).

1.6.2.5 Chronic Rejection

Chronic rejection can occur when the anti-rejection therapies can no longer inhibit the immune response against porcine xenografts. The reason for this could be minor mismatch in the MHC molecules. But it will not be discussed further in this thesis.

1.6.3 Methods for Generating Genetically Modified Pigs

The first transgenic pig was produced by microinjection of fusion gene into fertilized egg in 1985, however, this method had a low efficiency in living stock species[125]. Hence several techniques have been developed to achieve germ-line gene transfer. Techniques such as pronuclear DNA microinjection, viral vectors (including retroviral vectors, replication defective adenovirus vectors, cytomegalovirus vectors and other virus) sperm-mediated gene transfer (SMGT), and somatic cell nuclear transfer (SCNT) can randomly integrate the target gene into the porcine host genome to provide genetically modified pigs. Homologous recombination followed by SCNT can define the integration process[126, 127]. Such techniques are used for genetic modification of pigs, either by knockout or adding of extra human genes. Recently, the progress in gene-editing tool such as clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system enables more efficient and convenient production of genetically modified pigs[128].

1.6.4 Types of Genetically Modified Pigs

To overcome the barriers discussed above, significant effort has been spent on the genetic modification of pigs, either by gene knockout or transgenesis, or the combination of them. The types of genetically modified pigs currently available for xenotransplantation will be discussed in

the following section.

1.6.4.1 Xeno-antigen Knockout

Alpha-1,3-galactosyltransferase knockout

Porcine foetal fibroblasts express alpha-1,3-Galactosyltransferase on the cell surface, which is encoded by GGTA1 gene and transfers galactose from UDP-galactose to an acceptor molecule. GGTA1 has 6 exons, from 4 to 9, in which the endogenous ATG translation initiation codon in exon 4 or the catalytic domain in exon 9 could be targeted to inactivated GGTA1[129].

Some scholars used a highly inbred, major histocompatibility complex-defined miniature pig line to produce GGTA1 knockout descendant. They used one male and three female fetuses at day 37 of gestation to produce donor cell lines. The endogenous GGTA1 allele was replaced homogenously by pGalGT, a gene trap targeting vector, which included 21kb of homologous sequences of GGTA1. Then the coding region upstream of the catalytic domain was disrupted by insertion of a selection cassette consisting of a Bip internal ribosome entry site followed by G418 resistance sequences (as shown in Figure 1-6). Healthy heterozygous knockout pigs were produced in Large White gilts as surrogate mothers[116].

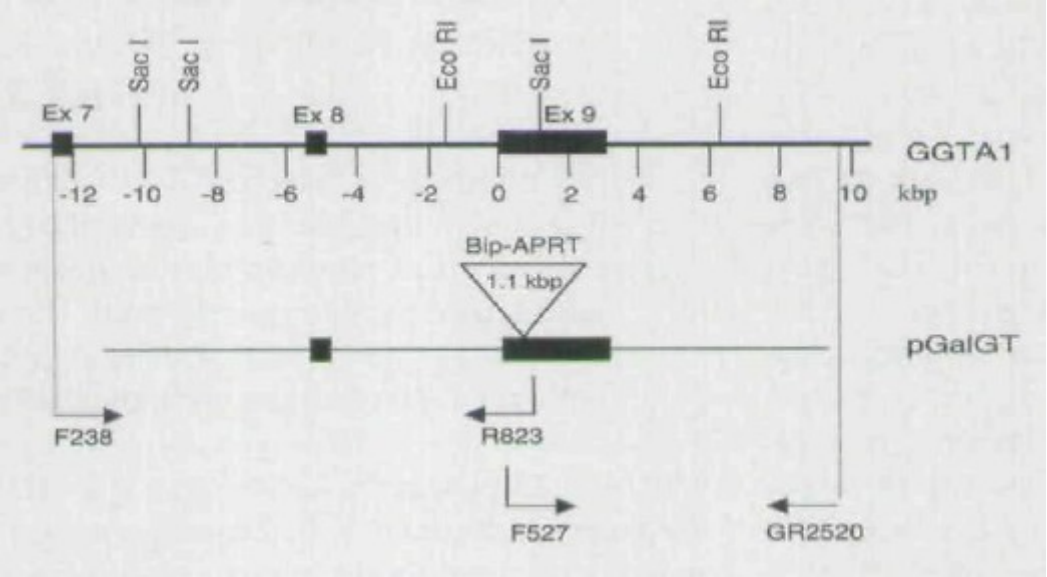

Figure 1-6. pGalGT targeting vector and genomic PCR assays for targeting *(Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, ImGS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS. 2002. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295:1089–1092.)*.

The cell clones showed a spontaneous loss of the wild-type allele by loss of heterozygosity (LOH) at the GGTA1 locus in those heterozygous animals with inbred background. The deletion of the wild-type allele, somatic crossing over or gene conversion might result in the LOH. GGTA1 deficient pigs with two distinct LOH genotypes could be produced from those gene-targeted heterozygous cells lacking GGTA1 expression by somatic cell nuclear transfer[130].

In another attempt, two alpha-1,3-GalT knockout vectors, pPL654 and pPL657, were constructed from isogenic DNA of two primary porcine fetal fibroblasts, SLA1-10 and PCFF4-2 cells. Those 6.8kb genomic fragments include most of intron 8 and exon 9. The unique EcoRV site at the 5' end of exon 9 was converted into a SalI site by inserting a 1.8kb IRES-neo-poly A fragment. Heterozygous GGTA1 knockout pigs were produced by somatic cell nuclear transfer[115].

Phelps *et al* found that Toxin A from *Clostridium difficile* could be used to select for cells that

lack the second allele of the gene, due to the high affinity to alpha-1,3-Gal epitopes of the toxin. The knockout of the second GGTA1 allele resulted from a missense T-to-G single point mutation at the second base of exon 9. The pigs appeared healthy after somatic cell nuclear transfer[131].

In another trial, the GGTA1 targeting vector pGT-Dtneo, which consists of 5 kb homology at the 5' end from exon 8 through 230 bp of exon 9 followed by a neomycin selection cassette including the phosphoglycerate kinase promoter and bgh (bovine growth hormone) polyadenylation site and 2 kb homology at the 3' end from 231 bp of exon 9 to 1,680 bp after the stop codon, was used to produce heterozygous GGTA1 mutant pigs. For negative selection, a copy of the diphtheria toxin gene with a Pol 2 promoter and SV 40 polyadenylation sequence was cloned upstream of exon 8 (as shown in Figure 1-7). The foetal fibroblasts from a 30-day-old embryo were transfected with linearized targeting vector using lipofectamine 2000[132]. Heterozygous mutant pigs were produced by somatic cell nuclear transfer using targeted cells. Approximately 1-2% of the normal level α-gal antigen was detected in pig cells using fluorescence-activated cell sorter (FACS) analysis with mouse anti-gal monoclonal antibodies and sensitized human serum[132].

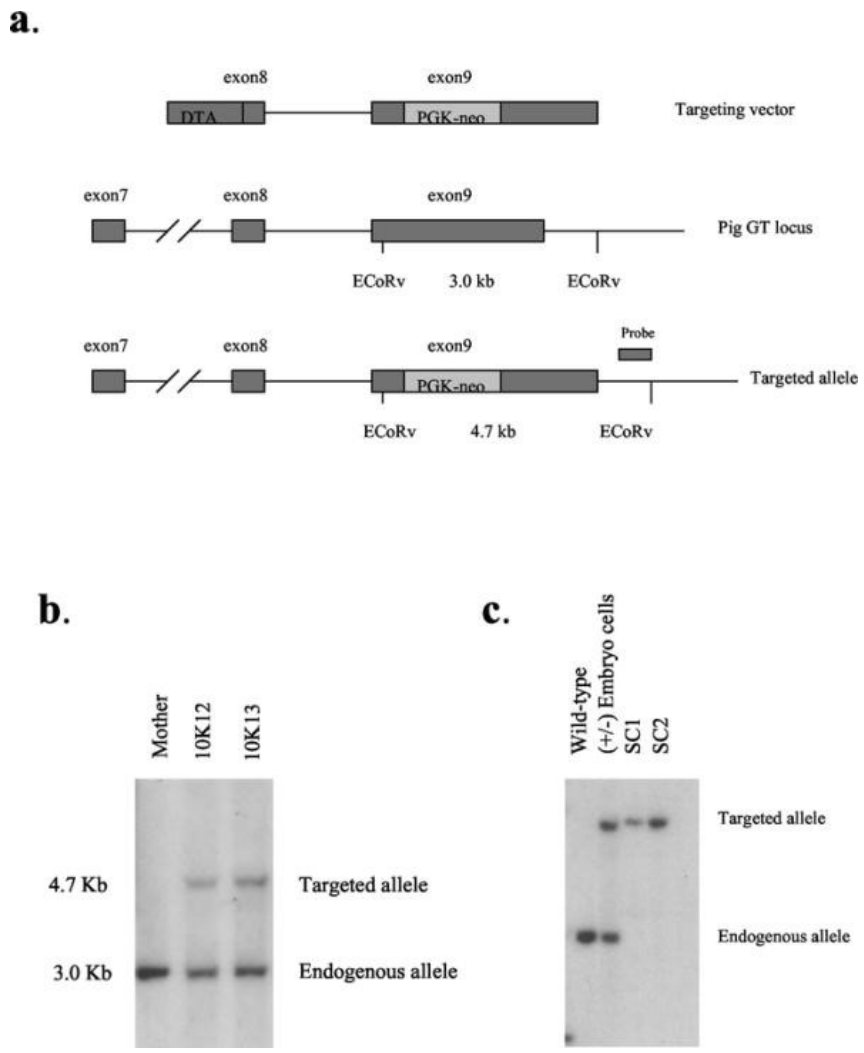

Figure 1-7. (a) Targeting vector, pig [alpha]1-3 galactosyltransferase (GT) locus and the targeted allele showing position of restriction enzyme sites for EcoRV and location of probe used for Southern Blot analysis. (b) Representative GT (+/-) pigs (Southern blot). (lane 1) DNA from the sow; (lanes 2 and 3) GT (+/-) offspring labeled 10K12 and 10K13. DNA has been digested with EcoRV, and the position of the targeted and endogenous alleles and appropriate sizes are indicated. (c) Representative GT (-/-) cells (Southern blot). Conditions are as described in part b. (lane 1) normal pig DNA; (lane 2) DNA from the GT (+/-) fetal fibroblast cells; (lanes 3 and 4) DNA from two selected cells (SC1 and SC2). (*Sharma A, Naziruddin B, Cui C, Martin MJ, Xu H, Wan H, Lei Y, Harrison C, Yin J, Okabe J, Mathews C, Stark A, Adams CS, Houtz J, Wiseman BS, Byrne GW, Logan JS. 2003. Pig cells that lack the gene for alpha 1-3 galactosyltransferase express low levels of the gal antigen. Transplantation 75:430–436.*

Harrison *et al.* in Australia transfected XhoI-linearized targeting vector pKO28 to porcine fetal fibroblasts isolated from a single fetus obtained on day 25 of gestation before somatic cell nuclear transfer using a promoterless construct approach[133].

A further experiment modified GGTA1 using a homologously dependent strand transferase protein named drosophila recombination-associated protein (DRAP) and DNA oligonucleotides. This method produced piglets with markedly reduced alpha-1,3-galactosyl sugar epitopes without any selection steps[134]. All the GalTKO pigs described above showed decreased or no expression of alpha-Gal. However, few studies focus on the function of the organs from these pigs. Also, other transgenic pigs have been created to alleviate the potential HAR.

Alpha-1,2-fucosyltransferase (Alpha-1,2-FT)

Another approach developed to overcome HAR is the expression of human alpha-1,2-fucosyltransferase (alpha-1,2-FT). The transgenic expression of alpha-1,2-FT inhibits the activity of alpha-1,3-galactosyl- transferase through competing for the common acceptor substrate, N-acetyl lactosamine. Also, alpha-1,2-FT synthesizes the O blood group antigen and H epitope (H antigen shares a common precursor with alpha-1,3-GalT epitope in human)[135], which were responsible for a concomitant decrease in the level of alpha-1,3-GalT epitope expression[136].

In Sharma *et al.*'s experiment, the alpha-1,2-FT cDNA from human A431 cell line was integrated into vectors containing a 500-bp chicken beta-actin promoter or a 4.3-kb $H2k^b$ promoter for transgenic expression (as shown in Figure 1-8). Also, a 900-bp HindIII/KpnI fragment of human alpha globulin gene containing splice and polyadenylylation information was used to produce transgenic pigs. The results showed that alpha-1,2-FT competed with alpha-1,3-GT in the cells of transgenic pigs through the synthesis of H antigen hence decreased the binding to xeno-

reactive natural antibodies to reduce HAR[137].

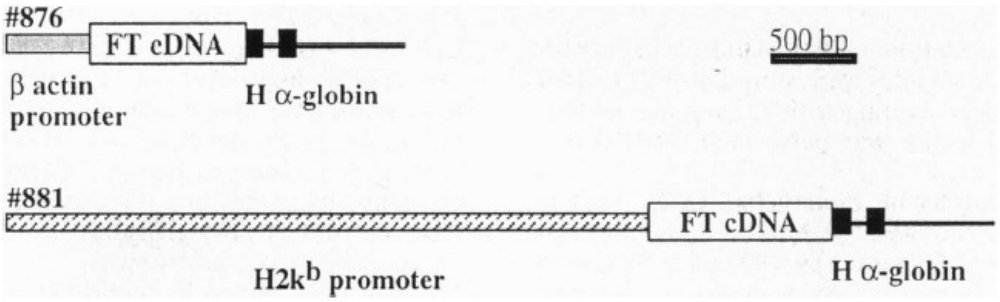

Figure 1-8. Expression constructs for alpha (1,2) FT production in transgenic animals. A 1.1-kb fragment containing human alpha (1,2) FT cDNA was fused to either 0.5 kb of chicken 3-actin promoter (construct 876) or to 4.3 kb of murine H2kb promoter (construct 881). A 900-bp fragment of human a-globin gene comprising 2 exons and an intron with 400 bp of 3' untranslated region was ligated at the 3' end to provide splice and poly(A) signals. (*Sharma A, Okabe J, Birch P, McClellan SB, Martin MJ, Platt JL, Logan JS. 1996. Reduction in the level of Gal(alpha1,3) Gal in transgenic mice and pigs by the expression of an alpha (1,2) fucosyltransferase. Proc Natl Acad Sci USA 93: 7190–7195*.)

In another attempt in the USA, the cDNA of human alpha-1,2-FT was integraded to the 3’ end of CMV promoter into a pGEM7Z-derived expression vector, BamHI/XhoI sites of APEX-1, which contained the polyadenylation signal of SV40. Transgenic pigs were produced using embryo microinjection. The transgenic expression of alpha-1,2-FT resulted in a remarkable reduction in the expression of alpha-1,3-GalT epitope, thus increased resistance to human serum mediated cytolysis[138].

β-D-mannoside-1,4-Nacetylglucosaminytransferase (GnT-III)

In addition to alpha-1,2-FT, β-D-mannoside-1,4-Nacetylglucosaminyltransferase III (GnT-III)

competes with alpha-1,3-GT for the terminal glycosylation to reduce its antigenicity to human xenoactive natural antibodies. Miyagawa *et al*. integrated cDNA of human GnT-III into the pCX vector containing a β-actin promoter and a CMV enhancer[139]. Microinjection and embryo transfer were used to produce transgenic pigs. The endothelial cells from those pigs showed remarkable decrease in antigenicity and complement-mediated or natural killer cell-mediated pig cell lyses[139].

1.6.4.2 Knock-in of Human Complement Regulatory Proteins

The complement regulatory proteins, either in soluble or membrane-bound forms, can down-regulate the complement cascade to reduce cell damage in physiological conditions[140]. Due to the homologous restriction of complement regulatory proteins, the expression of them in pigs is relatively ineffective to regulate human complement after transplantation[141]. Hence the use of cobra venom factor to inactivate complement component C3b or soluble complement receptor 1 to combine CR1 was introduced to the pig-to-primate recipients and the results consolidated the role of complement regulatory proteins in preventing HAR[142, 143]. Yet the continual systemic inhibition of complement is impractical due to the unacceptable high risk of infection in the recipients. To overcome the hyperacute rejection in pig-to-primate xenotransplantation, transgenic pigs expressing human membrane-bound complement-regulatory proteins like CD46, CD55, CD59 and the combination of them have been generated.

Human CD46

Human CD 46 (hCD46), also known as membrane cofactor protein (MCP), encodes a 55- to 65-kD type I membrane protein which regulates the complement system by inactivation of complement component C3b and C4b (provided by RefSeq, Jun 2010)[144]. The human CD46

gene has 14 exons, spanning more than 40 kb genomic sequences[145]. The alternative splicing of exons 7-14 is responsible for the expression of several isoforms. Some isoforms are involved in the protection against HAR through the alternative activation pathway[146, 147]. Several groups have tried to produce transgenic pigs with expression of human CD46 by microinjection of yeast artificial chromosome DNA containing CD46 gene, or co-injection of a CD46 minigene constructed with chick lysozyme matrix attachment DNA, or microinjection of a glycosyl phosphatidyl-inositol anchored CD46 hybrid cDNA. However, the expression of CD46 in the transgenic animals was relatively low and insufficient[148-150].

In 2001, Diamond *et al.* reported transgenic pigs with expression of human CD46 and demonstrated its ability *in vivo* to regulate complement activation and thus inhibit hyperacute rejection in a pig-to-nonhuman-primate transplantation (Figure 1-9). They used a 60-kb genomic sequence containing the human CD46 gene isolated from a P1 phage library. Transgenic pigs were produced by microinjection of the gene into the male pronuclei of fertilized porcine oocytes[151].

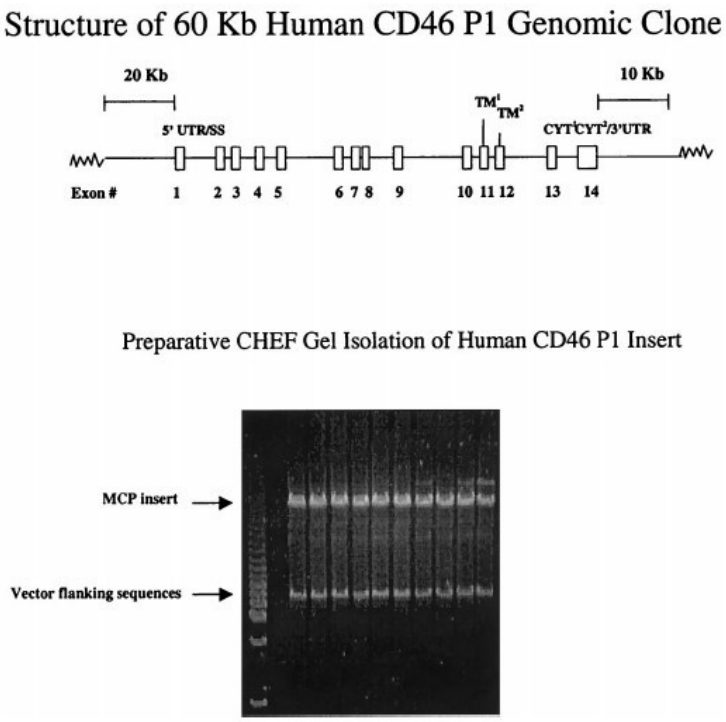

Figure 1-9. The human genomic CD46 gene was isolated from flanking vector sequences by restriction digest with Mlu I. The DNA was size-fractionated on a clamped homogenous electric field (CHEF) gel, and the 60-kb restriction fragment was isolated for microinjection. (*Diamond LE, Quinn CM, Martin MJ, Lawson J, Platt JL, Logan JS. 2001. A human CD46 transgenic pig model system for the study of discordant xenotransplantation. Transplantation 71:132–142.*)

In another attempt, Loveland *et al* used an engineered CD46 minigene to produce high-level expression pigs. The ~8kb minigene was constructed by involving the sequences that may optimize the promotor through increasing the 5'-end sequence to 636bp or by adding a shortened SV40 poly A sequence. The minigene was microinjected into the fertilized eggs by pronuclear injection and then transferred to the oviducts of recipient gilts. The transgenic pigs showed highly efficient CD46 function in controlling both the classical and alternative pathways[152].

Human CD55 (Decay Accelerating Factor)

Human CD55 (hCD55), also named human decay accelerating factor (hDAF), is a 70kDa glycoprotein involved in the regulation of the complement cascade. It disrupts the cascade and protects cells from damage through the acceleration of its decay by binding to complement proteins. (provided by *RefSeq, Jul 2014*)

Expression of human decay accelerating factor in various cells of the transgenic pigs was examined. A cDNA fragment containing the 5'-end untranslated region, the signal peptide sequence, the first exon and 0.4 kb of the first intron of the hDAF gene and the last 10 exons of the gene was microinjected into the pig oocytes. The transgenic pigs showed high level of hDAF expression in endothelial cells, vascular smooth muscle cells and squamous epithelial cells[153].

In another attempt, Murakami *et al.* generated hDAF transgenic pigs with hybrid genes containing hDAF gene regulated by porcine membrane cofactor protein (pMCP) promoters. The transgenic pigs were produced through microinjection and showed tissue-specific expression of the transgene[154].

In 2002, some scholars used sperm-mediated gene transfer (SMGT) rather than microinjection of exogenous DNA into male pronuclei of the zygote. The sperm cells from selected pig donors were used for transferring hDAF minigene into oocytes by artificial insemination to produce transgenic pigs[155].

Human CD59 (MAC-inhibitory Protein)

Human CD59 (hCD59) gene encodes an 18- to 20-kD glycosyl-phosphatidylinositol-anchored cell surface glycoprotein regulating complement-mediated cell lysis. It is expressed in various tissues and inhibits the formation of the complement membrane attack complex (MAC) by binding to complement C8 and/or C9 during the assembly of this complex[156, 157]. Evidence showed

that the high-level expression of human CD59 could protect porcine tissue from human serum complement system after xenotransplantation and the protection was proportional to the amount of the expression of human CD59 on the surface of the transgenic cell[158].

In 1994, after introducing T4 DNA polymerase and dNTPs into the 5'-end 4-nucleotide overhang, Fodor *et al.* subtracted a human CD59 cDNA fragment from a human CD59-pcDNAI-Amp expression plasmid and then inserted the cDNA into exon 1 of the murine H2Kb-gene right at the 12 nucleotides downstream of the transcriptional starting site[159]. The H2Kb-human CD59 DNA was used for embryo injection. High level of human CD59 was detected in a variety of porcine cell types and protected those cells from anti-porcine antibodies and human complements[159].

Due to the low efficiency in producing transgenic livestock using microinjection of DNA constructs into pronuclei of zygotes, Niemann *et al* generated transgenic pigs expressing human CD59 cDNA under the transcriptional control of the human cytomegalovirus (CMV) immediate early promoter followed by a SV40 poly A signal. The fibroblasts and endothelial cells from the transgenic pigs showed high level of human CD59 expression and the transgene provided protection against hyperacute rejection[160].

Combination of Human Complement Regulatory Proteins

CD55 and CD59 double transgenic cell lines were produced by the co-injection of the transgene into porcine aortic endothelial cells. The human CD59 transgene consists of a chick β-actin promoter and splicing and polyadenylation signal sequences. The human CD55 transgene is constructed with a human CD55 cDNA fragment regulated by mouse $H2K^b$ promoter and $H2K^b$ polyadenylation signal sequences. Low level expressions of CD55 and CD59 were described to

significantly influence the humoral immune response[161].

In another approach, double transgenic pigs for human CD55 and CD59 were produced using a human elongation factor 1α gene promoter. The expression of both genes mainly in endothelial cells improved the resistance to complement-mediated lysis[161].

CD46, CD55 and CD59 triple transgenic pigs were produced when the heterozygous hCD55 transgenic pigs were used to breed with hCD59 transgenic pigs or hCD59 plus hCD46 double transgenic pigs. The expression of the three genes were found in most of the tissue and exerted greater degree of human complement regulation than each single gene alone[162].

Cowan *et al.* produced CD55, CD59 and the enzyme alpha-1,2-fucosyl- transferase (H-transferase, HT) triple transgenic pigs. H-2K^{b}/HT, composed of an enhancer from the murine T cell receptor alpha chain gene linked to a 4.2-kb murine H-2K^{b} promoter fragment, hybrid intron, human HT cDNA, and SV40 early polyadenylation signal, and H-2K^{b}/CD55 and H-2K^{b}/CD59, both of which were produced by replacing the HT cDNA in H-2K^{b}/HT with human CD55 and CD59 cDNAs respectively, were introduced into the transgenic pigs by pronuclear microinjection of equimolar combinations of these constructs. High expression of CD55 and CD59 but relative low expression of HT in various tissues were observed. The transgenic pigs were protected from hyperacute rejection but developed coagulopathy manifested by thrombocytopenia and abnormalities in coagulation[162].

1.6.4.3 Anti-thrombosis Strategies

As mentioned previously, islets transplanted into the portal vein of patients with T1DM are destroyed by IBMIR, which is characterized by activation of complement, platelets, and the coagulation pathway. In addition, the coagulation control is critical for the long-term xenograft survival after HAR and AHXR. Genetically modified pigs that express anti-human thrombotic

molecules have been created to abrogate this kind of response against xenografts.

Human CD39

CD39, also named ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD-1), can rapidly hydrolyze ATP and ADP to AMP; AMP is hydrolyzed by ecto-5'-nucleotidase to adenosine, an anti-thrombotic and anti-inflammatory mediator. Regarded as the dominant vascular ectonucleotidase modulating hemostasis and thrombotic reaction, expression of CD39 decreases rapidly in xenografts after transplantation[163, 164]. Inversely, the decrease in expression of CD39 is associated with the sequestration of platelet-rich fibrin thrombi in the xenograft microvasculature and hence increases the sensitivity to vascular injury[165, 166]. Furthermore, the administration of soluble CD39 can completely inhibit ADP-induced platelet aggregation[167].

Wheeler *et al.* reported the function of hCD39 expression in a transgenic swine model of myocardial ischemia/reperfusion injury, in which anti-coagulation is critical in the treatment (Figure 1-10). Transgenic pigs were produced by somatic cell nuclear transfer. Myocardial ischemia/reperfusion injury was induced by intracoronary balloon inflation in the left anterior descending artery. The results showed that the expression of CD39 in transgenic pigs has the potential to reduce myocardial ischemia/reperfusion injury[168].

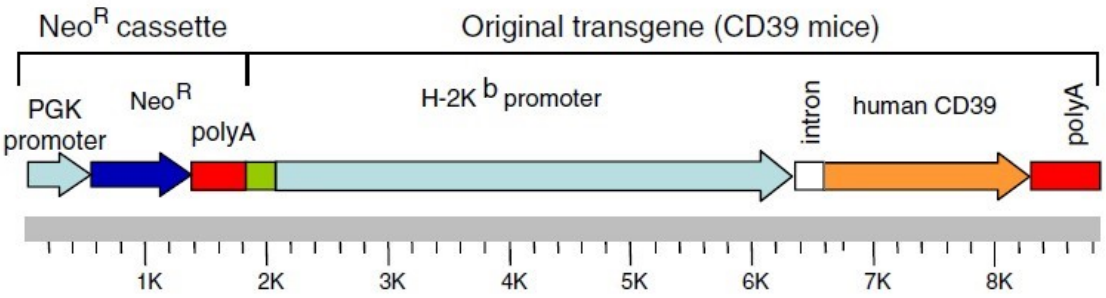

Figure 1-10. Expression of hCD39 in swine. A. Schematic of CD39 transgene (*Wheeler, D. G., Joseph, M. E., Mahamud, S. D., Aurand, W. L., Mohler, P. J., Pompili, V. J., & Gumina, R. J. (2012). Transgenic swine: expression of human CD39 protects against myocardial injury. Journal of molecular and cellular cardiology,52(5), 958-961*.).

Also, the hCD39 is used for generating transgenic pigs in combination with other genes. GalTKO, hCD55, hCD59, hCD39, and hHT transgenic pigs were produced following Cowan's procedures[169]. The transgenic pigs developed moderately improved graft survival with rejection at about two weeks post-transplantation while the wild type group experienced acute humoral rejection at about four days post-transplantation. No PERV transmission was detected in any recipients. This topic will be further discussed in the following section[169].

Human Tissue Factor Inhibitor

Tissue factor, synthesized and secreted by various cells like islets and inflammatory cells such as neutrophils, monocytes, and platelets, is thought to be involved in the initiation of IBMIR. Ji *et al.* investigated the mechanism of xeno-rejection by IBMIR in tissue factor gene knockdown islets. Neonatal islet cell clusters (NICC) from 1- to 3-day-old pigs were harvested after 3 days of culture after the isolation procedures then transfected with siRNA specific for tissue factor or a nonspecific control siRNA. Reduction in tissue factor gene and protein expression were observed. Formation

of blood clots, platelet activation, thrombin generation, and complement activation were decreased after exposure to human ABO compatible blood *in vitro*[170]. Otherwise from inhibiting production of tissue factor by silencing gene expression, another direction is through the inhibition of the tissue factor pathway. Tissue factor pathway inhibitor-1 (TFPI-1) has two isoforms, TFPIα and TFPIβ. TFPIα contains three Kunitz-type domains, among which K1 links to the active site of factor VIIa in the fVIIa/TF complex and K2 connects to and inhibits factor Xa while K3 has no inhibitory function[171]. TFPIβ has two domains connected to endothelial cells through a GPI linkage[172]. The sequence of pig TFPI cDNA was produced by simultaneous 5 ¢ /3 ¢ rapid amplification of cDNA ends from pig aortic endothelial cells (PAEC). The human TFPIα open reading frame (ORF) fused to the glycosyl phosphatidylinositol (GPI) was replaced with the pig TFPIa ORF to generate TFPIα-GPI expression vectors using the plasmid pEF-BOS-FLAG. Primer sets pTFPI-MluI-F/TFPI-MluI-R and hTFPI-MluI-F/TFPI-MluI-R were used to replicate pig and human TFPIa-GPI fusion ORFs respectively. Results showed that pig or human TFPIα bound to human factor Xa and inhibited human factor VIIa/tissue factor complex and that no obvious incompatibilities were observed between the recombinant pig TFPI and human tissue factor pathway[173].

Human Thrombomodulin

Human thrombomodulin (hTM) has several beneficial effects in controlling coagulation and cell protection. It can regualte the molecular incompatibility in protein C activation and acts as a physiological regulator only when thrombin is formed[174, 175]. This mechanism is different from that of tissue factor pathway inhibitor or CD39, which inhibits direct prothrombinase activity[176] and exhibits anti-inflammatory properties[174, 177]. As pig thrombomodulin was not able to

efficiently convert human protein C into activated protein C, which can inhibit factor Va and VIIIa to regulate anticoagulation activity, the expression of hTM in transgenic pigs is essential for the further application of xenotransplantation[178].

Yazaki *et al.* produced hTM transgenic pigs using pCAGGS expression vector and pPGK-puro vector. Briefly, the fibroblasts from the fetus of Landrace/Yorkshire pig were electroporated with HEPES buffer containing pCAGGS/hTM expression vector (including the cytomegalovirus enhancer and chicken beta-actin promoter) and pPGK-puro vector. After cell culture and isolation, the nuclei of the fibroblasts were transferred into the oocytes by direct intracytoplasmic injection to produce hTM transgenic pigs. Expression of hTM was detected in endothelial cells of kidney and liver, platelets, mononuclear cells, aortic endothelial cells but not in red blood cells. The hTM expressed in aortic endothelial cells, with a comparable level as that of human umbilical vein endothelial cells, was able to covert protein C into activated protein C. Soluble hTM was detected in pig plasma and activated thromboplastin time (APTT) was slightly prolonged, but no increase in bleeding tendency was observed[179].

Furthermore, hTM was introduced into pigs with genetic background of GalT knockout/hCD46 to produce triple transgenic pigs. Compared to GalT knockout/hCD46 cells, the GalT knockout/hCD46/hTM cells have a higher level of hTM expression and increase activated protein C elimination by reducing adhesion and aggregation. Hence the expression of hTM could be applied to reduce hyperacute thrombosis and protect the xenograft after xenotransplantation[180].

In another attempt, hCD59/hCD55/hTM triple transgenic pigs were produced under the control of cytomegalovirus promoter. Fibroblast cells were obtained from a hCD59/hCD55 transgenic pig and transfected with a vector under the control of the cytomegalovirus promoter.

Somatic cell nuclear transfer was used to produce CD59/hCD55/hTM triple transgenic pigs. Expression of hTM was found in all organs of the recipient and the hTM cofactor activity was significantly increased in fibroblasts of transgenic pigs compared to wild-type porcine fibroblasts[181]. Although knock-in of these genes into pig genome has been confirm, the characterization of organ function remains further investigation.

1.6.4.4 Anti-immune Cell Strategies

As mentioned above, either the innate immune cells such as NK cells and macrophages or adaptive immune cells are involved in the rejection of the xenotransplant. Several strategies have been established to overcome these obstacles. Here I mainly discuss the transgenic pigs produced for human TNF alpha-related apoptosis inducing ligand (TRAIL), HLA-E/beta-2-microglobulin and CTLA-4 Ig, which have potential anti-rejection property.

Human TNF alpha-related Apoptosis Inducing Ligand

TNF alpha-related apoptosis inducing ligand (TRAIL) is a type II membrane protein. Five receptors for TRAIL have been reported, in which TRAIL-R1 and TRAIL-R2 induce apoptosis by cytoplasmatic death domains, while TRAIL-R3 and TRAIL-R4 function as decoy receptors and TRAIL-R5 is different from the other four receptors as a soluble decoy receptor[182, 183]. The binding of TRAIL receptors does not result in cell death in normal cells but induces apoptosis in tumor cells[184]. Also, evidence has shown that TRAIL has other immune effects in regulating the rejection of pig-to-primate xenograft. First, TRAIL can induce primary plasma cell apoptosis and shorten the lifespan of neutrophils[185-187]. Second, TRAIL could also inhibit T cell proliferation regardless of the differentiation status or antigen specificity of T cells[188, 189]. Third, expression of TRAIL may inhibit the primary T-cell-mediated graft rejection in corneal

allotransplantation[190]. Hence, the expression of TRAIL in transgenic pigs can be a strategy to protect the pig xenograft against cell-mediated rejection in non-human primate recipients.

Klose *et al*. reported transgenic pigs produced by microinjection of an expression vector for human TRAIL under the control of murine H-2K^b promoter. The 4.3-kb construct sequences consisted of a genomic H-2K^b (mouse class I MHC) promoter sequence, human TRAIL cDNA and beta-globin splice polyadenylation signal sequences. The expression of TRAIL was detected in the membrane fractions in various tissues including lung, spleen and others in the transgenic pigs. Induction of apoptosis without any detrimental side effects was shown in Jurkat and Hut 78.2 cells by inducing apoptosis[191].

In another attempt Kemter *et al.* produced human TRAIL transgenic pigs on a GGTA1 knockout/hCD46 transgenic background under the control of either murine H-2K^b promoter or a cytomegalovirus enhancer/chicken beta-actin (CAG) promoter. The results showed that the CAG-human TRAIL transgenic pigs had higher level of TRAIL expression in various tissues than the H-2K^b-human TRAIL transgenic pigs. The dendritic cells but not fibroblasts from the transgenic porcine decreased the proliferation of human PBMCs without any evidence of apoptosis. Hence the expression of human TRAIL could be applied to other transgenic methods to regulate T cell response in pig-to-primate xenograft model[192].

HLA-E/beta-2-microglobulin

HLA-E is assembled in the endoplasmic reticulum and is a stable trimeric complex consisting of an HLA-E heavy chain, beta-2-microglobulin and a peptide derived from the leader sequence of some MHC class I molecules[193]. Two alleles of HLA-E have been found, in which HLA-E*0103 has a higher cell surface expression level than HLA-E*0101 due to a single difference at

amino acid position 107[194]. As the peptides produced by the signal sequence of HLA-E do not loan on HLA-E, the expression of HLA-E, the binding affinity to CD94/NKG2 dimers as well as its ability to reverse NK-mediated lyses depend on the nature of the peptides in the trimeric complex[195]. Transgenic methods could be applied to produce pigs expressing HLA-E for xenotransplantation.

Lilienfeld *et al.* produced HLA-E transgenic pig endothelial cell lines by Ca_2PO_4-transfection followed by limiting dilution cloning. The expression of HLA-E single chain trimers (SCT) consisted of a canonical HLA-E binding peptide antigen, the mature human beta-2-microglobulin and the mature HLA-E heavy chain was detected in the cell lines. Level of protection of porcine endothelial cells from human NK cell-mediated cytotoxicity was related to the intensity of HLA-E expression on cell surface. Moreover, the protection effect was specifically inhibited by blocking the HLA-E specific NK inhibitory receptor CD94/NKG2A. Hence the stable expression of HLA-E on porcine endothelial cell surface can be applied to produce transgenic pigs for further pig-to-primate xenotransplantation experiments[122].

In another attempt, Weiss *et al.* reported the production of transgenic pigs against human NK cell-mediated cytotoxicity by pronuclear microinjection of the genomic fragments of HLA-E with HLA-B7 signal sequence and human beta-2-microglobulin into zygotes. The 330 bp HLA-B7 cDNA sequence was isolated by polymerase chain reaction (PCR) using primer pair B7-5UT (5-GATATCTAGAAGCCAATCAGCG-3) and Eex2as 5-GCGGCCGGGCCGGG ACACGGAAGTGTGGAAATACTTCAAGG-3) and was inserted into the vector pCR2.1-Topo. The vector was integrated into the 7.7 kb genomic HLA-E Hind III/Bgl II fragment (HLA-E*01033) using the Xba I and Eag I restriction sites. Human beta-2-microglobin was produced following Pacasova's method[196]. After isolation and purification, the sequence obtained above was

microinjected into the oocytes to produce transgenic pigs. Consistent expression of HLA-E and human beta-2-microglobin was detected in peripheral blood mononuclear cells and cells in organ like heart and kidney. The lymphoblasts and endothelial cells isolated from the HLA-E/human beta-2-microglobin transgenic pigs were protected from human NK cell-mediated cytotoxicity, depending on the level of CD94/NKG2A expression on the NK cells[197].

CTLA4-Ig

Cytotoxic T lymphocyte-associated antigen 4 immunoglobulin (CTLA4-Ig), a T cell surface antigen linked with the Fc portion of human immunoglobulin G (IgG1), can inhibit the B7/CD28 costimulatory interaction by binding to B7 on antigen presenting cells to down-regulate the T cell response[198]. Hence the expression of CTLA4 can be applied to downregulate the CD4 and CD8 T cell response. Evidence showed that short-term treatment with human CTLA4-Ig could increase the survival of porcine islets in non-human primates after xenotransplantation[199].

A 1.6 kb Ssc1/Mlu1 restriction fragment containing the extracellular region of pCTLA4 cDNA, followed by a linker to CH2 and CH3 regions of human IgG1, was constructed into an Invitrogen vector pCpG-vitro-neo-LacZ, which consists of a cytomegalovirus enhancer, chicken β-actin promoter, rabbit globin splice site and β-globin/21q21 matrix at 5'- and 3'-end respectively. Then the DNA fragment was transferred into primary fetal fibroblasts from wild type or alpha-1,3-GalT knockout pigs. The expression of CTLA4-Ig was detected and resulted in a significantly compromised humoral immune status[200].

Mulley's group described the transgenic expression of CTLA4-Ig in porcine endothelial cells. The cDNA for the extracellular domain of murine CTLA4-Ig and Fc portion of human IgG1 were transduced into the lentiviral entry vector pENTRTM11 in pig endothelial cell line. The results

showed that the expression of CTLA4-Ig inhibits indirect xenogeneic T cell proliferation and cytokine production including interferon γ, interleukin 4 and interleukin 10[201].

In another attempt in 2001, Zhai *et al.* transfected the adenoviral vector (Adv-GFP) with the pig CTLA4-Ig fusion gene (Adv-pCTLA4-Ig) into the islets isolated from adult pigs. The genetically modified islets were transplanted into the renal capsule of diabetic rats 48 hours after the transfection. Various level of interleukin 4 and interferon γ were observed in different groups of rats after transplantation. The direct pathway in antigen-mediated T cell activation was inhibited[202].

1.6.4.5 Porcine Endogenous Retrovirus Knockout

Another aspect we need to focus on is the zoonosis between swine and human recipient. The transmission of most porcine microbes can be prevented by using specified pathogen free condition in breeding. Porcine Endogenous Retrovirus (PERV) is a gamma retrovirus and integrated in the porcine genome, in which up to 50 pro-viral loci has been discovered. Three replication-competent subtypes of PERV have been identified due to the variation in the envelope gene sequence. PERV A and PERV B can infect human cells *in vitro* while PERV C can only infect porcine cells[203, 204]. In addition, some scholars found that the recombinant PERV A/C can infect human cells[205]. Further, evidence showed that porcine endothelial cells and bone marrow cells release particles and infects human cells[206]. Yet to date, no evidence has been found that PERV infection appears in human transplanted with living pig organs, tissues or cells[207]. It has been reported that PERV is associated with tumorigenesis and immunodeficiencies in recipients[208], hence different methods have been developed to prevent the PERV transmission. Those strategies include the selection of lower-PERV-producer pigs for transmission[209], the use of anti-PERV vaccine[210].

The most promising methods have been developed to prevent PERV transmission in

xenotransplantation is RNA interference (RNAi) technology. The mechanism of RNAi remains highly conserved in plants, flies, worms and mammals. The double strand RNA can be cleaved by ribonuclear III-like enzyme Dicer into smaller fragments. The target RNA is degraded by the nuclease in silence complex induced by siRNA and brought to target RNA by complementary siRNA which recognizes and hybridize to target sequences[211].

Some scholars used an efficient small interfering RNA (siRNA) to target a highly conserved region in the PERV DNA to inhibit the expression of all PERV subtype. Primary porcine fibroblasts were transduced with lentiviral vector pLVTHM-pol2, which expresses a small hairpin RNA (shRNA) corresponding to the PERV pol2 sequence. The transgenic pigs with remarkable inhibited expression of PERV were produced by somatic nuclear transfer cloning[212].

Another attempt in Missouri used two small interfering RNAs (siRNA) which were expressed as shRNA) against PERV gag and pol medal-genes under the control of a RNA polymerase III (pol III) or a pol II promoter to knock down the PERV. Porcine fibroblasts were transfected with both shRNA and used for somatic cell nuclear transfer. The stable long-term expression of anti-PERV siRNAs was observed in all tissues of transgenic pigs. Yet very low level of expression of PERV was detected in control pigs, which made it hard to assess the knockdown of PERV *in vivo*[213]. Though different technologies have been applied to the knockout of PERV in transgenic pigs, it still leaves the possibility of regenerating replication-competent PERV by spontaneous recombination[214].

1.6.4.6 Use of CRISPR-Cas in Creating GMP

In 2013, four groups of researchers demonstrated the use of CRISPR-Cas9 system for multiple genome engineering[128, 215-217]. Thereafter, this new gene-editing tool has been used in creating GMP. Yang et al. reported the eradication of all PERVs in a pig kidney epithelial

immortalized cell line with a more than 1,000 fold reduction in PERV transmission to human cells[218]. Two years later, the same group used CRISPR-Cas 9 to inactivate all PERV genotypes in pig primary cell line and produced PERV-inactivated pigs through somatic cell nuclear transfer[219, 220]. Though it is still not confirmed if PERV would infect human *in vivo*, infected human cells pass PERV to human cells *in vitro*[219]. With the advancement above, more genetic modifications could be applied to the PERV-inactivated pigs.

1.7 OBJECTIVES AND GENERAL OUTLINE OF THESIS

With the global increase in T1DM-related morbidity, the urge to find the ultimate solution for the disease drives progression in the field. Compared to the traditional insulin replacement therapy, islet transplantation is a more physiologic approach to prevent complications of T1DM. In this approach the damaged beta cells are replaced by new beta cells in the islet transplant and beta cells are capable of sensing the changes in blood glucose levels. Islet transplantation also has advantage over pancreas transplantation as it involves a minimally invasive surgery with less treatment morbidity. However, the widespread application of islet transplantation is currently limited by the shortage of human donors and rejection of the islet transplant. Pigs are being considered an attractive option for an alternative source of islets because pigs can provide an abundant supply of islets and they are not exposed to compromising conditions such as co-morbidity, brain death, and cold ischemic injury as many deceased human donors[106]. Pigs can be housed in pathogen-free facilities, which would allow for an on-demand source of islets with limited risk of pathogen transmission[106]. They can also be genetically modified to change the expression of proteins, which would allow for the procurement of less immunogenic tissue. Thus, the overall aim of this thesis project is to gain more understanding on pig islets, in which four projects were performed to

answer the following questions: 1) How do human immune cells respond to stimulation by pig islet cells? Would some small inhibitory molecules have the potential to inhibit this response? 2) What would be the pattern of recognition and rejection of neonatal pig islets in a humanized mouse model? 3) In terms of pig islet development, what are the molecules that help form the oval- to round-shape of islets? 4) If genetic modifications are applied to the pig, would pig islets function be affected?

In CHAPTER THREE, the pattern of proliferation of human PBMC from individuals with T1DM stimulated with neonatal pig islet cells was studied. Previously, our lab demonstrated that NPI trigger human CD4+ T cell proliferation, in which PBMC were isolated from non-diabetic individuals[221]. My goal was to investigate the proliferation of PBMC from individuals with T1DM stimulated with NPI cells. In this chapter, we also investigated the potential anti-rejection property of two small inhibitory molecules, Suramin and Direct Red 80 (DR80). Small molecules disrupt protein-protein interaction by binding to the small gaps in protein[222]. Suramin is a derivative of napthalenesulphonic acid with a molecular weight of 1429.17 g/mol (Molecular structure seen in Figure 1-11). Evidence showed that it inhibits enzymes involved in intracellular mitogenic signal transduction, such as protein kinase C[223]. It also interrupts heparinase, an important enzyme responsible for tumor metastasis and angiogenesis[223], the anti-tumor effect of which was demonstrated in some solid tumors (*https://www.clinicaltrials.gov/ct2/show/NCT00066768*). In addition, several studies revealed that the anti-proliferative property of Suramin was through the co-stimulatory blockade of CD40-CD150 interaction[224, 225]. DR80 is an organic dye used in textile industry with a molecular weight of 1373.07 g/mol (Figure 1-12). Margolles-Clark and associates demonstrated that DR80 also inhibited CD40-CD150 interaction in human immune cells[226]. In this chapter, we

investigated if Suramin and DR80 would have anti-proliferative effect in human PBMC from individuals with T1DM when stimulated with neonatal pig islet cells.

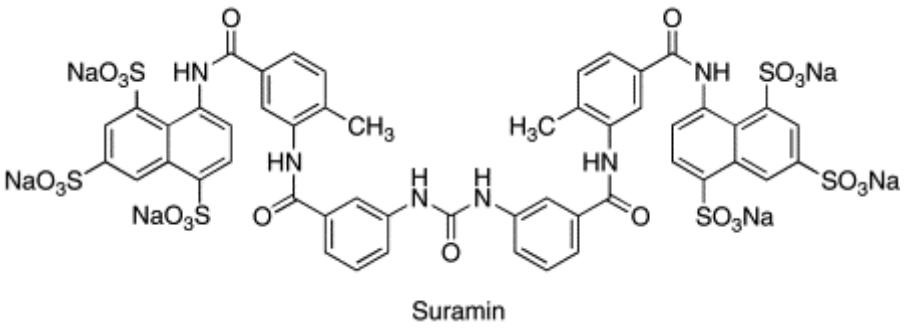

Figure 1-11. Molecular structure of Suramin (*Avendaño, C., and J. C. Menéndez. "Drugs that inhibit signaling pathways for tumor cell growth and proliferation." Medicinal Chemistry of Anticancer Drugs (2008): 251-305*.)

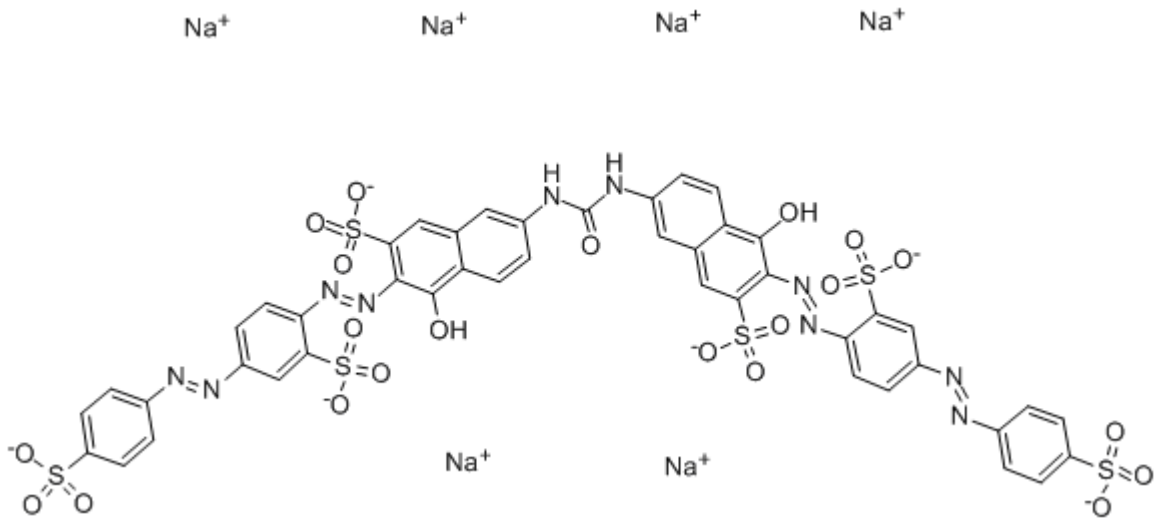

Figure 1-12. Molecular structure of Direct Red 80 (*https://www.chemicalbook.com/ ChemicalProductProperty_EN_CB3126376.htm*)

In CHAPTER FOUR, we examined the rejection of neonatal pig islets by human cells in a humanized NSG mouse model. A previous study revealed that NPI cells induce human CD4 T cell response *in vitro[221]*. In pig-to-rodent models, the rejection of pig islets is also by CD4 T cells[227, 228], while in pig to non-human primate model, multiple cells such as macrophages, CD 4 and CD8 T cells are involved in the rejection[229-231]. However, few research studies have focused on how neonatal pig islet xenografts would be rejected by human immune cells in rodent model. Hence, we generated a humanized model by the injection of PBMC from individuals with or without T1DM. Also, we studied the tempo of NPI rejection as well as the cells involved in this rejection. In addition, we reconstituted the mice with NOD splenocytes as a control to see if there exist hierarchy order in islet xenograft rejection.

In CHAPTER FIVE, we characterized the function of islets from different genetically modified pigs. GalTKO pigs that express human CD46 and/or human CD39 have been created and are available through Revivicor, Inc. These multiple gene transgenic pigs lack the enzyme alpha 1, 3-galactosyltransferase. Thus, these pigs lack the expression of xenoantigen alpha Gal[232], which is a carbohydrate that triggers a strong humoral (antibody) immune response against pig tissues[233-240]. The human CD46 transgene has been introduced into pig donors in an attempt to circumvent the IBMIR[241]. This reaction involves activation of the complement and coagulation cascades, which results in clot formation and infiltration of leukocytes into the islets[242], leading to disruption of islet integrity and islet destruction. CD46 is a complement regulatory protein that is believed to assist in the avoidance of complement mediated early destruction of islets upon transplantation into the portal vein. Ectonucleoside triphosphate diphosphohydrolase -1 (CD39) acts as an anti-coagulant[243, 244]. Our collaborators have demonstrated the benefit of introducing CD46 in terms of complement activation in islets, but not in the clotting time of human blood when

pre-incubated with islets that express CD39[245]. To implicate these GMP islets in the clinic, another question that needs to be answered is whether the genetic modification would affect the function of islets. It is our hypothesis that genetic modification of pigs will not affect the cellular composition and function of islets and that GalTKO, GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal transgenic pig islets will be able to reverse diabetes after transplantation.

In CHAPTER SIX, we turned our focus on islet development. As described before, sporadic islet cell clusters develop into oval or round shaped islets of Langerhans in the pancreas as pigs mature. The communication between cells through intercellular adhesion molecules may play a role in this process. Hence, we examined the expression of E-, VE- and N-Cadherin in neonatal pig islets of different ages at different time points of culture. The pattern of expression of these molecules might correlate with islet morphology development.

With all the efforts above, we hope to put islet xenotransplantation a step forward into clinical use, as an alternative treatment for T1DM.

Chapter 2 MATERIALS AND METHODS

2.1 Mice

Six-week-old male NOD.SCID gamma (NSG) mice (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and used as recipients of an islet transplant. All animals were housed in specific pathogen-free condition in the Health Sciences Laboratory Animal Services Facility of the University of Alberta. Mice were fed standard laboratory food, given water ad libitum, and cared for according to the guidelines established by the Canadian Council on Animal Care. Animals used for research were approved by the University of Alberta Animal Care and Use Committee.

Diabetes in recipient mice was induced by a single intraperitoneal injection of 180mg/kg body weight of streptozotocin (STZ, Sigma, St Louis, MO, USA) 5-7 days before transplantation. Glucose levels of these mice were monitored for blood collected from the tail vein and a precision glucose meter (ONETOUCH, Ultra, Lifescan, Milpitas, CA, USA). NSG mice that had two consecutive non-fasting blood glucose levels of ≥20mmol/l within one week after STZ injection were considered diabetic and candidates for islet transplantation.

2.2 Neonatal Pig Pancreas Procurement

One-, 3-, 7- and 10-day-old Duroc/Landrace Large White F1 neonatal pigs (University of Alberta Swine Research Centre, Edmonton, AB, Canada) of either sex were housed under “High Health” (free of diseases of significant economic impact) condition following the guidelines set by the Canadian Council on Animal Care. Peripheral blood was collected for pig peripheral blood mononuclear cells (PBMCs) isolation and pancreases from these pigs were procured at the Ray Rajotte Surgical-Medical Research Institute of the University of Alberta and stored in Hank’s

Balanced Salt Solution (HBSS, Sigma, St. Louis, MO, USA) on ice prior to islet isolation. A small piece of tissue from tail pancreas was collected for immunohistochemistry analysis.

Pancreases from five to 7-day-old genetically modified pigs (GMP, Duroc x Large White x Landrace) were procured at Revivicor, Inc. (Blacksburg, VA, USA) or at Exemplar Genetics (Sioux Center, IA, USA). Alpha-Gal was homogenously knockout in all GMP. Human CD46 was systemically expressed while human CD39 was islet-specific expressed (Figure 5-7). Pancreases from similar age of WT pigs (Duroc x Large White x Landrace) were procured at the Ray Rajotte Surgical-Medical Research Institute of the University of Alberta. Genetically modified pig pancreases were contained in University of Wisconsin Solution and packed in foam insulated container with ice pack and were shipped to the University of Alberta immediately after procurement by a courier service within 22 to 27 hours. The temperature of the foam-insulated container was monitored during transportation, which fluctuated between 1.7 and 4.4 °C. WT pig pancreases underwent mock shipment with the same condition and length of time in the transport container.

2.3 Islet Isolation and Culture

Islets were isolated from the pancreas tissues using a mechanical and enzymatic digestion method. Briefly, upon receipt of the pancreases, they were placed in cold Hank's Balanced Salt Solution (HBSS, Sigma, St. Louis, MO, USA) and chopped into small pieces. The chopped pancreas tissues were digested in 25 ml of HBSS containing 1.0 mg/ml collagenase type XI (Sigma). After approximately 15 minutes of digestion at 37°C water bath, the digested tissues were filtered through a nylon screen (500 μm) and washed 3 times using cold HBSS. Thirty-five ml of digested tissues in supplemented Ham's F10 medium (Gibco, Burlington, ON, Canada) were then

cultured in 150 mm x 15 mm polystyrene culture dishes at 37ºC, 5% CO_2 and 95% air for 7 days. Ham's F10 medium was supplemented with 10 mmol/l glucose, 50 µmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, QC, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade, Sigma), 2 mmol/l L-glutamine, 10 mmol/l nicotinamide (BDH Biochemical, Poole, Dorset, UK), 100 U/ml penicillin and 100 µg/ml streptomycin. At one, three, 5 days of culture, media were changed to remove the exocrine cells and to provide fresh media to the islets in culture. Islets were collected on day 7 of culture to use for further experimentation.

To characterize the postnatal islet development, pictures of islet morphology was taken using a MS-3 Microscopy on day 0, 1, 3, 5 and 7 of culture; and 200, 500 and 2,000 islet equivalent (IEQ) were collected for immunohistochemistry, RT-PCR and western blot analysis, respectively, on day 0, 1, 3, 5 and 7 of culture.

2.4 Isolation of Human and Pig PBMC

The obtainment of human blood from individuals with or without T1DM for peripheral blood mononuclear cells (PBMC) isolation was approved by the Research Ethics Boards at University of Alberta (Pro00002005, Mechanism of newborn pig islet xenograft rejection by type 1 diabetic human immune cells). Approximately 45 ml of peripheral blood was collected from T1DM volunteers by the nurse in Clinical Research Unit of the Alberta Diabetes Institute using vacuum tubes with heparin. PBMC were isolated immediately after the blood draw using a density gradient centrifugation method. Briefly, fresh blood containing heparin was diluted with normal saline at a ratio of 1:1. Ten ml of lympholyte cell separation medium (Cedarlane, Burlington, Ontario, Canada) was added to a 50 ml conical tube, followed by slowly adding 30 ml of the diluted blood to the top of lympholyte. Balanced tubes were spun at 1500 rpm (CRU-5000 Centrifuge from Beckman

Coulter, Indianapolis, IN, USA) with no brake briefly for 30 minutes at room temperature. The PBMC layer was the third "cloudy" layer from the bottom and was carefully removed with a sterile Pasteur pipette into a new 50 ml conical tube. PBMC were washed with saline twice and spun at 1500 rpm for 5 minutes at room temperature. PBMC were stained with Trypan Blue and counted and suspended in 10% dimethyl sulfoxide (DMSO, Sigma) in fetal bovine serum (FBS, Gibco) and stored in cryotubes in liquid nitrogen. Pig peripheral blood mononuclear cells (pPBMC) were isolated following the same protocol used in isolating human PBMC.

2.5 Isolation of NOD Splenocyte

Spleen was procured from non-diabetic NOD mice and placed into a small Petri dish containing sterile saline. The spleen was cut into small pieces using autoclaved forceps and scissors, then milled between the rough sides of two sterile autoclaved slides. The dissociated tissue was rinsed with 10 ml sterile saline from the slides into the Petri dish. The cell suspension was transferred to a 50 ml conical tube and left on ice for 5 minutes to allow the large pieces of tissue to settle. Supernatant of cell suspension was transferred to another 50 ml conical tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 5 ml of red blood cell lysing buffer for 4 minutes to remove red blood cells. Saline was added to stop lysing, and the cell suspension was centrifuged at 1500 rpm for 5 minutes. Isolated NOD splenocytes were washed with saline twice, then were counted and suspended in 10% dimethyl sulfoxide (DMSO, Sigma) in fetal bovine serum (FBS, Gibco) and stored in cryotubes in liquid nitrogen.

2.6 Islet Dissociation

Neonatal pig islets were dissociated into single islet cells following the Rayat's Lab protocol. Briefly, after 7 days of culture and purification, islets were collected and suspended in 10 ml of dissociation medium. One ml of trypsin (1 mg/ml, Gibco) and 500 µl of DNase I (0.4 mg/ml, Roche, Mannheim, Germany) were added to islet suspension. Islets were dissociated by pipetting islet suspension at 37°C water bath for 8 minutes. After most of the islets were dissociated into single cells, the cell suspension was filtered through a 70 µm Nylon Mesh (Fisherbrand, Ottawa, Ontario, Canada). Filtered cells were washed with supplemented Ham's F10 medium twice, counted, assessed for viability and used for further experiments.

2.7 One-way Mixed Lymphocyte Reaction

One-way mixed lymphocyte reaction (MLR) was set up in a 200 µl system in a 96-well non-tissue culture treated flat bottom plate. Human PBMC served as responder cells and were plated into culture at 0.5 million cells/50 µl medium/well. Dissociated islet cells or pig PBMC used as stimulator cells were irradiated at 9 Rad/min for 2 minutes and 37 seconds and plated into the MLR system at 0.3 million cells/50 µl medium/well. One hundred µl of supplemented RPMI-1640 medium (Gibco, supplemented with 2g/l sodium bicarbonate, 10% fetal bovine serum, 100U/ml penicillin and 100µg/ml streptomycin) was added to each well so that the total volume for each well was 200 µl. Cells were cultured in an incubator at 37°C with 5% CO_2, 95% air. Response of human PBMC after stimulation with Concanavalin A (ConA, 100µg/ml, Sigma) was also measured as control. Briefly, half million responder cells were suspended in 180 µl of supplemented RPMI-1640 medium and plated into culture. Twenty µl of ConA was added to the culture such that total volume would be 200 µl. Controlled cells were cultured in the same condition as previously described.

The human immune response against neonatal pig islets or pig PBMC was measured using β emission which was expressed as counts per minute (CPM). Briefly, at 24 hours prior to cell collection, ten µl of [^{3}H]-thymidine was added to the MLR culture system. The [^{3}H]-thymidine in tritium was integrated into the gap of newly synthesised DNA of proliferated cells. At days 1, 2, 3, 4, 5, 6, and 7 of MLR setup, gross morphology of cells was analyzed by microscopy, and thereafter the cells in 96-well plates were collected using a Tomtec Cell Harvester 96 (Tomtec, Inc. Hamden, CT, USA). The proliferation of human PBMC was detected using a MicroBeta TriLux machine (PerkinElmer, Woodbridge, Ontario, Canada). Such that, the proliferation curve of human PBMC in the MLR system could be obtained. Based on this proliferation curve, the day with the maximum response was chosen to test the effect of Suramin or DR80 on human PBMC proliferation.

To investigate the effect of Suramin or DR80 on human PBMC proliferation, the responder cells were stained with 5µM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Eugene, Oregon, USA) for 5 minutes and washed with 5% FBS in PBS twice before use. At the end point of study, cells were collected and analyzed by flow cytometry on an LSR-II machine (BD, Mississauga, Ontario, Canada). Cell proliferation was determined with software FCS Express 5.0 and expressed as proliferation index.

2.8 Preparation of Suramin and Direct Red 80

Suramin and direct red 80 (DR80) were weighed and dissolved in supplemented RPMI-1640 medium following the concentration series showed in Table 1. Drug solutions were filtered through a 0.2µm filter before added to the cell culture.

Table 2-1. Preparation of Suramin and/or DR80

Suramin/DR80	Working Concentration (µg/ml)	Final Concentration (µg/ml)
1	2000	1000
2	1000	500
3	500	250
4	250	125
5	125	62.5
6	62.5	31.2
7	31.2	15.6
8	15.6	7.8
9	0	0

2.9 Transmission Electron Microscopy

NPI xenografts were chopped into small pieces and washed with 1x PBS twice. Tissues were fixed with a modified Karnovsky's fixative (Ted Pella Inc., Redding, CA, USA), which contains 2% paraformaldehyde, 2% glutaraldehyde in 0.1M sodium cacodylate buffer. Samples were rinsed 3 times with 0.1M sodium cacodylate buffer quickly, followed by spinning in a swing bucket rotor at 4°C at 1000 x g for 5 minutes, 3000 g for 5 minutes, 6000 g for 5 minutes and 12000 g for 5 minutes to make a hard pellet. The pellet was fixed with 1% osmium tetroxide (Ted Pella Inc., Redding, Canada) in 0.1M sodium cacodylate buffer for one hour on ice in the dark fume hood. Then the pellets were quickly rinsed in water twice and stained with 1% uranyl acetate (Ted Pella Inc.) in water for 1 hour on ice at dark. The pellets were dehydrated through a series of graded ethyl alcohol on ice: 70% ethanol, rinsed twice, 5 minutes each; 90% ethanol, rinsed twice, 5

minutes each; 100% ethanol, rinsed three times, 5 minutes each. The pellets were infiltrated in propylene oxide (Ted Pella Inc.) twice with 5 minutes each time, followed by a two-hour stay in a 50:50 mixture of propylene oxide/resin under vacuum. Then the pellets were placed in a fresh change of 100% embedding media for an hour and then transferred to the Epon 812 beams (EMS # 14120) for polymerization. Thin sectioning was prepared with UltracutE Reichert Jung followed by imaging at a Philips 410 TEM, equipped with Mega View III soft imaging system.

2.10 Microarray Analysis

Microarray analysis was performed to compare the difference in gene expression in islets from transgenic GalTKO, GalTKO/hCD46, GalTKO/hCD46/hCD39 pigs and wild type pigs. Total RNA was extracted from neonatal pig islets using Trizol agent following the manufacturer's protocol (Invitrogen, Burlington, ON, Canada). One hundred ng of each total RNA was labelled using the Affymetrix GeneChip 3' IVT Plus labelling kit (P/N 902395) and the Affymetrix GeneChip 3' IVT Plus Reagent Kit Manual (Thermo Fisher Scientific Inc., Fife, WA, USA). Poly A controls were added to each sample at the beginning of the labelling. Then 10 µg of fragmented, biotin-modified cRNA was hybridized to the Affymetrix GeneChip Porcine Genome Array (Ref 900624) in the Affymetrix GeneChip Hybridization Oven 645 at 45ºC, 60rpm for 16 hours. Hybridization controls were added to each sample before the arrays were hybridized. The arrays were washed and stained with Affymetrix GeneChip Wash and Stain Kit (P/N 900720) on the Affymetrix GeneChip Fluidics Station 450. The arrays were then scanned on the Affymetrix GeneChip Scanner 3000 (7G) and the raw data were generated. The Affymetrix® Expression Console™ Software and Transcriptome Analysis Console (TAC) Software were used to analyze gene level normalization, signal summarization and gene level differential expression. To further investigate which biological

processes were involved in the differences in gene expression, functional analysis of all up- or down-regulated genes was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

2.11 RT-PCR

The total RNA was extracted from 200 IEQ of neonatal pig islets using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and the concentration of RNA was measured by NanoDrop (ThermoFisher, Canada). Two hundred nanograms of total RNA was used to construct cDNA using 5X All-In-One RT MasterMix (Applied Biological Materials, Vancouver, BC, Canada). Gene sequences for *CDH1* (E-cadherin), *CDH2* (N-cadherin) and *CDH5* (VE-cadherin) were obtained from Gene Bank in Pubmed and primers were designed using Custom Assay Design Tool in Taqman RT-PCR Assays (ThermoFisher). PCR amplifications were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan gene expression assays (Applied Biosystems).

2.12 Western Blot

Total protein from 2000 IEQ were extracted using RIPA buffer with 0.1% proteinase inhibitor according to the manufacturer's protocols. BCA protein kit was used to quantify protein concentration following the manufacturer's protocol. Western blot was performed using Western Simple kit (ProteinSimple, San Jose, California, USA). Briefly, protein lysates were standardized to 200 μg/ml and mixed with MasterMix with 4:1 ratio, denatured in 95°C for 5 minutes. E-cadherin, VE-cadherin and N-cadherin were detected using a monoclonal mouse anti E-cadherin primary antibody (1:50 dilution, Life Technologies), a monoclonal rabbit anti VE-cadherin primary

antibody (1:100 dilution, Abcam) and a monoclonal rabbit anti N-cadherin primary antibody (1:50 dilution, Abcam) for 30 minutes, respectively, followed by secondary anti mouse (10µl, ProteinSimple) or anti rabbit (10µl, ProteinSimple) antibody for 30 minutes. The intensity of binding was visualized by chemical luminance.

2.13 Islet Transplantation

NOD.SCID gamma mice that had two consecutive non-fasting blood glucose levels of ≥ 20mmol/l within one week after a single STZ injection were transplanted with 2000 islet equivalents (IEQ) under the left kidney capsule. Briefly, the mouse was anesthetized with isoflurane (2-4% for induction and 0.5-2% for maintenance of general anaesthesia) and hair at the left renal region was shaved. The surgical field was treated with 10% povidone-iodine and 70% ethanol. An approximate 1.5 cm incision on the skin and muscle was made to expose the kidney. Neonatal pig islets were aspirated into polyethylene tubing (PE-50 tube) and spun to form a pellet of islets. Islets were gently placed under the left kidney capsule with the aid of a micromanipulator syringe. Once the islets were in place, the kidney bearing the islet transplant was placed back into the peritoneal cavity, and muscles and skin were sutured. After recovery from surgery, recipient mice were returned to the housing facility and monitored daily. Blood glucose levels of recipient mice were measured once per week as described above. When mice achieved normoglycemia, they were grouped as follows: (1) non-reconstituted (n=5); (2) reconstituted with T1D PBMC (n=3) or (3) NT1D PBMC and euthanized at 1-week post-reconstitution (n=3); (4) reconstituted with T1D PBMC (n=4) or (5) NT1D PBMC and euthanized at 2-week post-reconstitution (n=4); (6) reconstituted with T1D PBMC (n=3) or (7) NT1D PBMC and euthanized at 3-week post-reconstitution (n=3); (8) reconstituted with NOD splenocytes (n=3).

2.14 Intraperitoneal Glucose Tolerance Test

Islet-transplant recipient mice that had maintained stable normal blood glucose levels (<10.0 mmol/l) for two consecutive weeks were fasted for approximately 16 hours, with unlimited supply of drinking water. Glucose tolerance test was performed by injection of 50% dextrose solution (3.0 g/kg body weight) into the peritoneum of recipient mice and their blood glucose levels measured at 0, 15, 30, 60 and 120 minutes after administration of glucose. The mice were returned to the housing facility and their blood glucose levels measured once per week until the end of the study (>100 days post-transplantation). For recipient mice that maintained normal blood glucose levels, the kidney that contained the islet xenograft was removed at the end of the study. The blood glucose levels of these mice were monitored after removal of the kidney and once they became diabetic (>10.0 mmol/l), they were euthanized. For those mice that remained diabetic at the end of the study, they were euthanized and the kidney that contained the islet xenograft was procured.

2.15 Reconstitution with Human PBMC or NOD Splenocytes

PBMCs from T1DM or NT1DM individuals or NOD splenocytes were recovered from liquid nitrogen in RPMI-1640 medium (Gibco, supplemented with 2g/l sodium bicarbonate, 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin) in 37°C water bath for 40 minutes. Fifteen million PBMCs from T1DM or NT1DM individuals or 25 million NOD splenocytes were injected into NSG mouse through tail vein at 2 weeks after the IPGTT. Blood glucose levels were measured weekly as described before. At 1, 2 and 3 weeks after the reconstitution with PBMCs from T1DM or NT1DM individuals, kidneys that contained the islet xenografts were removed. The blood glucose levels of these mice were monitored after removal of the kidney and once they became

diabetic (>10.0 mmol/l), they were euthanized.

2.16 Immunohistochemistry Analysis

Pancreatic tissues, islets or islet grafts from recipient mice were fixed in Zinc fixative solution for 24 hours, washed three times with 70% ethanol and stored in the same solution until processed for histology.

The presence of endocrine hormones and islet precursor cells in 5μm sections of kidney tissues that contained the islet xenograft was determined following our standard protocols. Briefly, guinea pig anti-pig insulin (1:1000 dilution, Dako Corporation, Carpinteria, CA, USA) or mouse anti-pig glucagon (1:5000 dilution, Sigma) primary antibodies or goat anti-somatostatin (1:1000 dilution, Sigma) primary antibodies were used to detect the presence of endocrine hormones. After washing the tissue sections with PBS, biotinylated goat anti-guinea pig IgG (1:200 dilution, Vector Laboratories, Burlingame, CA, USA) and biotinylated goat anti-mouse IgG (1:200 dilution, Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA) and horse anti-goat secondary antibodies (1:200 dilution, Vector Laboratories) were added to the respective samples.

To detect the islet precursor cells or E-, VE-, N-cadherin expression, heat mediated antigen retrieval with sodium citrate buffer at pH=6 was performed using a domestic microwave. Expression of cytokeratin-7 (CK-7) was detected by incubation of tissue sections with a monoclonal mouse anti-human CK-7 primary antibody (1:200 dilution, Dako Corporation) for 30 minutes and biotinylated goat anti-mouse IgG secondary antibody (1:200 dilution, Vector Laboratories). Expression of E-, VE-, N-cadherin was detected by incubation of tissue sections with a monoclonal mouse anti E-cadherin primary antibody (1:25 dilution for 1.5 hours at room temperature, Life Technologies, Rockford, IL, USA), a monoclonal rabbit anti VE-cadherin

primary antibody (1:3000 dilution overnight at 4°C, Abcam, Cambridge, United Kingdom) and a monoclonal rabbit anti N-cadherin primary antibody (1:1000 dilution overnight at 4°C, Abcam), respectively. The innervation in pancreas was detected with a monoclonal mouse anti-PGP9.5 primary antibody (1:2000 dilution, Abcam, Toronto, ON, Canada). Tissue sections were washed and incubated with biotinylated goat anti mouse IgG antibody (1:200 dilution, Jackson ImmunoResearch Laboratories, Inc.) or biotinylated goat anti rabbit IgG antibody (1:200 dilution, Vector Laboratories).

To detect the human immune cells in the islet graft, an anti-human CD45 (1:500) antibody was used to identify the leukocyte common antigen. Macrophage, human CD4 T cells, human CD8 T cells were detected using rat anti-human MAC-2 (Galectin-3, a marker for activated macrophages) antibody (1:1000), anti-human CD4 (1:500) and anti-human CD8 antibody (1:250), respectively, followed by secondary anti-rat antibody, anti-rabbit antibody and anti-mouse antibody.

The avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories) and 3,3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA, USA) enzyme-substrate method was used to visualize the positive reaction (brown colour) in all tissue sections. After immunostaining, the tissue sections were counterstained with Harris' haematoxylin and eosin.

2.17 Statistical Analysis

Data were presented as means ± SD. Statistical differences between groups were sought using *t*-test or one-way ANOVA in Stata 13.0 for Windows. A p value of <0.05 was considered statistically significant.

Chapter 3 DIRECT RED 80 AND SURAMIN INHIBIT HUMAN IMMUNE-MEDIATED RESPONSE TO NEONATAL PIG ISLETS IN VITRO

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3.1 INTRODUCTION

Neonatal pigs are being considered as an alternative source of islets for transplantation. However, due to the expression of xeno-antigen on neonatal pig islets (NPI)[240], the potential hyperacute rejection as well as cell-mediated rejection remain major barriers for the application of NPI in the clinic. For example, previous study demonstrated that NPI cells trigger human CD4+ T lymphocyte proliferation *in vitro*[246]. A potential solution to overcome this obstacle is to understand the mechanism whereby human immune cells recognize and reject pig islet cells, based on which anti-rejection strategies can be developed to overcome the human immune-mediated response to pig islet cells. Of anti-rejection strategies, traditional calcineurin inhibitors have hepatotoxicity or nephrotoxicity; newly emerged monoclonal antibodies are target-specific and stable in human serum, however, several disadvantages of monoclonal antibodies limit their clinical usage, such as poor oral bioavailability, difficulty and expense of manufacture, poor permeability into cells[247]. Therefore, the field necessitates the search for anti-rejection reagents of other sources.

Some small-molecule inhibitors are found to have anti-rejection effects. Small inhibitory molecules (by their name, the molecular weight is usually less than 900 daltons) are molecules that specifically inhibit or disrupt a specific protein-protein interaction by binding to specific area in protein with high affinity[247]. Suramin is a derivative of napthalenesulphonic acid with a molecular weight of 1429.17 g/mol. Clinically, Suramin has been used for treatment of human sleeping sickness caused by trypanosomes and for treatment of river blindness[248], although the detailed mechanism is not clear. Several studies show the anti-rejection property of Suramin through the blockade of CD40-CD154 costimulatory interaction[224, 225, 249]. Suramin has also been demonstrated to have anti-proliferative effect by interruption of growth factors binding to

their cell surface receptors[250-252]. Direct Red 80 (DR80) is an organic dye with a molecular weight of 1373.07 g/mol, which is commonly used in textile industry. Margolles-Clark *et al.* showed the inhibitory effect of DR80 in blocking CD40-CD154 interaction, with IC50 of 1.8μM in human cells[249]. Together, these studies suggest the inhibitory effect of Suramin and/or DR80 in preventing immune rejection.

Thus, the purpose of this study is to investigate the human immune response to neonatal pig islet cells *in vitro* and to examine the effect of Suramin and DR80 on the proliferation of human peripheral blood mononuclear cells (PBMC) after stimulation with pig islet cells. Specifically, we focus on immune cells from individuals with type 1 diabetes mellitus (T1DM), as the goal of the study is to forward transplantation of NPI into patients with T1DM. We hypothesized that proliferation of human PBMC from individuals with T1DM will be inhibited by Suramin or DR80.

3.2 RESULTS

Gross morphology of human PBMC from individuals with T1DM in response to different stimulators. In the absence of stimulation, proliferation of PBMC from T1DM individuals did not occur following 7 days of culture (Figure 3-1A to 3-1G). In the contrast, cell clusters were identified under microscopy on day 1 after ConA was added to the MLR system (Figure 3-1H). The size of these cell clusters was increased on day 2 (Figure 3-1I) and day 3 (Figure 3-1J) and largest on day 7 (Figure 3-1N). However, the number of the cell clusters was reduced following 7 day of culture (Figure 3-1H to 3-1N). Maximal number of cell clusters appeared to be on day 3 after ConA stimulation (Figure 3-1J). When stimulated with dissociated NPI cells, the change in size and number of proliferated cells was not obvious under microscopy following 7 days of culture (Figure 3-1O to 3-1U). Aggregation of dissociated islet cells was observed (Figure 3-1O to 3-1U), which was different from the responded cell clusters when human PBMC were stimulated with ConA. When stimulated with pig PBMC, human PBMC cluster was not observed on days 1 to 4 after experimental set-up (Figure 3-1V to 3-1Y). Rather, cell clusters were clearly identified at later days (Figure 3-1Z, 3-1A1, 3-1B1). These results suggest robust proliferation of human PBMC in early days when stimulated with non-antigen-specific stimulation with ConA, but in later days when stimulated with antigen-specific pig cells. This response appears to be restricted to human PBMC stimulated with pig PBMC but not NPI.

Proliferation curve of human PBMC in response to different stimulators. As cells divide, the [^{3}H]-thymidine is integrated into the newly synthesized DNA. Hence, the proliferation of human PBMC was indicated by beta emission of [^{3}H]-thymidine, which was expressed as counts per minute (CPM). Without stimulation, proliferation of human PBMC was low during culture on

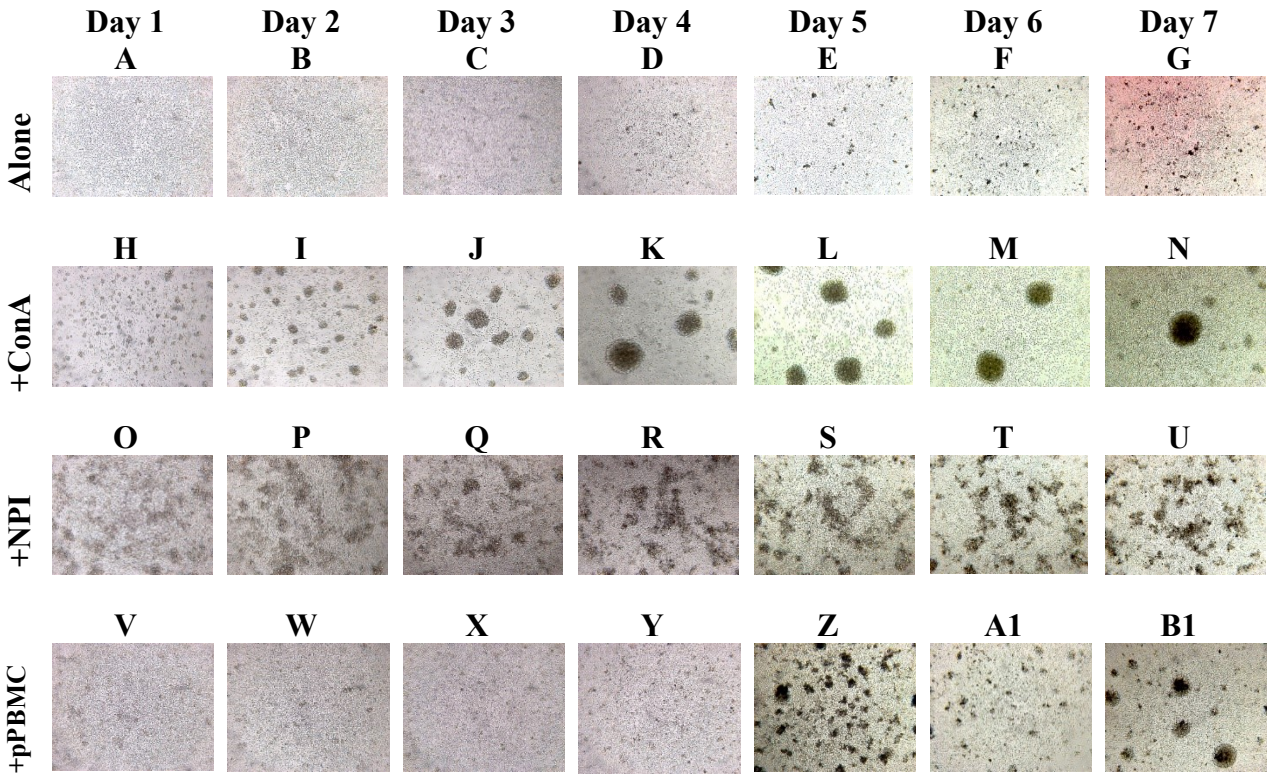


Figure 3-1. Gross morphology of PBMC from individuals with T1DM with or without stimulators concanavalin A (ConA), dissociated neonatal pig islet cells (NPI) or pig PBMC (pPBMC). **A-G:** Human PBMC alone on days 1 to 7 in culture after experiment set-up. **H-N:** Gross morphology of human PBMC in response to ConA stimulation on day 1 to 7 in culture. **O-U:** Gross morphology of human PBMC in response to dissociated NPI stimulation on days 1 to 7 in culture. **V-B1:** Gross morphology of human PBMC in response to pPBMC stimulation on days 1 to 7 in culture.

day 1 (266±131 CPM, n=12, Figure 3-2A). A slight increase in the proliferation of human PBMC was observed on day 7 of culture (1,563±1,278 CPM, n=12, Figure 3-2A). In contrast, robust proliferation was observed on day 1 (13,873±9792 CPM, n=12, Figure 3-2B) when human PBMC were stimulated with ConA. Maximum proliferation was observed on day 3 after ConA was added, which was significantly different compared to that on day 1 (39,383±16,634 CPM, n=9, $p<0.01$, Figure 3-2B). This proliferation was decreased on day 4 (14,417±15,947, n=14, $p<0.01$) and day

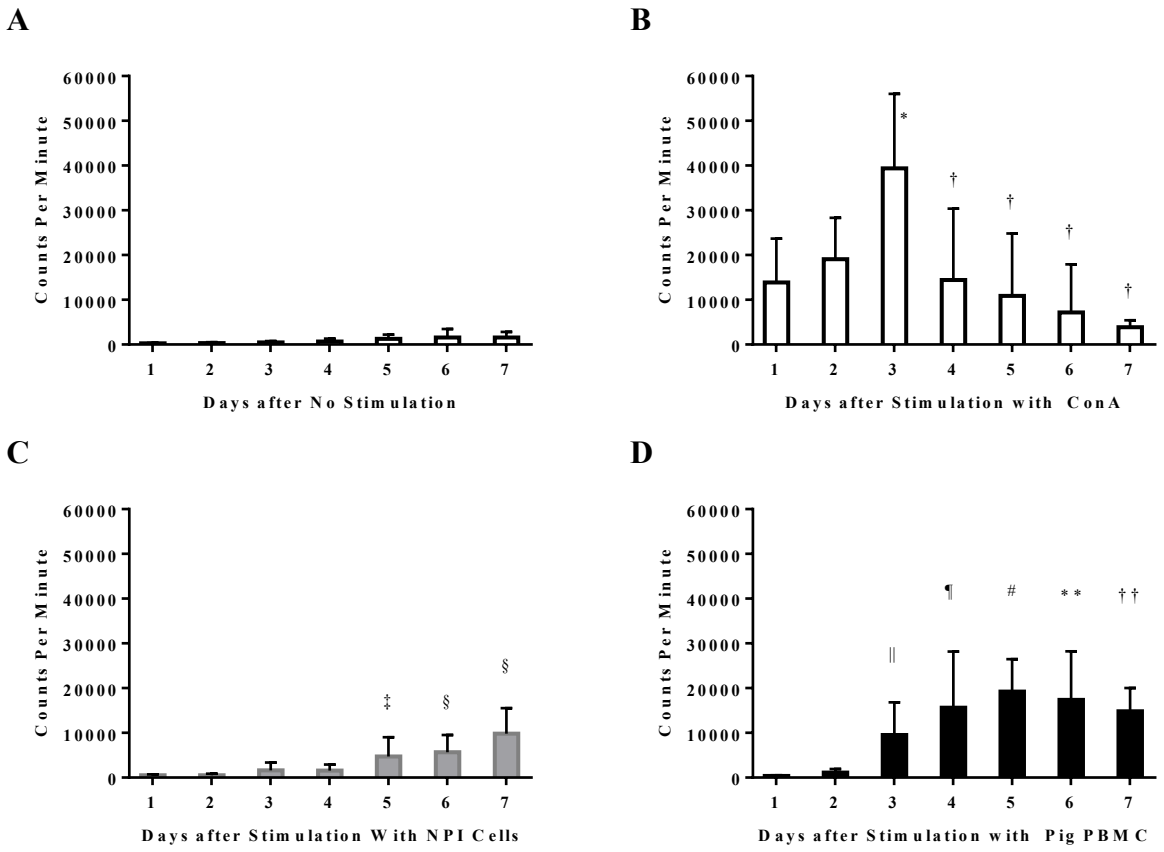


Figure 3-2. Proliferation of human peripheral blood mononuclear cells (PBMC) from individuals with type 1 diabetes mellitus in response to concanavalin A (ConA) or neonatal pig cells. **A:** Proliferation of human PBMCs without stimulation in culture. **B:** Proliferation of human PBMC when stimulated with ConA in culture. **C:** Proliferation of human PBMC when stimulated with dissociated neonatal pig islet cells in culture. **D:** Proliferation of human PBMC when stimulated with pig PBMC in culture. Symbols: *, $p<0.01$, day 3 vs day 1; †, $p<0.01$, day 4 vs day 3, day 5 vs day 3, day 6 vs day 3, day 7 vs day 3; ‡, $p<0.05$, day 5 vs day 1; §, $p<0.01$, day 6 vs day1, day 7 vs day1; ||, $p<0.05$, day 3 vs day 1, ¶, $p<0.01$, day4 vs day 1; #, $p<0.01$, day 5 vs day 1; **, $p<0.01$, day 6 vs day 1; ††, $p<0.01$, day 7 vs day 1

5 (10,866±13,972 CPM, n=14 $p<0.01$), day 6 (7,169±10,733 CPM, n=14, $p<0.01$) and lowest on day 7 (3,857±1,591 CPM, n=12, $p<0.01$) compared to that on day 3. When stimulated with dissociated neonatal pig islet cells, proliferation of human PBMC was low (506±224 CPM, n=8,

Figure 3-2C) on day 1 and thereafter, a slight increase was observed on day 4 (1,565±1,336 CPM, n=20). The proliferation was significantly increased on day 5 (4,742±4,284 CPM, n=20, $p<0.05$) and highest proliferation was observed on day 7 (9,838±5,681 CPM, n=9, $p<0.01$), compared to that of day 1. When stimulated with pig PBMC, proliferation of human PBMC was higher on day 3 compared to that on day 1 (9,549±7,262 CPM versus 373±171 CPM, n=14, $p<0.05$, Figure 3-2D). The proliferation was significantly more on day 4 (15,610±12,569 CPM, n=12, $p<0.01$), day 5 (19,200±7,263 CPM, n=14, $p<0.01$), day 6 (17,390±10,820 CPM, n=13, $p<0.01$) and day 7 (14,821±5,202 CPM, n=13, $p<0.01$) in culture compared to that of day 1. There was no significant difference in the proliferation of human PBMC when stimulated with pig PBMC among day 4, day 5, day 6 and day 7 in culture. Together, the above data showed the proliferation curve of human PBMC in response to different stimulators. Similar to morphology changes in culture, it appears that human PBMC responds robustly to pig PBMC but not NPI.

Suramin inhibited the proliferation of human PBMC from individuals with T1DM in response to ConA, dissociated NPI cells and pig PBMC stimulation. Based on the proliferation kinetics above, day 3 was used as the time point to test the effect of Suramin and DR80 on human PBMC proliferation when stimulated with ConA and day 7 was used as the time point when human PBMC was stimulated with dissociated NPI cells or pig PBMC. The proliferation of human PBMC was followed labelling of cells with CFSE and expressed as proliferation index (PI). PI is the total number of divisions divided by the number of cells that went into division. In the absence of Suramin, human PBMC from individuals with T1DM proliferated (11.7±3.5 PI, n=5, Figure 3-3A) in response to ConA. The proliferation index was decreased when the concentrations of Suramin increased, which was significantly lower when 125 μg/ml, 250 μg/ml or 500 μg/ml of Suramin was

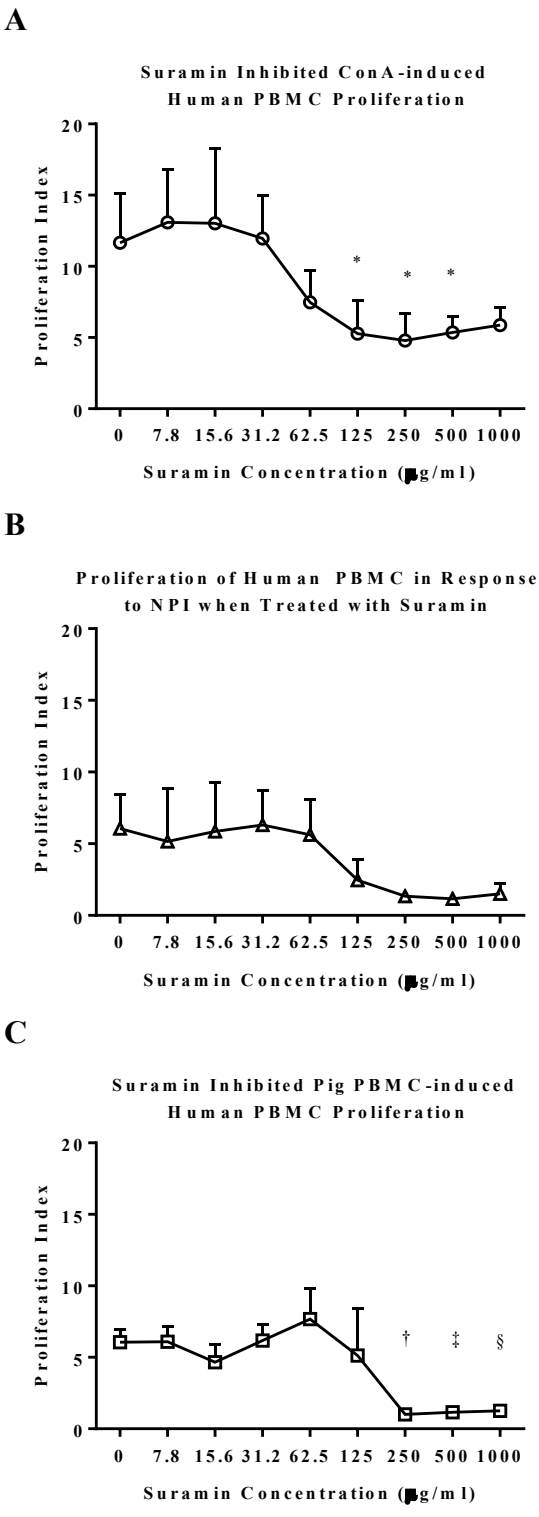


Figure 3-3. Suramin inhibited the proliferation of human PBMC from individuals with T1DM in response to **A:** concanavalin A; **B:** dissociated neonatal pig islet cells; **C:** pig PBMC stimulation.

Proliferation of human PBMC was analyzed on day 3 after stimulation with ConA, and on day 7 after stimulation with NPI cells or pig PBMC. Data are expressed as mean±SD. Symbols: *, $p<0.05$, 125 µg/ml vs 0 µg/ml, 250 µg/ml vs 0 µg/ml, 500 µg/ml vs 0 µg/ml; †, $p<0.01$, 250 µg/ml vs 0 µg/ml; ‡, $p<0.01$, 500 µg/ml vs 0 µg/ml; §, $p<0.01$, 1,000 µg/ml vs 0 µg/ml

applied (5.3±2.3 PI, n=5, $p<0.05$; 4.8±1.9 PI, n=5, $p<0.05$; 5.4±1.2 PI, n=5, $p<0.05$, respectively, Figure 3-3A), compared to that observed without Suramin. Suramin inhibited Con A-induced proliferation of human PBMC with IC_{50} dose of 62.5 µg/ml (Figure 3-4).

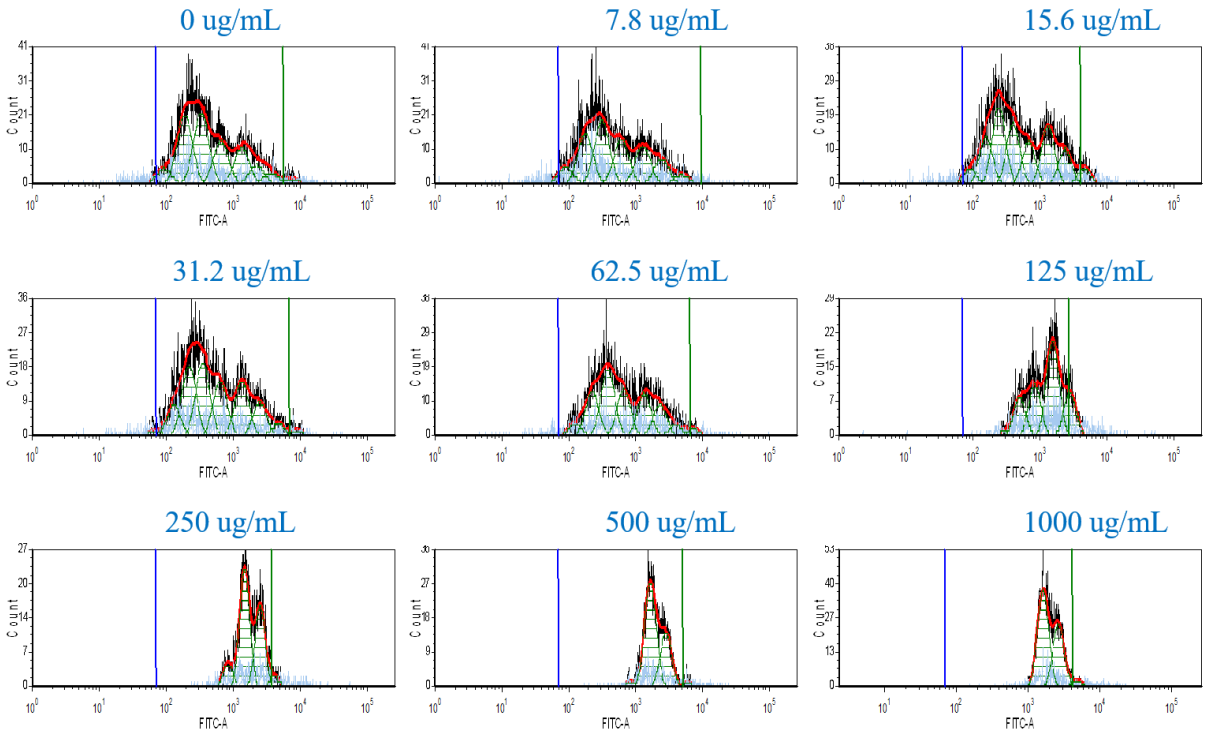


Figure 3-4. Dose dependant effect of Suramin on the proliferation of human PBMC from individuals with T1DM when stimulated with concanavalin A. Green line represents non-divided positive stained cells. Blue line represents non-stained negative control cells.

In the absence of Suramin, human PBMC from individuals with T1DM also proliferated (6.1±2.4 PI, n=4, Figure 3-3B) in response to dissociated NPI cells. The proliferation index was decreased when the concentrations of Suramin increased, which was lower when 125 µg/ml of Suramin was applied, but this was not found to be statistically significant (2.5±1.5 PI, n=4, p>0.05, Figure 3-3B), compared to without Suramin. Though not significantly different, Suramin appeared to suppress NPI cell-induced proliferation of human PBMC with IC_{50}~62.5 to 125 µg/ml (Figure 3-5).

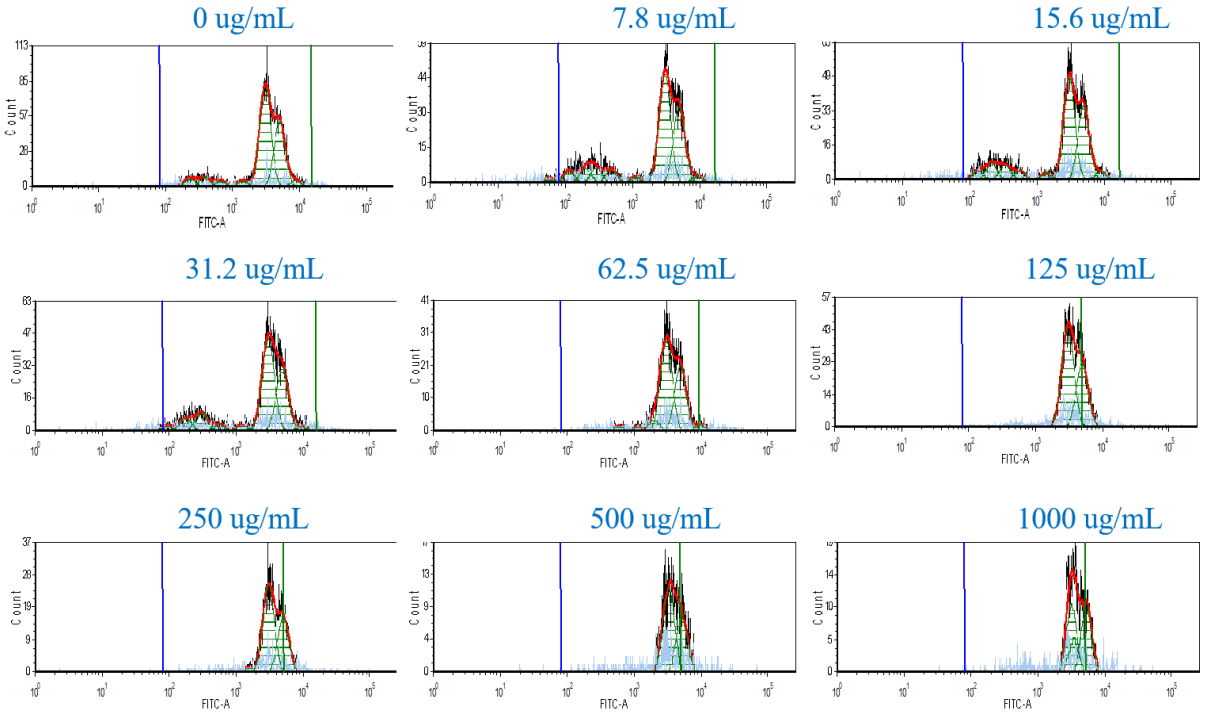


Figure 3-5. Dose dependant effect of Suramin on the proliferation of human PBMC from individuals with T1DM when stimulated with dissociated neonatal pig islet cells. Green line represents non-divided positive stained cells. Blue line represents non-stained negative control cells.

Further, unstimulated human PBMC from individuals with T1DM proliferated (6.1±0.9 PI, n=4, Figure 3-3C) in response to pig PBMC. The proliferation index was decreased when the concentrations of Suramin increased, which was significantly lower when 250 µg/ml (1.0±1.0 PI, n=4, p<0.01, Figure 3-3C), 500 µg/ml (1.2±0.2 PI, n=4, p<0.01) and 1,000 µg/ml (1.3±0.4 PI, n=4, p<0.01) of Suramin was applied, compared to that detected in the absence of Suramin. Suramin inhibited pig PBMC-induced proliferation of human PBMC with IC_{50}~125 to 250 µg/ml (Figure 3-6).

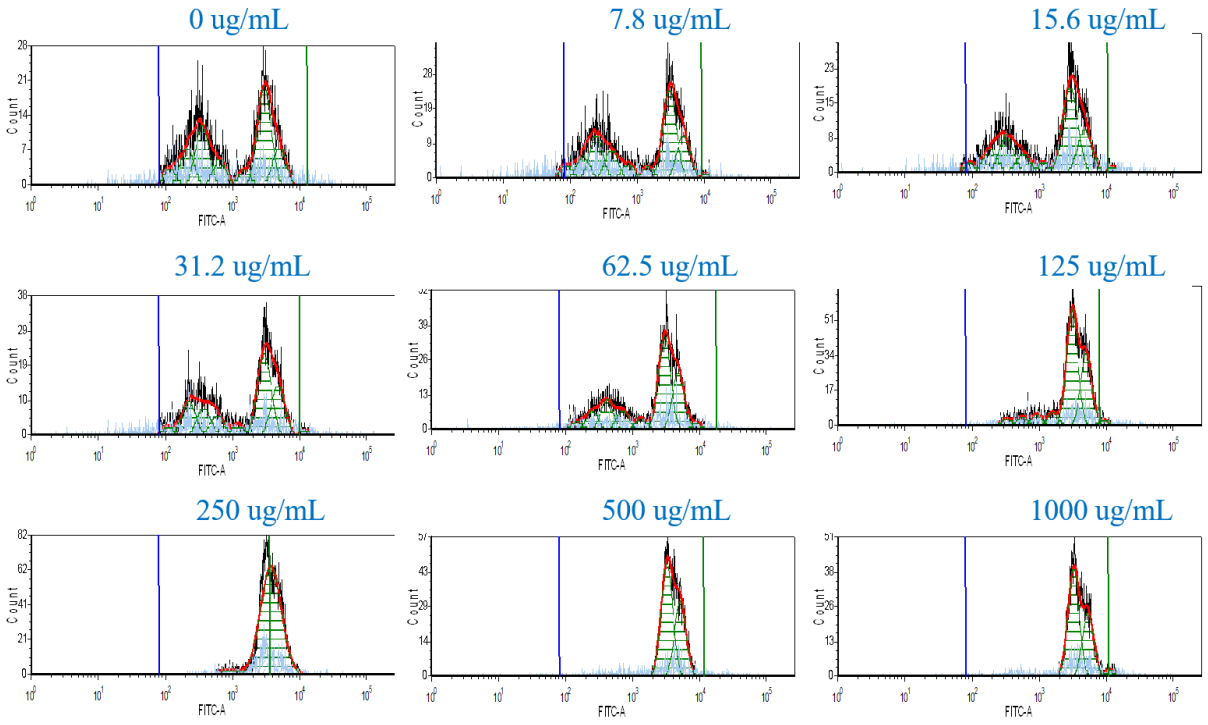


Figure 3-6. Dose dependant effect of Suramin on the proliferation of human PBMC from individuals with T1DM when stimulated with pig PBMC. Green line represents non-divided positive stained cells. Blue line represents non-stained negative control cells.

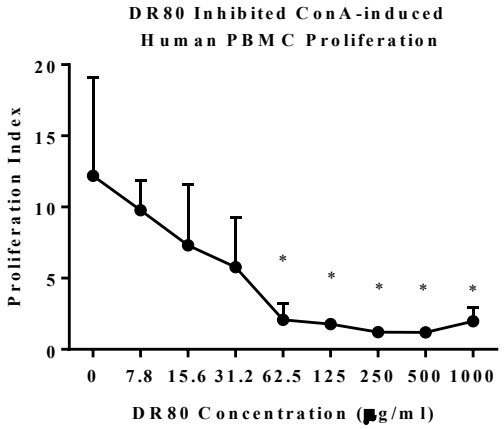
Direct Red 80 inhibited the proliferation of human PBMC from individuals with T1DM in response to ConA, dissociated NPI cells and pig PBMC. Human PBMC from individuals with T1DM were stimulated with ConA and the proliferation was observed (12.2±7.0 PI, n=4, Figure 3-7A). The proliferation index was decreased when concentrations of DR80 increased, which was significantly lower when 62.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml or 1000 μg/ml of DR80 were applied (2.1±1.1 PI, n=4, $p<0.01$; 1.8±0.1 PI, n=4, $p<0.01$; 1.2±0.2 PI, n=4, $p<0.01$; 1.2±0.2 PI, n=4, $p<0.01$; 2.0±1.0 PI, n=4, $p<0.01$, respectively, Figure 3-7A), compared to that seen without DR80. DR80 inhibited the proliferation of human PBMC stimulated with Con A with IC_{50}~31.2 to 62.5 μg/ml.

Similar trend was also observed when human PBMC were stimulated with NPI cells. In the absence of DR80, human PBMC from individual with T1DM proliferated (7.4±2.6 PI, n=6, Figure 3-7B) in response to dissociated NPI cells stimulation. The proliferation index was decreased when the concentrations of DR80 increased, which was significantly lower when 62.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml or 1000 μg/ml of DR80 were applied (3.3±2.1 PI, n=6, $p<0.01$; 1.5±0.2 PI, n=6, $p<0.01$; 2.1±1.2 PI, n=6, $p<0.01$; 1.9±0.9 PI, n=6, $p<0.01$; 2.3±1.4 PI, n=6, $p<0.01$, respectively, Figure 3-7B), compared to the proliferation observed without DR80. DR80 inhibited the proliferation of human PBMC stimulated with NPI cells with IC_{50}~31.2 to 62.5 μg/ml.

When human PBMC from individuals with T1DM were stimulated with pig PBMC, we observed proliferation of these cells (9.4±6.3 PI, n=6, Figure 3-7C). The proliferation index was decreased when the concentrations of DR80 increased, which was significantly lower when 62.5 μg/ml or 1000 μg/ml of DR80 was applied (2.4±1.7 PI, n=6, $p<0.05$; 1.5±0.9 PI, n=6, $p<0.01$, respectively, Figure 3-7C), compared to that observed in cells without DR80 treatment. DR80 inhibited the proliferation of human PBMC stimulated with pig PBMC with IC_{50}~31.2 to 62.5

μg/ml.

A


DR80 Inhibited ConA-induced
Human PBMC Proliferation
Proliferation Index
20
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10
5
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7.8
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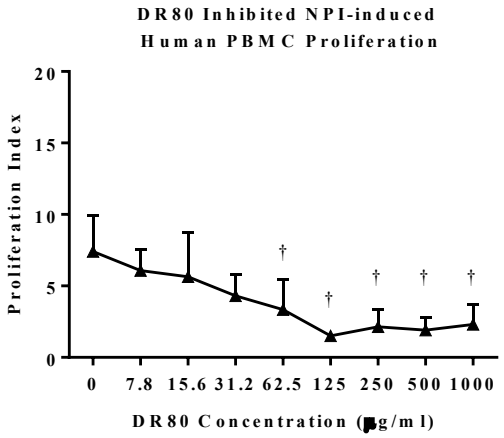
125
250
500
1000
DR80 Concentration (μg/ml)

B


DR80 Inhibited NPI-induced
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DR80 Concentration (μg/ml)

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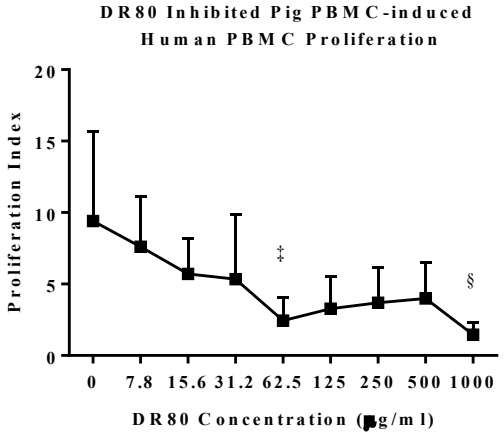
DR80 Inhibited Pig PBMC-induced
Human PBMC Proliferation
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62.5
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DR80 Concentration (μg/ml)

Figure 3-7. DR80 inhibited the proliferation of human PBMC from individuals with T1DM in response to **A:** concanavalin A; **B:** dissociated neonatal pig islet cells; **C:** pig PBMC stimulation. Proliferation of human PBMC was analyzed on day 3 after stimulation with ConA, and on day 7 after stimulation with NPI cells or pig PBMC. Data are expressed as mean±SD. Symbols: *, $p<0.01$, 62.5 μg/ml vs 0 μg/ml; 125 μg/ml vs 0 μg/ml; 250 μg/ml vs 0 μg/ml; 500 μg/ml vs 0 μg/ml; 1000 μg/ml vs 0 μg/ml; †, $p<0.01$, 62.5 μg/ml vs 0 μg/ml; 125 μg/ml vs 0 μg/ml; 250 μg/ml vs 0 μg/ml; 500 μg/ml vs 0 μg/ml; 1000 μg/ml vs 0 μg/ml; ‡, $p<0.05$, 62.5 μg/ml vs 0 μg/ml; §, $p<0.01$, 1000 μg/ml vs 0 μg/ml

3.3 DISCUSSION

Human immune cell-mediated response against NPI have been examined. In a previously study by Murray A *et al*., human PBMC from individuals without T1DM were co-cultured with neonatal pig splenocytes or dissociated NPI cells, and the maximum for both responses were closed and were observed on day 8 or day 9 in culture[246]. In terms of the type of immune cells involved in the response, they found that NPI cells induce $CD4^+$ but not $CD8^+$ T cell response *in vitro*, and NPI cells are not susceptible to NK cell or cytotoxic lymphocyte lysis[246].

This study showed the proliferation kinetics of human PBMC from individuals with type 1 diabetes mellitus in response to different stimulation. When stimulated with ConA, the maximum proliferation of human PBMC was observed on day 3 in the proliferation of these cells. The decrease thereafter may be explained by the exhaustion of nutrients necessary for cell growth. In terms of the ability to stimulate PBMC proliferation, ConA as a non-antigen specific stimulator[253], elicits a higher human PBMC proliferation response compared to antigen-specific stimulators such as neonatal pig PBMC or islet cells. The proliferation of human PBMC in response to NPI cells was not as strong as that of pig PBMC, which could be due to the lower percentage of antigen presenting cells in NPI compared to that in pig PBMC[254]. Also, when stimulated with pig cells, the maximal proliferation of human PBMC occurred at later days of experimental set-up, which suggested that antigen-specific stimulation occurs in a slower manner than non-antigen specific stimulation. These data together provided optimal timepoints for further experiments using such mixed lymphocyte reaction (MLR) system.

It has been reported that the CD40-CD154 interaction was blocked by Suramin in a concentration dependent manner with IC_{50} of 18.9-20.9 μg/ml, and DR80 with IC_{50} of 2.5 ug/ml in human immune cells[224, 249]. Whereas in this study, Suramin inhibited pig PBMC-induced

proliferation of human PBMC with IC_{50}~125 to 250 μg/ml. Although it was not statistically significant, Suramin showed some inhibition of NPI-induced human PBMC proliferation. This higher IC_{50} of Suramin on human PBMC proliferation when stimulated with pig PBMC compared to that when stimulated with NPI corresponded with previous data that pig PBMC stimulated more human PBMC proliferation than NPI did. The inhibitory effect of Suramin may be partially explained by the inhibition of protein kinase C, which is an enzyme involved in intracellular mitogenic signal transduction[223]. DR80 inhibited the proliferation of human PBMC stimulated with ConA, NPI cells or pig PBMC with IC_{50}~31.2 to 62.5 μg/ml. The inhibitory effect of DR80 in the proliferation of human PBMC from diabetic individuals is consistent with Margolles-Clark et al.'s study, in which they revealed that the inhibition is through the blockade of CD40-CD154 interaction[226]. It is notable that the IC50 observed in this study was higher than that in other studies[224, 249]. This variation may be due to the different parameters used to calculate the IC, in which the proliferation index was used in this study. Another possibility for this difference may lie in the immune response: a higher dose of Suramin or DR80 was necessary to inhibit the human immune response against NPI cells and/or pig PBMC.

A major limitation of this study is that we did not provide detailed phenotype of cells involved in the recognition and rejection of pig cells by human PBMC. Another limitation is that the inhibitory effect of Suramin/ DR80 against neonatal pig cells was only examined *in vitro*. Inclusion of animal model would strengthen the conclusion. Another future direction for this study would be the combination of these small inhibitory molecules with clinically available immunosuppressant to test if there exists a synergistic effect in preventing rejection of islet xenograft.

These results together demonstrate the inhibitory effect of DR80 and Suramin on the *in vitro* human immune response to pig cells. Further studies in the mechanism of these small inhibitory

molecules may offer options for effective anti-rejection therapies for islet xenotransplantation.

Chapter 4 REJECTION OF NEONATAL PIG ISLET XENOGRAFTS BY HUMAN IMMUNE CELLS IN VIVO

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4.1 INTRODUCTION

The shortage of human organ donors could be alleviated by the use of pigs as an alternative source of organ for islet transplantation. Pigs share similarity in morphology and physiology; they have short gestation period; they can be genetically modified to decrease immune rejection post-transplantation[106]. Specifically, neonatal pig islets (NPI) are of great interest as they are relatively easy to isolate, and they remain robust in culture. Also, NPI are more resistant to hypoxia-induced apoptosis due to the presence of immature insulin-low or insulin-negative precursor beta cells, which can differentiate into mature beta cells[255]. Though encouraging findings were shown in pig-to-non-human primate islet xenotransplantation[256], the rejection of pig islets remains a major obstacle for clinical application of NPI[257].

First, if NPI were to be transplanted via the intraportal route, the tissue factor that is expressed on pig islet cells may trigger instant blood-mediated inflammatory reaction[242, 258], which involves platelet aggregation, activation of coagulation and complement, and neutrophil infiltration, resulting in damage up to 60% of transplanted islets[259, 260]. Second, compared to other vascularized organs such as heart and lung, hyperacute rejection is rarely observed in adult pig islet xenograft[261] due to the loss of endothelial cells[262] and/or lower expression of α-Gal[263]. However, α-Gal expression in pig islets is age-dependent and higher level of this xeno-antigen is observed in NPI[263]. Therefore, α-Gal remains an obstacle for the application of NPI in the clinic due to the presence of naturally formed antibodies in human beings. Third, cellular rejection is another obstacle for pig islet transplantation into humans. *In vitro*, NPI induce human $CD4^+$ T cell but not $CD8^+$ T cell proliferation[221]. This is also evidenced in rodent models where the rejection of pig islets is mainly by $CD4^+$ T cells[227, 228]. In non-human primates, the rejection of pig islets involves macrophages, $CD4^+$ T cells and $CD8^+$ T cells[230, 264]. Also, this T cell mediated

rejection also induces response of other cell types such as NK cells and B cells. Most of these findings are found in pig-to-rodent or pig-to-nonhuman primate model. Very limited studies have investigated the human immune response to pig islets *in vivo*. Therefore, it necessitates a humanized model that can mimic human immune response against NPI.

Humanized mouse are immune-deficient mice that contain functional human genes, cells, tissues or organs[265]. NOD scid gamma mouse (NSG) is an immunodeficient mouse model developed by Jackson Laboratory (Bar Harbor, ME, USA). NSG is developed from strain NOD/ShiLtJ with the $Prkdc^{scid}$ mutation[266, 267] and the $Il2rg^{tm1Wjl}$ targeted mutation[268], resulting in absence of T cells, B cells and NK cells. The construction of humanized NSG mice is through intravenous or intraperitoneal injection of mature lymphoid populations such as peripheral blood mononuclear cells, lymph node cells or splenocytes[265]. Human islets are rejected within 17 days in NSG mice reconstituted with 2*10^5 hematopoietic stem cells[269]. However, few studies focus on how NPI are recognized and rejected in humanized NSG mice.

In this study, we characterized the human immune response against NPI using a humanized NSG model. We hypothesized that NPI would restore euglycemia in diabetic NSG mice, and that NPI would be rejected by the human immune cells when NSG mice were post-reconstituted with human immune cells.

4.2 RESULTS

Neonatal pig islets reversed hyperglycaemia in streptozotocin-induced diabetic NSG mice. All 28 NSG mice became diabetic (≥ 20.0 mmol/l) at 3 to 6 days after a single intraperitoneal injection of STZ. The average blood glucose level (BGL) of all mice was 7.4 ± 0.6 mmol/l prior to STZ injection, which increased after STZ injection and was 22.2 ± 3.9 mmol/l on the day of islet transplantation (Figure 4-1A). The average BGL of mice started to decrease from 3 weeks post-transplantation and reached below 10.0 mmol/l at 13 weeks post-transplantation. The body weight of mice was also measured during the study. Average body weight of NSG mice was 26.6 ± 1.7 g prior to STZ injection (8-week-old). Diabetic mice lost weight after the streptozotocin injection and had lowest average weight at 21.0 ± 2.2 g at 1-week post-transplantation. Thereafter, the average body weight of mice continuously increased and reached the level as prior to streptozotocin injection at 6-7 weeks post-transplantation (Figure 4-1A). For the group of mice that received no reconstitution (n=5), normal blood glucose level (normoglycemia) was achieved at 17-, 26-, 26-, 31- and 31-week post-transplantation (Figure 4-1B), respectively. At the end of study, the blood glucose level rose up to more than 20.0 mmol/l after the removal of the kidney bearing the islet xenograft (Figure 4-1B), indicating that the normoglycemic state in these mice was maintained by the islet transplant. For the group of mice that would be reconstituted with T1DM PBMC and euthanized at 1-week post-reconstitution (n=3), normoglycemia was achieved at 5-, 12- and 15-week post-transplantation (Figure 4-1C), respectively. For the group of mice that would be reconstituted with T1DM PBMC and euthanized at 2-week post-reconstitution (n=4), normoglycemia was achieved at 8-, 10-, 12- and 22-week post-transplantation (Figure 4-1D), respectively. For the group of mice that would be reconstituted with T1DM PBMC and euthanized at 3-week post-reconstitution (n=3), normoglycemia was achieved at 14-, 16- and 17-week post-

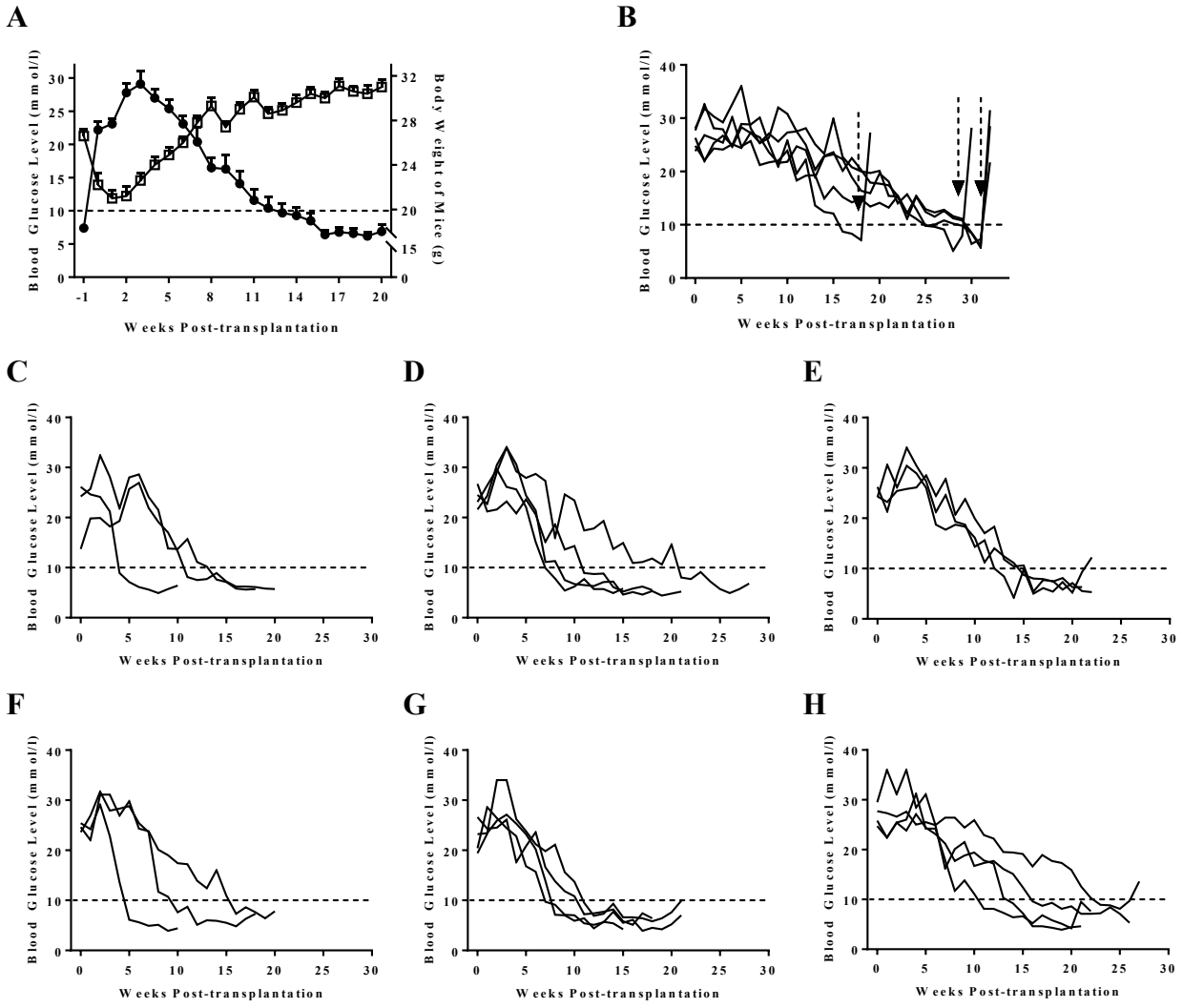

Figure 4-1. Blood glucose levels (BGL) of mice that would be reconstituted with human PBMC from individuals with (T1DM) or without (NT1DM) type 1 diabetes following the transplantation of neonatal pig islets. **A:** Average BGL (line with solid black dot symbols) and body weight (line with empty square box symbols) of mice after neonatal pig islet transplantation. **B:** BGL of mice without reconstitution. **C, D, E:** BGL of mice that would be euthanized at 1-, 2-, 3-week after reconstitution with T1DM human PBMC. **F, G, H:** BGL of mice that would be euthanized at 1-, 2-, 3-week after reconstitution with NT1DM human PBMC. The arrows facing down indicate the time when survival nephrectomy was performed to remove the kidney bearing the islet graft. Blood glucose levels below the dash line (10 mmol/l) represent normoglycemia and above the dash line represent hyperglycaemia.

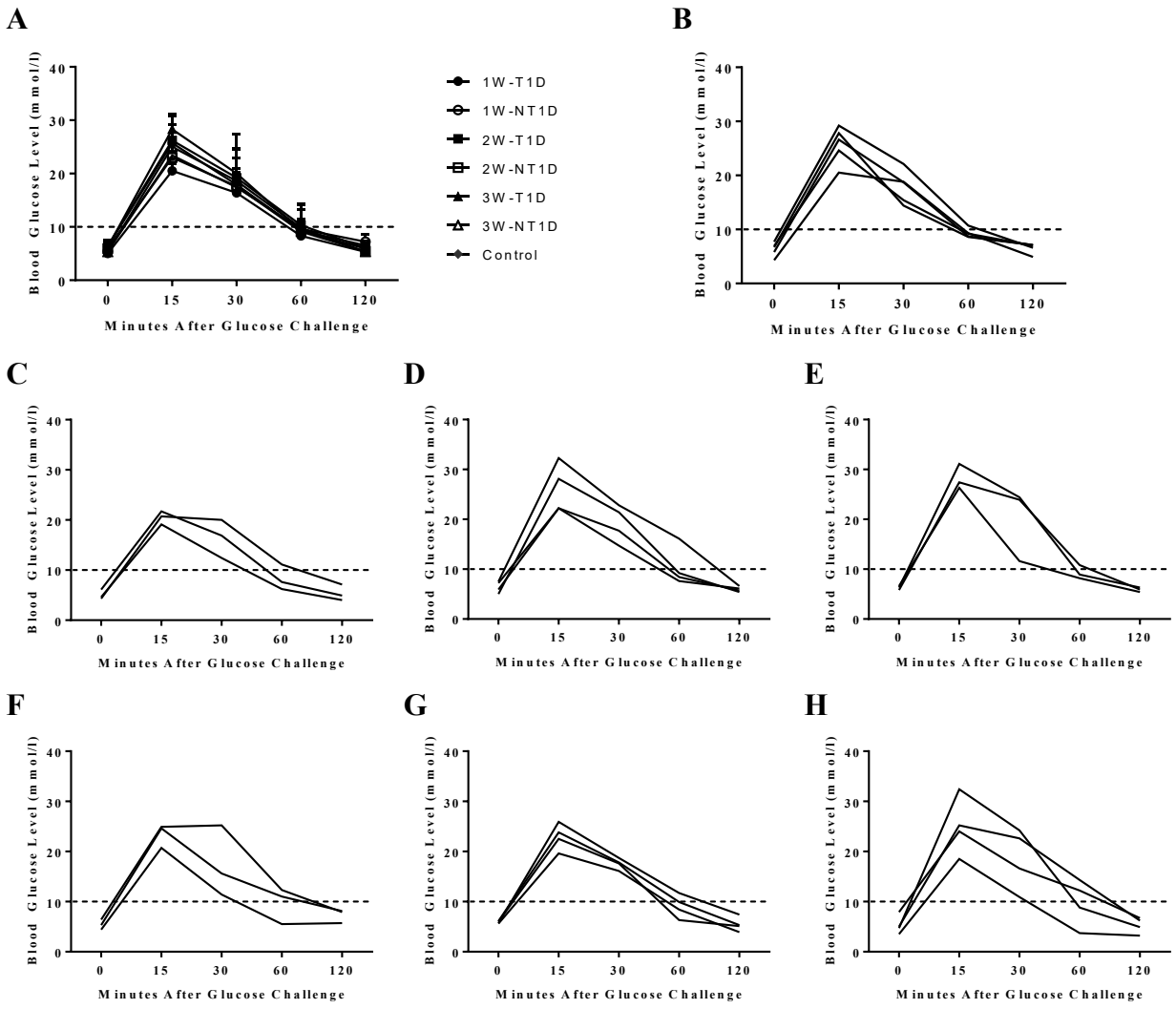

Figure 4-2. Intraperitoneal glucose tolerance test (IPGTT) data of mice that would be reconstituted with human PBMC from individuals with (T1DM) or without (NT1DM) type 1 diabetes following the transplantation of neonatal pig islets. **A:** Summary of IPGTT data of mice that received neonatal pig islet transplantation. **B:** IPGTT data of mice without reconstitution. **C, D, E:** IPGTT data of mice that would be euthanized at 1-, 2-, and 3-week after reconstitution with T1DM human PBMC. **F, G, H:** IPGTT data of mice that would be euthanized at 1-, 2-, and 3-week after reconstitution with NT1DM human PBMC. Blood glucose levels below the dash line (10 mmol/l) represent normoglycemia and above the dash line represent hyperglycaemia.

transplantation (Figure 4-1E), respectively. For the group of mice that would be reconstituted with NT1DM PBMC and euthanized at 1-week post-reconstitution (n=3), normoglycemia was achieved at 6-, 11- and 17-week post-transplantation (Figure 4-1F), respectively. For the group of mice that would be reconstituted with NT1DM PBMC and euthanized at 2-week post-reconstitution (n=4), normoglycemia was achieved at 8-, 9-, 12- and 12-week post-transplantation (Figure 4-1G), respectively. For the group of mice that would be reconstituted with NT1DM PBMC and euthanized at 3-week post-reconstitution (n=4), normoglycemia was achieved at 12-, 15-, 17- and 24-week post-transplantation (Figure 4-1H), respectively.

In intraperitoneal glucose tolerance test, BGL of mice in all groups described above peaked at 15 minutes after glucose injection, and continuously decreased to normoglycemic level at 60 to 120 minutes (Figure 4-2B, 4-2C, 4-2D, 4-2E, 4-2F, 4-2G and 4-2H), indicating that these mice had good response to glucose challenge. However, there was no significant difference between all the groups (Figure 4-2A). The BGL data post-transplantation and glucose tolerance test data together showed the therapeutic effect of neonatal pig islets in reversing hyperglycaemia in NSG mice.

In NSG mice that achieved normoglycemia post-transplantation, blood glucose level did not significantly increase at 1- to 3-week post reconstitution with PBMC from diabetic or non-diabetic individuals. At 1-week post-reconstitution with T1DM PBMC (Figure 4-3A) or NT1DM PBMC (Figure 4-3D), BGL remained below 10.0 mmol/l in all the mice. When survival nephrectomy was performed, mice in both groups became hyperglycaemic (Figure 4-3A, 4-3D). Similarly, the BGL of mice at 2-week post-reconstitution with T1DM PBMC remained below 10.0 mmol/l (Figure 4-3B). However, a slight increase of BGL (to 9.9 mmol/l) was observed in one of four mice at this time point when NT1DM PBMC were injected into mouse recipient of NPI, while

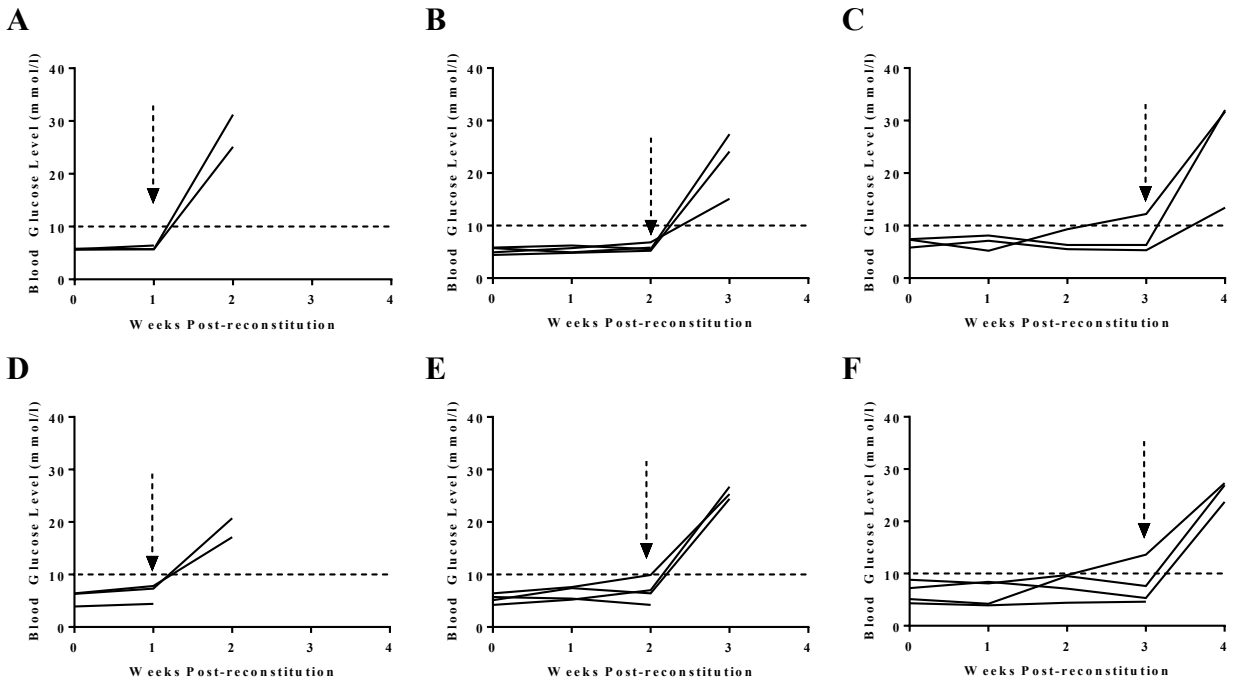

Figure 4-3. Blood glucose levels (BGL) of mice that were reconstituted with human PBMC from individuals with (T1DM) or without (NT1DM) type 1 diabetes following the transplantation of neonatal pig islets. **A, B, C:** BGL of mice at 1-, 2-, 3-week after reconstitution with T1DM human PBMC. **D, E, F:** BGL of mice at 1-, 2-, 3-week after reconstitution with NT1DM human PBMC. The arrows facing down indicate the time when survival nephrectomy was performed to remove the kidney bearing the islet graft. Blood glucose levels below the dash line (10 mmol/l) represent normoglycemia and above the dash line represent hyperglycaemia.

the other three mice remained normoglycemic (Figure 4-3E). BGL of these mice rapidly increased to more than 20 mmol/l after survival nephrectomy (Figure 4-3B, 4-3E). At 3-week post-reconstitution with T1DM PBMC (Figure 4-3C) or NT1DM PBMC (Figure 4-3F), increase in BGL was observed in 1 out of 3 mice (to 13.4 mmol/l) and 1 out of 4 mice (to 13.6 mmol/l), respectively. Though a slight increase in the BGL of 3 mice was also observed, the average blood glucose level of all the mice did not increase post-reconstitution at 1-, 2- or 3-week post-reconstitution.

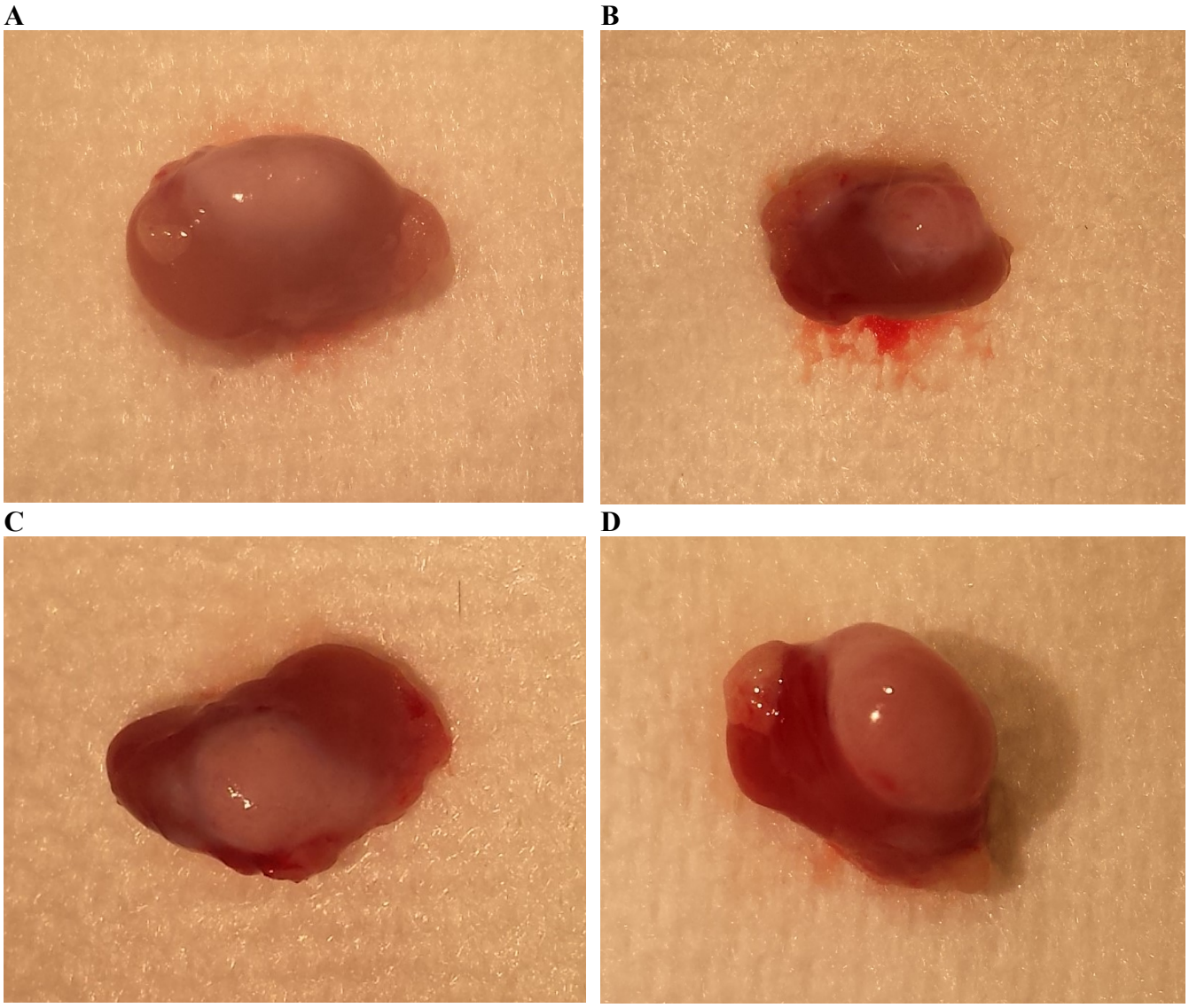

Figure 4-4. Representative islet xenograft morphology post-reconstitution. **A:** Islet xenografts from the mouse with no reconstitution; **B:** Islet xenograft from the mouse at 1-week post-reconstitution with human PBMC; **C:** Islet xenograft from the mouse at 2-week post-reconstitution with human PBMC; **D:** Islet xenograft from the mouse at 3-week post-reconstitution with human PBMC

Gross morphology, endocrine cell staining, and ultrastructure analysis suggested rejection of islet xenograft by injection of human immune cells in NSG mice. At 1-, 2- and 3-

week post-reconstitution, the kidney bearing islet xenografts were removed from the reconstituted mice. In the control mice that received no PBMC reconstitution, evenly distributed islet xenografts with smooth texture were seen under the kidney capsule, compared to the red kidney parenchymal (Figure 4-4A). However, in the mice at 1-week post-reconstitution with human PBMC from T1D or non-diabetic individuals, enlargement of the islet xenograft was seen under the kidney capsule, which was prominent to the adjacent kidney capsule (Figure 4-4B). The enlargement in islet xenografts became more obvious at 2-week post-reconstitution (Figure 4-4C). At 3-week post-reconstitution, islet xenografts were extremely enlarged and were hard when touched (Figure 4-4D). The gross morphology analysis showed the enlargement of islet xenografts from the mice that had been reconstituted with human PBMC as time progresses.

Massive insulin-positive cells were seen in islet xenografts at 1-week post-reconstitution with either T1DM PBMC (Figure 4-5A) or NT1DM PBMC (Figure 4-6A), while immune cell infiltration was also observed in both grafts. At 2-week post-reconstitution, insulin-positive cells became sporadic in the islet xenograft from the mice reconstituted with T1DM PBMC or NT1DM PBMC (Figure 4-5B, 4-6B). Immune cell infiltration was obviously seen in both grafts (Figure 4-5B, 4-6B). At 3-week post-reconstitution, immune cell infiltration in the islet xenografts became aggressive, and islet xenograft structure was destroyed under the kidney capsule with a few insulin-positive cells forming tubular structure (Figure 4-5C, 4-6C). Collectively, these results showed destruction of insulin-positive cells in islet xenografts from the mice receiving reconstitution with T1DM PBMC or NT1DM PBMC.

Glucagon staining revealed abundant sporadic positive cells at 1-week post-reconstitution with T1DM PBMC or NT1DM PBMC (Figure 4-5D, 4-6D). At 2-week post-reconstitution, glucagon-positive cells were hardly identified in the islet xenografts from one of the mice that had been

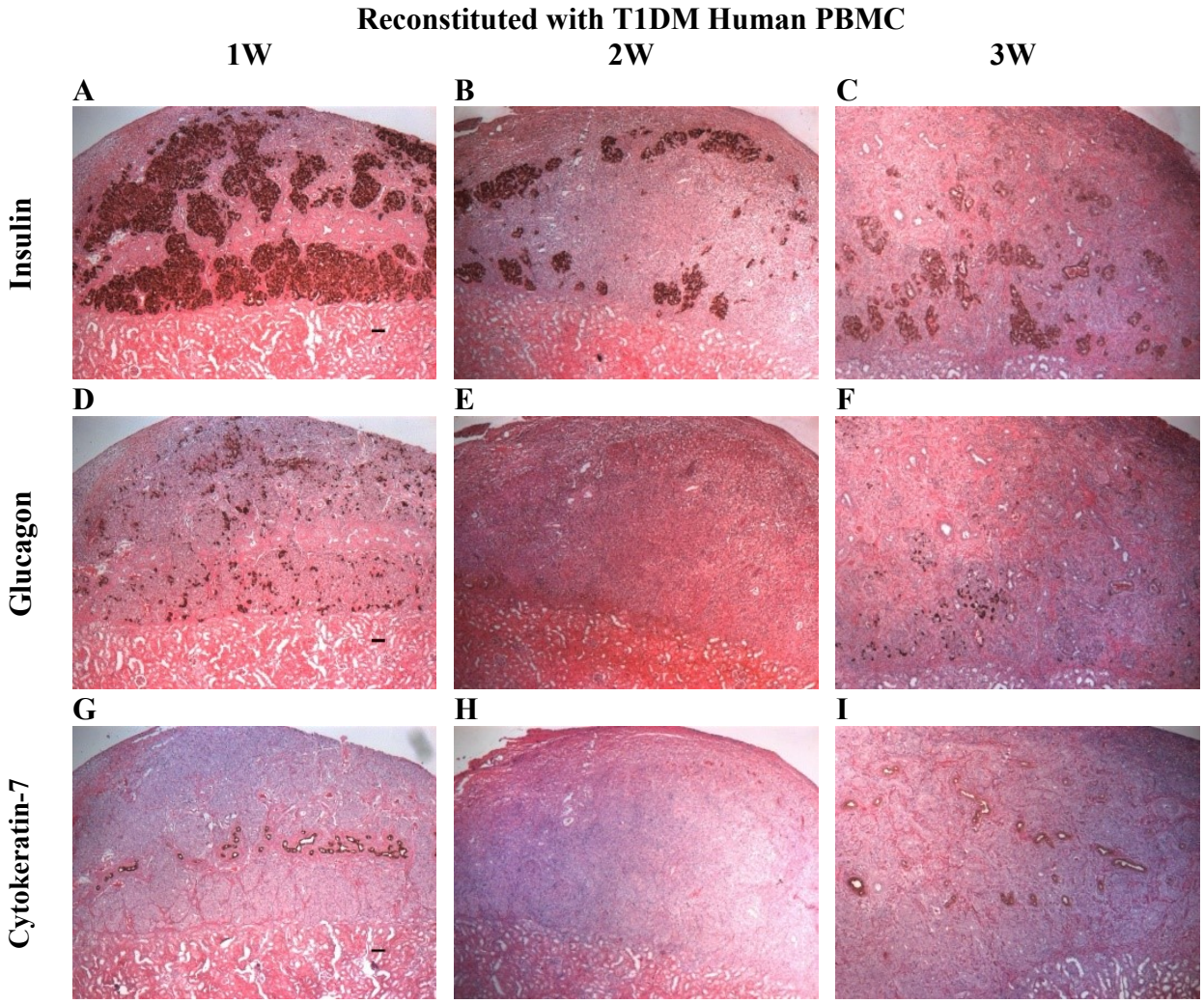

Figure 4-5. Representative images of insulin, glucagon and cytokeratin-7 (CK-7) stained islet xenograft from mice at 1-, 2- and 3-week post-reconstitution with human PBMC from individuals with T1DM. **A, B, C:** Insulin stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with T1DM human PBMC. **D, E, F:** Glucagon stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with T1DM human PBMC. **G, H, I:** CK-7 stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with T1DM human PBMC. Scale bar represents 100 µm.

reconstituted with T1DM PBMC (Figure 4-5E). This graft structure was damaged with massive immune cell infiltration (Figure 4-5E). Glucagon-positive cells were observed in xenografts from the mice reconstituted with NT1DM PBMC (Figure 4-6E). At 3-week post-reconstitution,

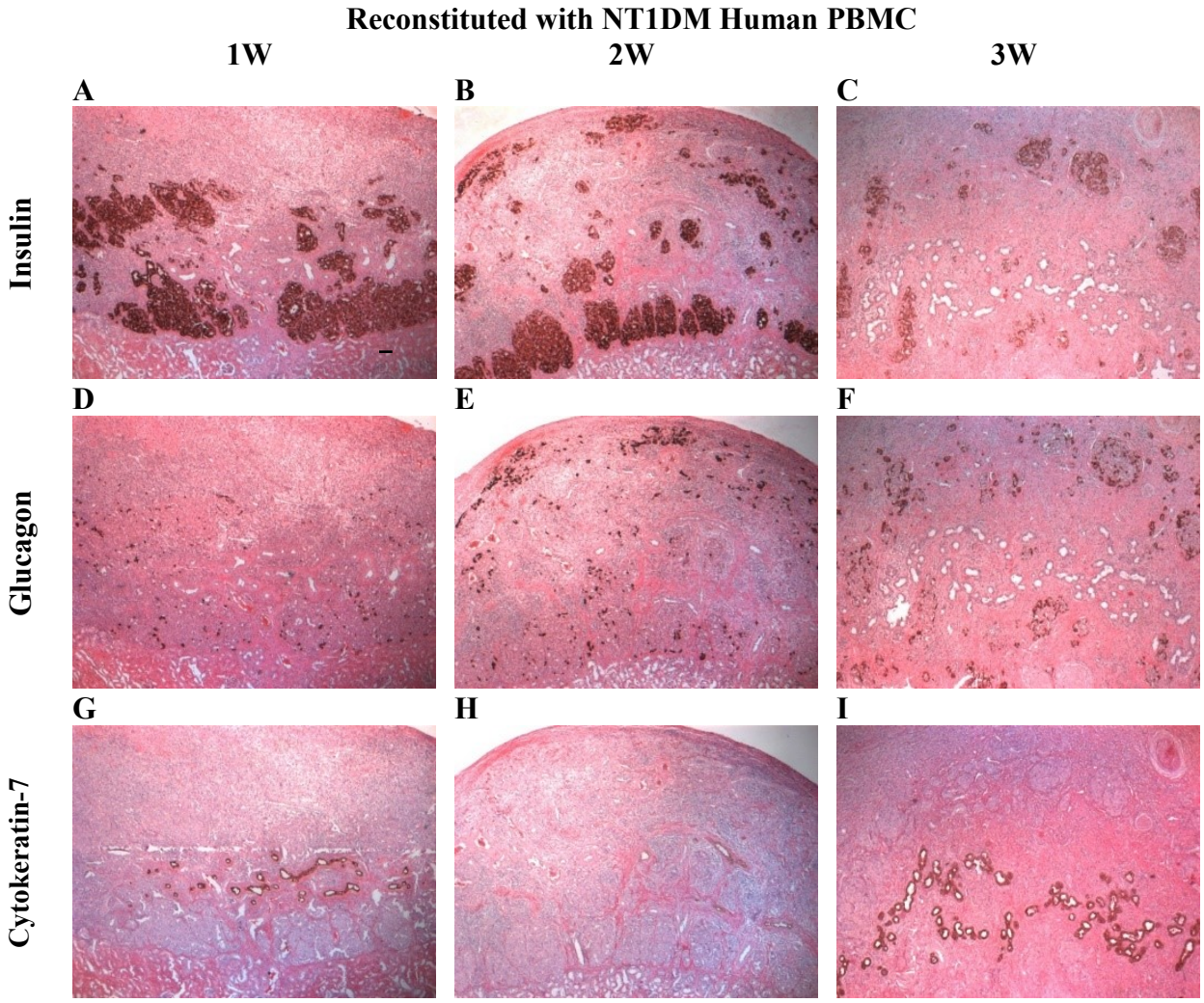

Figure 4-6. Representative images of insulin, glucagon and cytokeratin-7 (CK-7) stained islet xenograft from mice at 1-, 2- and 3-week post-reconstitution with human PBMC from non-diabetic individuals (NT1DM). **A, B, C:** Insulin stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with NT1DM human PBMC. **D, E, F:** Glucagon stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with NT1DM human PBMC. **G, H, I:** CK-7 stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with NT1DM human PBMC. Scale bar represents 100 μm.

glucagon-positive cells were identified in xenografts from the mice that had been reconstituted with T1DM PBMC (Figure 4-5F), which was less than that in the xenografts from the mice

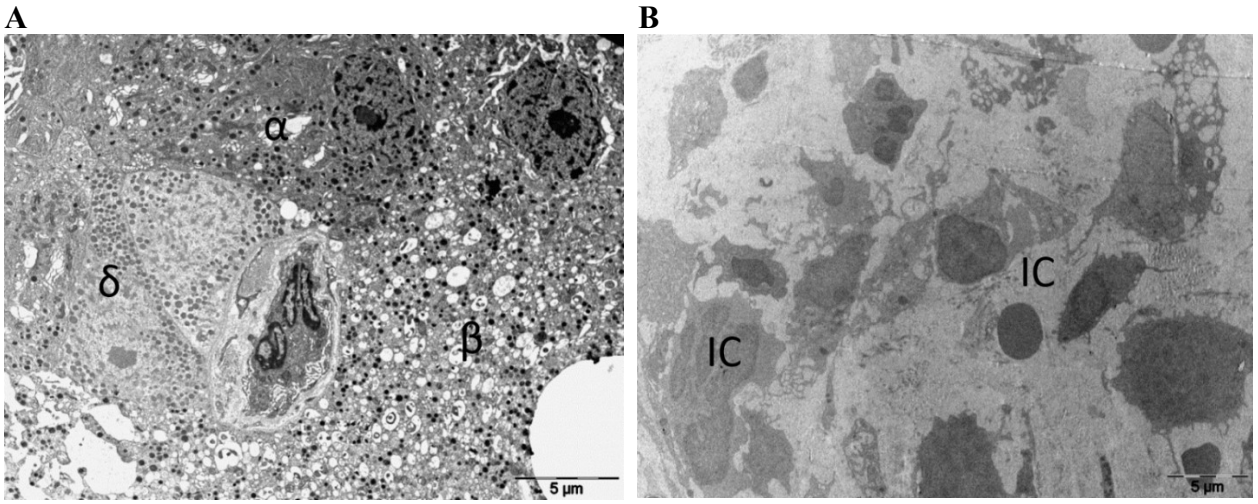

Figure 4-7. Representative transmission electron microscopy images of islet xenografts from the mice with or without reconstitution of human PBMC. **A:** Ultrastructure of an islet xenograft from a mouse that achieved normoglycemia after transplantation. **B:** Ultrastructure of islet xenografts from mouse at 3-week post-reconstitution with non-diabetic human PBMC. α: alpha cell; β: beta cell; δ: delta cell; IC: immune cell

reconstituted with NT1DM PBMC (Figure 4-6F).

Cytokeratin-7 (CK-7) is a marker for islet precursor cells. CK-7 positive cells, which appear around tubular structures, were identified in islet xenografts at 1-week post-reconstitution with T1DM PBMC (Figure 4-5G) or NT1DM PBMC (Figure 4-6G). However, CK-7 positive staining was hardly seen in the same graft (Figure 4-5H) that had no glucagon-positive cells at 2-week post-reconstitution with T1DM PBMC (Figure 4-5E). In the graft from the mice that received NT1DM PBMC injection, CK-7 positive cells were also identified at 2-week post-reconstitution (Figure 4-6H), but not as much as that in the grafts from the mice at 1-week post-reconstitution (Figure 4-5G, 4-6G). At 3-week post-reconstitution, CK-7 positive cells were seen in grafts from the mice receiving T1DM PBMC or NT1DM PBMC (Figure 4-5I, 4-6I).

Islet xenografts were also analyzed by transmission electron microscopy (TEM) to see the

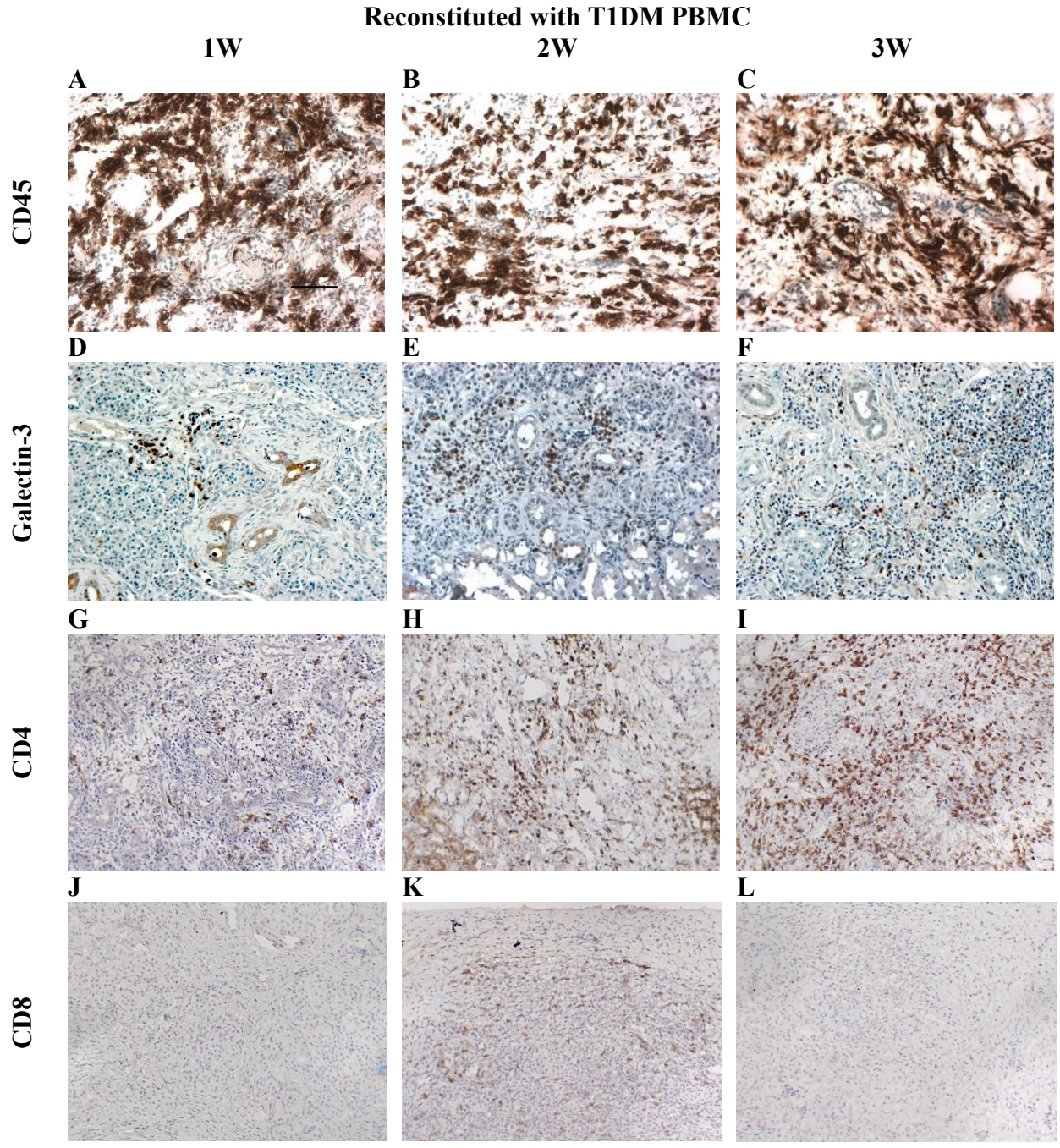

Figure 4-8. Representative images of human CD45, M2, CD4 and CD8a stained islet xenografts from mice at 1-, 2- and 3-week post-reconstitution with human PBMC from individuals with type 1 diabetes mellitus (T1DM). **A, B, C:** Human CD45 stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with T1DM PBMC. **D, E, F:** Galectin-3 stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with T1DM PBMC. **G, H, I:** Human CD4 stained islet

xenograft from mice at 1-, 2-, 3-week post-reconstitution with T1DM PBMC. **J, K, L:** Human CD8a stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with T1DM PBMC. Scale bar represents 100 μm.

microstructure. Endocrine cells such α cell or β cell containing granules in the cytoplasm were identified (Figure 4-7A) in islet xenograft from the mice that did not receive human PBMC. These granules contain insulin, glucagon or somatostatin. However, endocrine cells with granules were not seen in islet xenografts from mice at 3 weeks post-reconstitution with non-diabetic human PBMC (Figure 3-7B). Instead, immune cells such as macrophage and lymphocyte were present (Figure 4-7B).

Collectively, gross morphology, endocrine cell staining, and ultrastructure analysis together revealed the rejection of islet xenografts in NSG mice that had been reconstituted with human PBMC.

Macrophages, CD4 T cells and CD8 T cells may be involved in the rejection of islet xeongrafts in NSG mice. Human PBMC from T1DM or NT1DM individuals were injected into NSG mice via the tail vein. The presence of human immune cells in islet xenografts was indicated by the expression of CD45, also named leukocyte common marker. CD45-positive cells were identified in islet xenografts from the mice at 1-week post-reconstitution with T1DM PBMC (Figure 4-8A) or NT1DM PBMC (Figure 4-9A). The infiltration of human PBMC in islet xenografts indicated the success of the adoptive transfer. At 2-weeks post-reconstitution, human PBMC were also identified by CD45 staining in islet xenografts from mice reconstituted with T1DM PBMC or NT1DM PBMC (Figure 4-8B, 4-9B). At 3-weeks post-reconstitution, CD45-positive cells were observed in islet xenografts from mice reconstituted with T1DM PBMC or

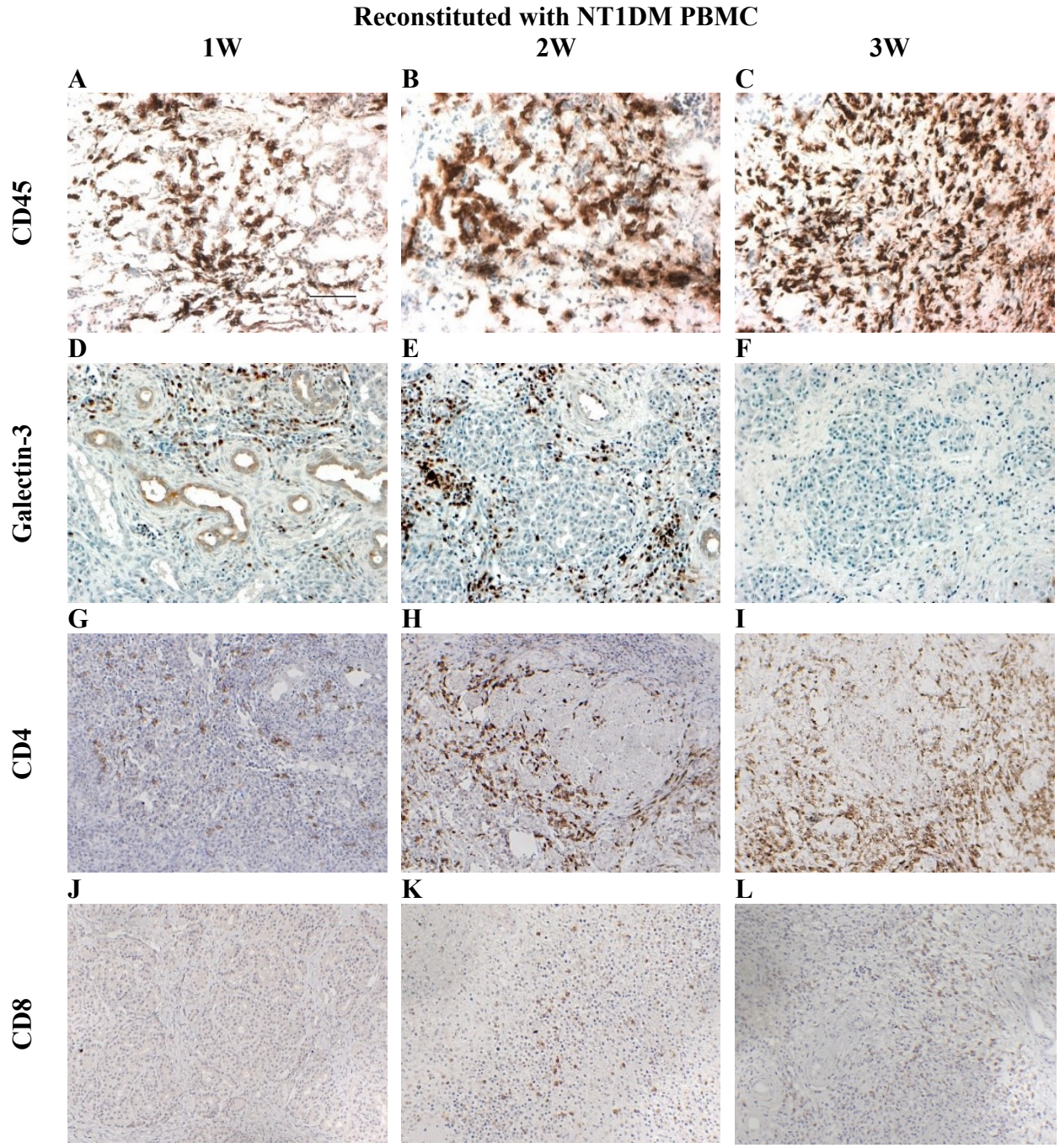

Figure 4-9. Representative images of human CD45, M2, CD4 and CD8a stained islet xenografts from mice at 1-, 2- and 3-week post-reconstitution with human PBMC from individuals without type 1 diabetes mellitus (NT1DM). **A, B, C:** Human CD45 stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with NT1DM PBMC. **D, E, F:** Galectin-3 stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with NT1DM PBMC. **G, H, I:** Human CD4 stained

islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with NT1DM PBMC. **J, K, L:** Human CD8a stained islet xenograft from mice at 1-, 2-, 3-week post-reconstitution with NT1DM PBMC. Scale bar represents 100 μm.

NT1DM PBMC (Figure 4-8C, 4-9C).

Anti-MAC-2 (Galectin-3) staining revealed the presence of activated macrophages in the islet xenografts from mice at 1-week post-reconstitution with T1DM PBMC (Figure 4-8D) or NT1DM PBMC (Figure 4-9D). These Galectin-3 positive cells were also present in the tubular structure within the graft. At 2- to 3-week post-reconstitution, activated macrophages were found to be surrounding the islet xenografts (Figure 4-8E, 4-8F, 4-9E, 4-9F).

Human CD4 T cells were also identified in the islet xenografts at 1-week post-reconstitution with T1DM PBMC (Figure 4-8G) or NT1DM PBMC (Figure 4-9G). Islet xenografts were surrounded and damaged as more CD4 T cells were recruited at 2-week post-reconstitution (Figure 4-8H, 4-9H). CD4 T cell infiltration became more apparent and destroyed islet xenografts, the margin of which were not clearly identified at 3 weeks post-cell reconstitution (Figure 4-8I, 4-9I).

At 1-week post reconstitution, human CD8 T cells were hardly present in islet xenografts (Figure 4-8J, 4-9J). However, CD8 T cell infiltration was observed in islet xenografts from mice at 2-week post-reconstitution with T1DM PBMC (Figure 4-8K) or NT1DM PBMC (Figure 4-9K). At 3-week post-reconstitution, CD8 T cells were also identified (Figure 4-8L, 4-9L), the number of which appeared to be less than that at 2 weeks post-reconstitution.

The tempo of rejection of neonatal pig islet xenografts varies as immune cells of different species were introduced into NSG mouse model. We next compared the rejection of NPI in NSG reconstituted with human PBMC or NOD splenocytes. In this experiment, all mice achieved

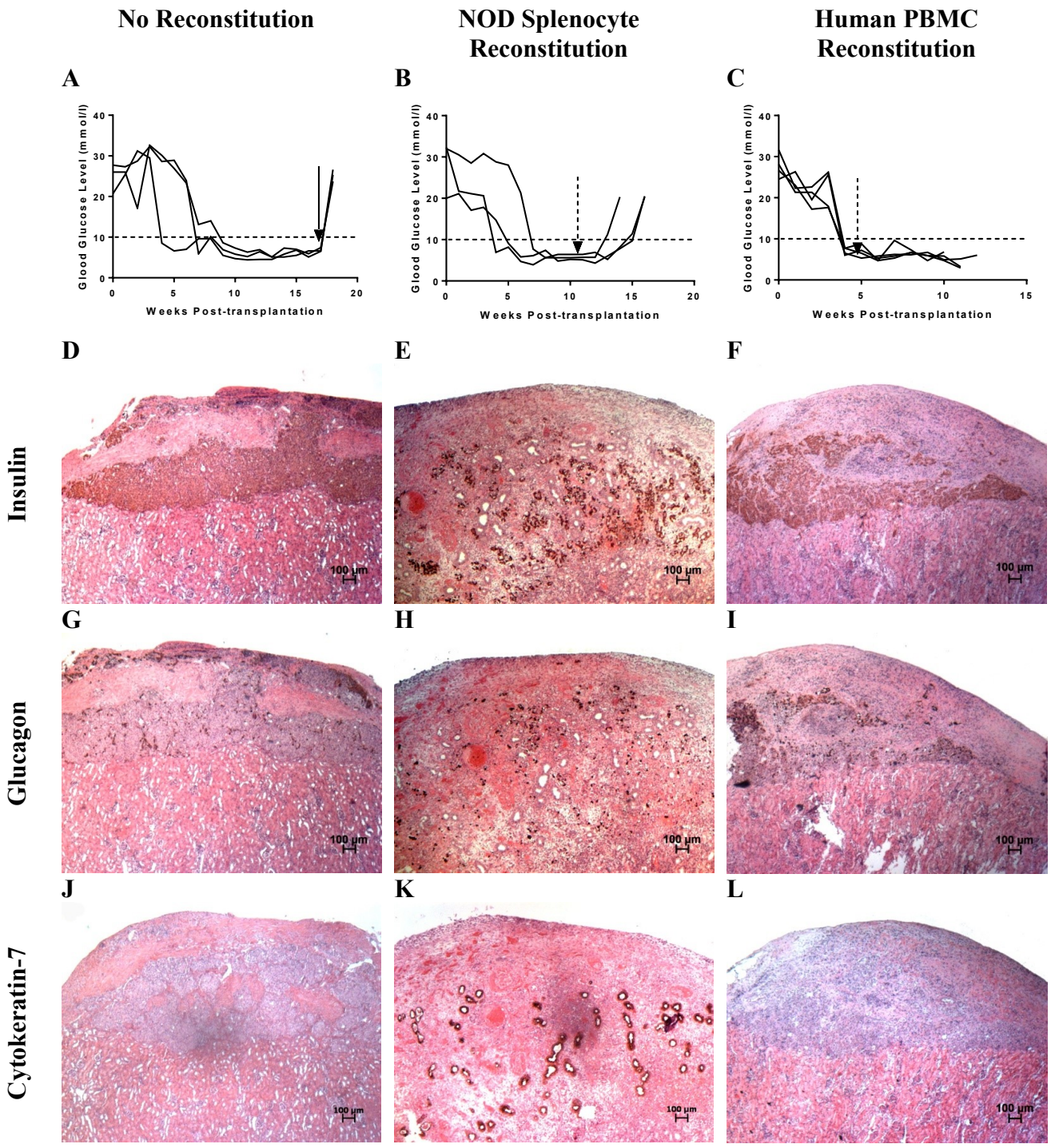

Figure 4-10. Comparison of NPI xenograft rejection in NSG mice reconstituted with human PBMC, or with non-obese diabetes (NOD) mouse splenocytes. **A, B, C:** Blood glucose level of mice that received neonatal pig islet transplantation followed by reconstitution with NOD splenocytes, human PBMC or that did not receive any immune cell injection. The arrow with black

solid line indicates the time when the kidney bearing islet xenografts in non-reconstituted mice was removed. The arrow with black dash line represents the time of reconstitution. **D, E, F:** Insulin stained in islet xenografts; **G, H, I:** Glucagon stained in islet xenografts; **J, K, L:** Cytokeratin-7 stained in islet xenografts. Scale bar represents 100 μm.

normoglycemia at 5-10 weeks post-transplantation (Figure 4-10A, 4-10B, 4-10C). For non-reconstituted mice, blood glucose levels remained normal until survival nephrectomy was performed and all mice became diabetic, indicating that the normoglycemic state in these mice was due to the transplanted islet xenografts (Figure 4-10A). All mice reconstituted with NOD splenocytes became diabetic at 2-3 weeks post-cell reconstitution (Figure 4-10B). However, none of the mice reconstituted with human PBMC became diabetic at 6 weeks post-cell reconstitution (Figure 4-10C). Immunohistochemistry revealed massive insulin-positive cells and numerous glucagon-positive cells in the islet grafts of non-reconstituted mice (Figure 4-10D, 4-10G). Few CK-7 positive cells were identified in these grafts (Figure 4-10J). Sporadic or no insulin- and glucagon-positive cells were observed in mice reconstituted with NOD splenocytes (Figure 4-10E, 4-10H). Immune cells were observed in the entire islet grafts while cytokeratin 7- (CK-7) positive cells were identified in one of the grafts (Figure 4-10K). The islet grafts from the mice reconstituted with human PBMCs contained insulin- and glucagon-positive cells, which was not as massive as that in the grafts of non-reconstituted mice (Figure 4-10F, 4-10I). Immune cell infiltration was seen while CK-7 positive cells were identified in two of the grafts (Figure 4-10L).

4.3 DISCUSSION

Islet transplantation is an attractive alternative treatment for patients with end stage type 1 diabetes mellitus. However, the further application of this treatment is limited by the shortage of human organ donors. To circumvent the shortage of organ donor, islets of other sources such as neonatal pig are being investigated for transplantation. NPI show encouraging results in reversing diabetes in rodent and non-human primate models. However, the potential immune rejection by human immune cells remains an obstacle for pig islet xenotransplantation and needs to be studied. Advancement in humanized rodent models such as humanized NSG mouse contributes to progress in research on how pig islets are recognized and rejected by human immune cells. For example, Lindeborg et al. demonstrated that adult pig islet cells activate human $CD4^+$ T cells through indirect pathway[270]. Very few studies focus on how NPI are rejected by human immune system.

In this study, we demonstrated that NPI reversed streptozotocin-induced hyperglycemia in NSG mice and showed stable function in response to glucose challenge. Adoptive transfer of human PBMC was performed to reconstruct humanized model in NSG mice. The anti-human CD45 staining indicated the presence of human immune cells in the xenograft. Contradictory to our expectation, blood glucose levels of reconstituted mice were hardly increased at 3 weeks post-reconstitution with T1DM or NT1DM PBMC. The underlying reasons for this phenomenon might be: 1) there may exist compensatory mechanism in the neonatal islet xenografts in response to immune damage, for example, there may be new islet cells transitioned from islet precursor cells; 2) though human immune cells were present in islet xenografts, there may be miscommunication of human immune cells in recognizing and rejecting NPI cells in NSG mice; and/or 3) the tempo of islet xenografts rejection in humanized NSG mice may require more time to occur.

To reveal the underlying mechanism, the immune rejection of NPI in humanized NSG mice

was characterized in different aspects: gross morphology of islet xenografts under kidney capsule, immunohistochemistry analysis of endocrine cells, and ultrastructure analysis by transmission electron microscopy. First, we observed the swelling of islet xenografts from the mice that had been reconstituted with human PBMC. This enlargement became more obvious as time increased, indicating the rejection of islet xenografts in humanized mice. Second, insulin-positive cells became less in the islet xenografts post-reconstitution while immune cell infiltration was observed within islet xenografts. CK-7 positive cells, served as islet precursor cells, continuously transformed into insulin-producing β cells. This may partially explain why reconstituted mice remained normoglycemic while islet xenografts were damaged by infiltrated immune cells. While evaluating the graft, we found no CK-7 positive cells in one of the grafts from the mice at 2 weeks post-reconstitution with PBMC from individuals with T1DM, whereas glucagon staining in the same graft was also found negative. Third, the rejection of islet xenografts in humanized NSG mice was also evidenced by transmission electron microscopy analysis. Different endocrine cells were identified in islet grafts from mice without reconstitution, which were not observed in islet grafts from mice reconstituted with human PBMC. Together, these findings indicated rejection of islet xenografts in humanized NSG mice.

We further evaluated the phenotypes of immune cells involved in the rejection of NPI in humanized NSG mice. In our previous *in vitro* studies, we demonstrated that neonatal pig islet cells induce human $CD4^+$, but not $CD8^+$ T cell proliferation[221]. Also, previous studies showed that cell-mediated xenograft rejection in the mouse depends on $CD4^+$ indirect response[271, 272]. In this study, we showed the infiltration of both human $CD4^+$ T cells, $CD8^+$ T cells and activated human macrophages in islet xenografts from the mice post-reconstitution with human T1D or NT1D PBMC. Rejection of grafts can be through either direct or indirect pathway. In our model,

direct recognition of antigen on pig islet xenografts by human $CD4^+$ T cells was less possible, as the low percentage of APC in NPI cell population. Another way that NPI would be rejected by human CD4+ T cells via the recognition of pig antigen present on human macrophages. However, the blood glucose levels of mice post-reconstitution with human PBMC remained low, indicating that there may exist miscommunication between NPI, antigen presenting cell and human effector T cells in this humanized model.

As documented, a limitation of humanized NSG model is that the engraftment of human immune cells is dominant with human $CD3^+$ T cells. This is in agreement with our finding that $CD4^+$ and $CD8^+$ T cells (which are also positive for CD3) were present in islet xenograft. Also, our results showed that other cells such as type 2 macrophages (M2 macrophages) were also involved in the rejection of NPI xenografts. M2 macrophages are being considered as benign opposites of classical macrophages and show anti-inflammatory property in wound healing and tissue repair[273, 274]. The presence of M2 macrophages in islet xenografts may have regulatory effects on NPI rejection, which may partially explain the slower tempo of rejection. Another limitation of this study is the development of graft-versus-host disease in the humanized NSG mice. Previous experiments in our lab showed that beyond this point reconstituted mice did not survive. Hence, we limited the tempo of observation to 3 weeks post-reconstitution. The graft-versus-host disease in humanized NSG model following human PBMC engraftment can be blocked by knockout of MHC class I and II expression with the retention of T-cell function[275]. This could be a future study.

We concluded from this study that rejection of NPI in NSG mice reconstituted with human PBMC was delayed compared to mice that were reconstituted with NOD splenocyte. There may exist a hierarchy in the rejection of NPI when reconstituted with immune cells of different species.

Chapter 5 THERAPEUTIC EFFECT OF GENETICALLY MODIFIED PIG ISLETS IN A MODEL OF ISLET TRANSPLANTATION

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5.1 INTRODUCTION

Islet transplantation is a more physiologic approach to prevent complications of type 1 diabetes compared to insulin injection because the damaged beta cells are replaced by new beta cells in the transplanted islets, which are able to efficiently sense the changes in blood glucose levels. The shortage of human organs and the continuous use of immunosuppressive drugs to prevent rejection of the islet transplant remain major limitations in the widespread application of islet transplantation to human patients with type 1 diabetes. Pigs are being considered an attractive alternative source of islets because they share similarity in morphology and physiology with humans. Since pigs have a relative short gestation period, they are an excellent source of abundant supply of islets. Pigs are also not exposed to compromising conditions such as co-morbidity, brain death, and cold ischemic injury as many deceased human donors. Pigs can be housed in pathogen-free facilities, which would allow for an on-demand source of islets with limited risk of pathogen transmission as well as they can be genetically modified to produce less immunogenic tissues [276]. An example would be *alpha-1,3-galactosyltransferase* gene knock-out (GalTKO) pigs, which lack the expression of xenoantigen galactose-α-1,3-Galactose (α-Gal) [131, 232]. The knock-out of *alpha-1,3-galactosyltransferase* gene can potentially reduce the hyperacute natural antibody mediated immune response against pig islets after transplantation in human patients. Finally, unlike human islets, pig islets do not develop amyloid at long-term post-transplantation [277].

Currently, islets are being transplanted into human patients through the portal vein, in which the islets first encounter the blood making them susceptible to damage induced by the instant blood mediated inflammatory reaction (IBMIR) [241]. IBMIR involves the activation of complement and coagulation cascades that result in blood clot formation and infiltration of leukocytes into the islets [242], which lead to the disruption of islet integrity. Thus, circumvention of IBMIR remains

one of the major areas of research in the field. Since IBMIR involves complement activation, the introduction of human complement regulatory genes in GalTKO pigs is an attractive approach to evade both αGal-mediated hyperacute rejection and IBMIR. CD46 also called membrane cofactor protein is a complement regulatory protein that can be expressed in pig islets to avoid their early destruction upon transplantation into the portal vein. Another attractive molecule that could help attenuate the damage induced by IBMIR on islets is CD39, also called ectonucleoside triphosphate diphosphohydrolase-1, which acts as an anti-coagulant [278, 279]. Expression of human CD39 (hCD39) has been demonstrated to be beneficial to islets since the clotting time of human blood when pre-incubated with islets that expressed CD39 was significantly prolonged, when compared to wild type (WT) pig islets [245]. In addition, GalTKO neonatal pig islets that expressed human CD46 with or without co-expression of human CD39 were found to display lower levels of soluble component Bb compared to WT pig islets when they were exposed to fresh human blood *in vitro* indicating that the activation of complement pathways were reduced [280]. The time to coagulation after exposure to human blood was found not to be significantly different between GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pig islets however; they were significantly reduced compared to the WT neonatal pig islets [280]. Collectively these results indicate that expression of human complement regulatory and anti-coagulant molecules on pig islets may reduce their susceptibility to damage induced by IBMIR.

As we contemplate on moving forward to the clinical application of pig islet xenotransplantation, it is important that the highest quality of islets be maintained when pigs are being genetically modified. In this study, we characterized the islets from GalTKO, GalTKO/hCD46, and GalTKO/hCD46/hCD39 neonatal pigs and compared them with isles from WT pigs. We hypothesized that genetic modification of pigs would not affect the cellular

composition and function of islets and that GalTKO, GalTKO/hCD46, and GalTKO/hCD46/hCD39 neonatal pig islets would equally be able to reverse hyperglycemia compared to WT pig islets after transplantation into diabetic mouse recipients.

5.2 RESULTS

Microarray analysis showed that less genes were up- or down-regulated in islets as more genetic modifications were introduced in pigs compared to WT pig islets. The gene expression in islets from GalTKO, GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pigs were compared with islets from WT neonatal pigs, using the Affymetrix® Expression Console™ Software and Transcriptome Analysis Console (TAC) Software, with a cutting point of $p < 0.05$, fold ration (FR) >2. The changes in gene expression were visualized using volcano plots for GalTKO (Figure 5-1A), GalTKO/hCD46 (Figure 5-1B) and GalTKO/hCD46/hCD39 (Figure 5-1C) pig islets. After ruling out the uncharacterized loci, 215 genes were found to be up-regulated, and 161 genes were down-regulated in GalTKO neonatal pig islets (Figure 5-1D). Sixty-two genes were up-regulated, and 117 genes were down-regulated in GalTKO/hCD46 neonatal pig islets. Thirty-seven genes were up-regulated, and 25 genes were down-regulated in GalTKO/hCD46/hCD39 neonatal pig islets (Figure 5-1D).

The top 30 genes that had been altered in GalTKO, GalTKO/hCD46 or GalTKO/hCD46/hCD39 pig islets were then compared (Figure 5-2). As expected, *GGTA1* (glycoprotein, alpha-galactosyltransferase 1), which is responsible for the expression of α1,3 Gal epitopes on cell surface of almost all mammals but humans, apes and Old-World monkeys [131, 281], was down-regulated in GMP islets, confirming the knockout of α-Gal. *CAND1* (Cullin-Associated Neddylation-Dissociated Protein 1) was down-regulated in GalTKO and GalTKO/hCD46 pig islets. This gene encodes an essential regulator of Cullin-RING ubiquitin ligases, which are involved in ubiquitinylation of proteins degraded by the Ub proteasome system. Knockdown of this gene in pre-adipocytes has been shown to result in blockade of adipogenesis [282]. *ECI2* (enoyl-CoA delta isomerase 2), which encodes for a member of the

hydratase/isomerase superfamily is down-

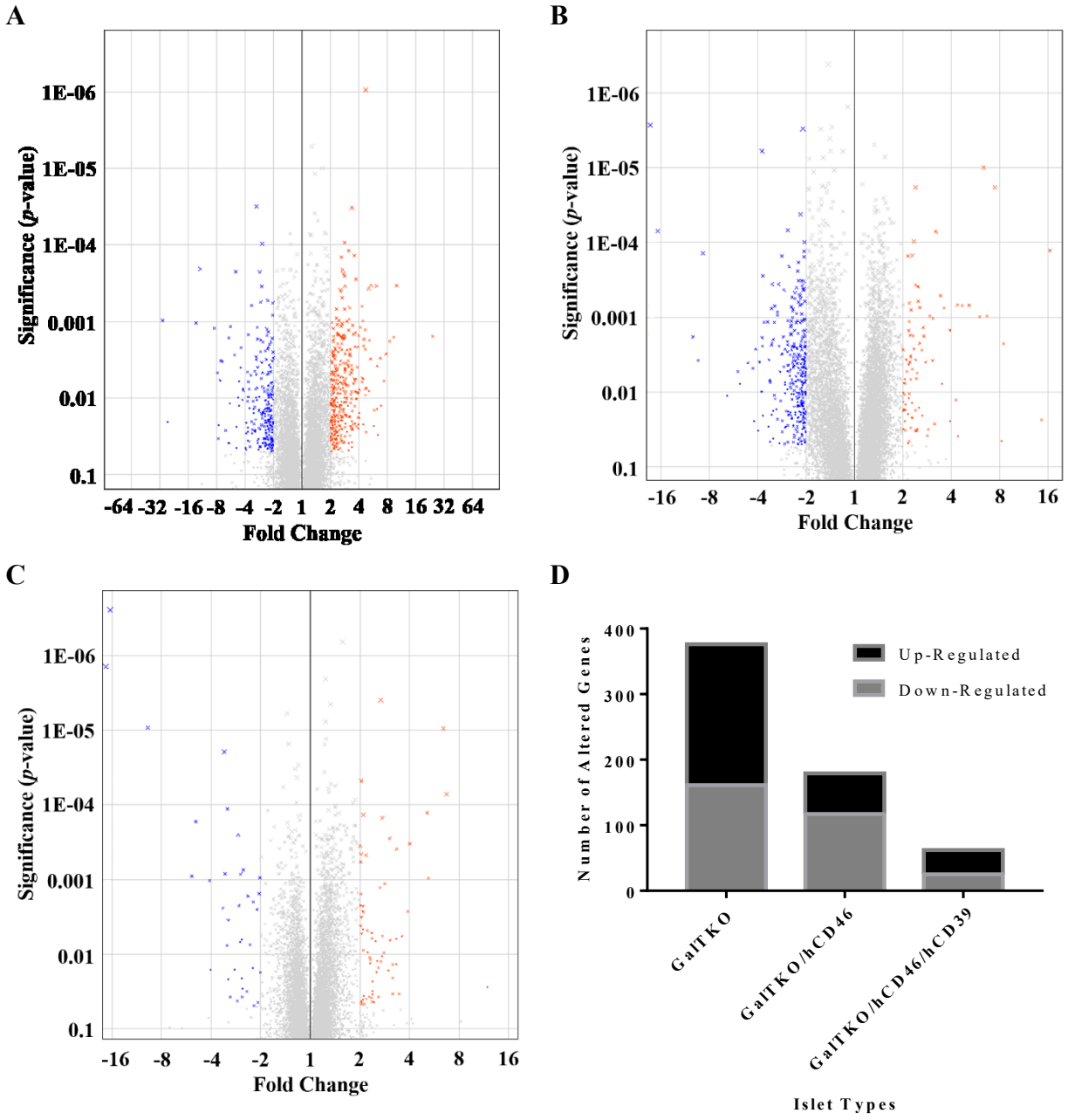

Figure 5-1. Microarray analysis of islets from GalTKO, GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pigs compared to islets from WT neonatal pigs. **A:** Volcano plots for the up- or down-regulated genes in GalTKO pig islets. **B:** Volcano plots for the up- or down-regulated genes in GalTKO/hCD46 pig islets. **C:** Volcano plots for the up- or down-regulated genes in GalTKO/hCD46/hCD39 pig islets. Red x represents up-regulated genes and blue x represents

down-regulated genes. **D:** A comparison of the number of the up- and down-regulated genes in GalTKO, GalTKO/hCD46 and GalTKO/hCD46/hCD39 pig islets compared to WT pig islets.

regulated in GalTKO/hCD46 and GalTKO/hCD46/hCD39 pig islets. The protein encoded by this gene is a key mitochondrial enzyme involved in beta-oxidation of unsaturated fatty acids. The enzyme catalyzes the transformation of 3-cis and 3-trans-enoyl-CoA esters arising during the stepwise degradation of cis-, mono-, and polyunsaturated fatty acids to the 2-trans-enoyl-CoA intermediates [283]. In addition, *ATP6V1C2* (ATPase, H^+ transporting, lysosomal 42kDa, V1 subunit C2), which encodes a component of vacuolar ATPase (V-ATPase); a multi-subunit enzyme that mediates acidification of eukaryotic intracellular organelles was down-regulated in GalTKO/hCD46 and GalTKO/hCD46/hCD39 pig islets (Figure 2). V-ATPase dependent organelle acidification is necessary for intracellular processes as protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation [284].

The common up-regulated genes in GMP islets compared to WT pig islets include *FOS* (FBJ murine osteosarcoma viral oncogene homolog), *EGR1* (Early Growth Receptor 1) and *ZFP36* (Zinc Finger Protein 36). *FOS*, which encodes leucine zipper proteins that can dimerize with proteins of the JUN family [285], was upregulated in all three GMP islets. FOS proteins have been found to be involved in cell proliferation, differentiation, and transformation [286]. *EGR1* encodes for EGR1 protein, a transcriptional regulator that mediates the response to growth factors, DNA damage [287], and ischemia [288]. EGR1 also plays a role in the regulation of cell survival, proliferation and cell death [289]. *ZFP36* gene encodes for Tristetraprolin (TTP), also known as ZFP36 homolog that binds to AU-rich elements (AREs) in the 3'-untranslated regions (UTRs) of the mRNAs of some cytokines and promotes their degradation [290].

Several solute carrier genes were also found to be up-regulated in GMP islets compared to

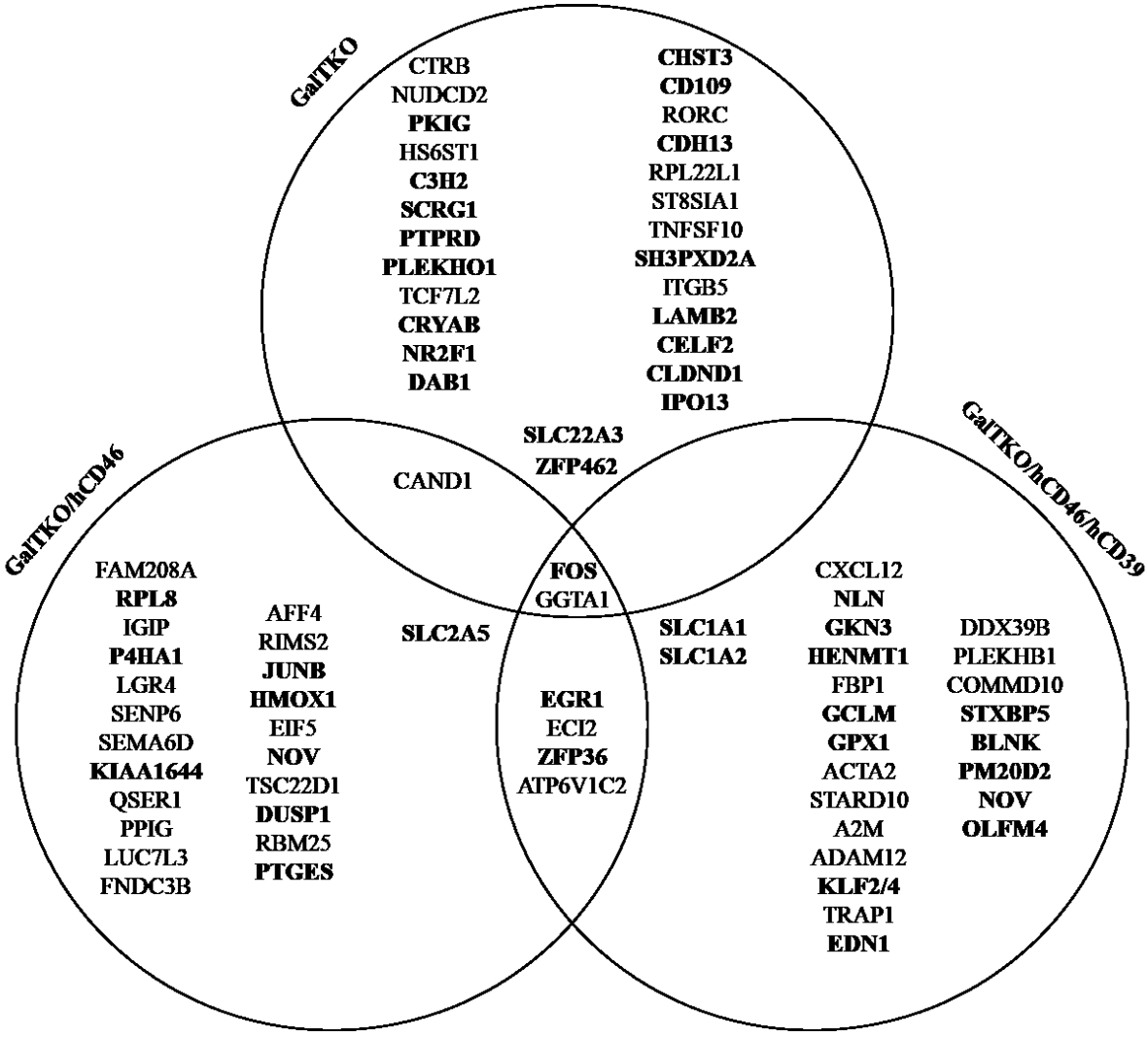

Figure 5-2. Top 30 genes that are up- or down-regulated in islets from GalTKO, GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pigs compared to WT neonatal pig islets. Up-regulated genes are in bold font, while down-regulated genes are in regular font.

WT pig islets. Such genes include *SLC22A3* (solute carrier family 22 member 3) which encodes SLC22A3, also known as OCT-3 (organic cation transporter 3) or EMT (extraneuronal monoamine transporter), was up-regulated in GalTKO pig islets. SLC22A3 is critical for the elimination of many endogenous small organic cations as well as a wide array of drugs and environmental toxins

[291, 292]. *SLC2A5*, which encodes GLUT-5 that is responsible for fructose uptake in small intestine [293], was up-regulated in GalTKO/hCD46 pig islets. *SLC1A1* and *SLC1A2*, which encode for SLC1A1, also known as excitatory amino-acid transporter 3 (EAAT3) and SLC1A2 also known as EAAT2 and glutamate transporter 1 (GLT-1), were both up-regulated in GalTKO/hCD46/hCD39 pig islets. EAAT3 is a member of the high-affinity glutamate transporters, which play an essential role in transporting glutamate across plasma membranes in neurons [294].

The functional analysis of all the up- or down- regulated genes in GMP islets was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8. Various KEGG pathways were identified (Table 1). In GalTKO pig islets, 25 and 21 genes involved in cancer and PI3K-Akt signalling pathways, respectively were among the most altered and significantly ($p<0.01$) different pathways detected compared to WT pig islets. Genes involved in focal adhesion, extracellular matrix (ECM)-receptor interaction, small cell lung cancer pathway, toxoplasmosis, and peroxisome proliferator-activated receptors (PPAR) signalling pathway were also among the genes that were significantly different in GalTKO pig islets compared to WT pig islets. In GalTKO/hCD46 pig islets, there were a number of pathways where the levels of gene expression were different compared to WT pig islets, but they were not found to be statistically significant. Pathways in cancer, regulation of actin cytoskeleton, human T-Lymphotropic Viruss Type 1 (HTLV-1) infection, focal adhesion, central carbon metabolism in cancer and hypoxia inducible factor 1 (HIF-1) were among the pathways that involved most of the altered genes in GalTKO/hCD46 islets. In GalTKO/hCD46/hCD39 pig islets, tumour necrosis factor (TNF) signalling pathway and salmonella infection pathway both involving 6 genes were found to be significantly ($p<0.05$ and $p<0.01$, respectively) different when compared to WT pig islets. A number of altered genes involved in chemokine signalling pathway, legionellosis, rheumatoid

arthritis and nuclear factor-kappa B signalling pathway were also identified to be statistically different from those detected in WT pig islets ($p<0.05$, Table 5-1).

Table 5-1. KEGG pathways that have been altered in islets from GalTKO, GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pigs compared to islets from WT pigs.

KEGG Pathways	Gene Count	Benjamini Corrected *p*-value
GalTKO		
Pathways in cancer	25	0.004
PI3K-Akt signalling pathway	21	0.006
Focal adhesion	15	0.008
ECM-receptor interaction	11	0.003
Small cell lung cancer	11	0.003
Toxoplasmosis	11	0.008
PPAR signalling pathway	9	0.009
GalTKO/hCD46		
Pathways in cancer	10	0.390
Regulation of actin cytoskeleton	8	0.230
HTLV-1 infection	8	0.380
Focal adhesion	7	0.420
Central carbon metabolism in cancer	6	0.120
HIF-1 signalling pathway	6	0.210
GalTKO/hCD46/hCD39		
TNF signalling pathway	6	0.015
Salmonella infection	6	0.008
Chemokine signalling pathway	5	0.041
Legionellosis	5	0.012
Rheumatoid arthritis	5	0.030
NF-kappa B signalling pathway	5	0.028

GMP islets showed therapeutic effect on STZ-induced diabetic NSG mice. All NSG mice that received a single intraperitoneal injection of STZ became diabetic at 3 to 6 days after injection. Recipients of WT pig islets (n=5, Figure 5-3A) achieved normal blood glucose levels at 5 to 7

weeks post-transplantation. Mice that received GalTKO pig islets (n=4) showed a slight delay (9 to 16 weeks post-transplantation) in achieving normal blood glucose levels compared to mice that received WT pig islets (Figure 5-3B). Eleven out of 15 mice that were transplanted with GalTKO/hCD46 pig islets achieved normal blood glucose levels at 7 to 19 weeks post-transplantation (Figure 5-3C). Three mice in this group remained diabetic until the end of the study while one mouse was found dead before achieving normoglycemia. However, the blood glucose levels of these mice appeared to decrease with time post-transplantation. Two of the six mice that received islets from GalTKO/hCD46/hCD39 neonatal pigs reached normal blood glucose levels at 9 and 14 weeks after transplantation (Figure 5-3D). One of these mice was found dead at 28 weeks post-transplantation. Another mouse showed continuous decrease in blood glucose level with time post-transplantation and had 15.6 mmol/l blood glucose level at the end of the study (>100 days post-transplantation). The remaining three mice had blood glucose levels >20 mmol/l throughout the study period (Figure 5-3D).

In terms of the average time when mice in each group achieved normal blood glucose levels, we observed a delay in the reversal of diabetes in recipients of GalTKO (12 weeks post-transplantation), GalTKO/hCD46 (22 weeks post-transplantation), and GalTKO/hCD46/hCD39 (>25 weeks post-transplantation) pig islets compared to recipients of WT (6 weeks post-transplantation) pig islets (Figure 5-4A). There were no significant differences in the average time when mice achieved normal blood glucose levels and the average blood glucose levels between GalTKO and WT pig islet recipients. The average blood glucose levels of the mice that received GalTKO/hCD46 or GalTKO/hCD46/hCD39 islets were significantly higher compared to the average blood glucose levels of the mice transplanted with WT pig islets after 7 weeks post-transplantation ($p<0.05$ and $p<0.01$, respectively, Figure 5-4A). The average blood glucose levels

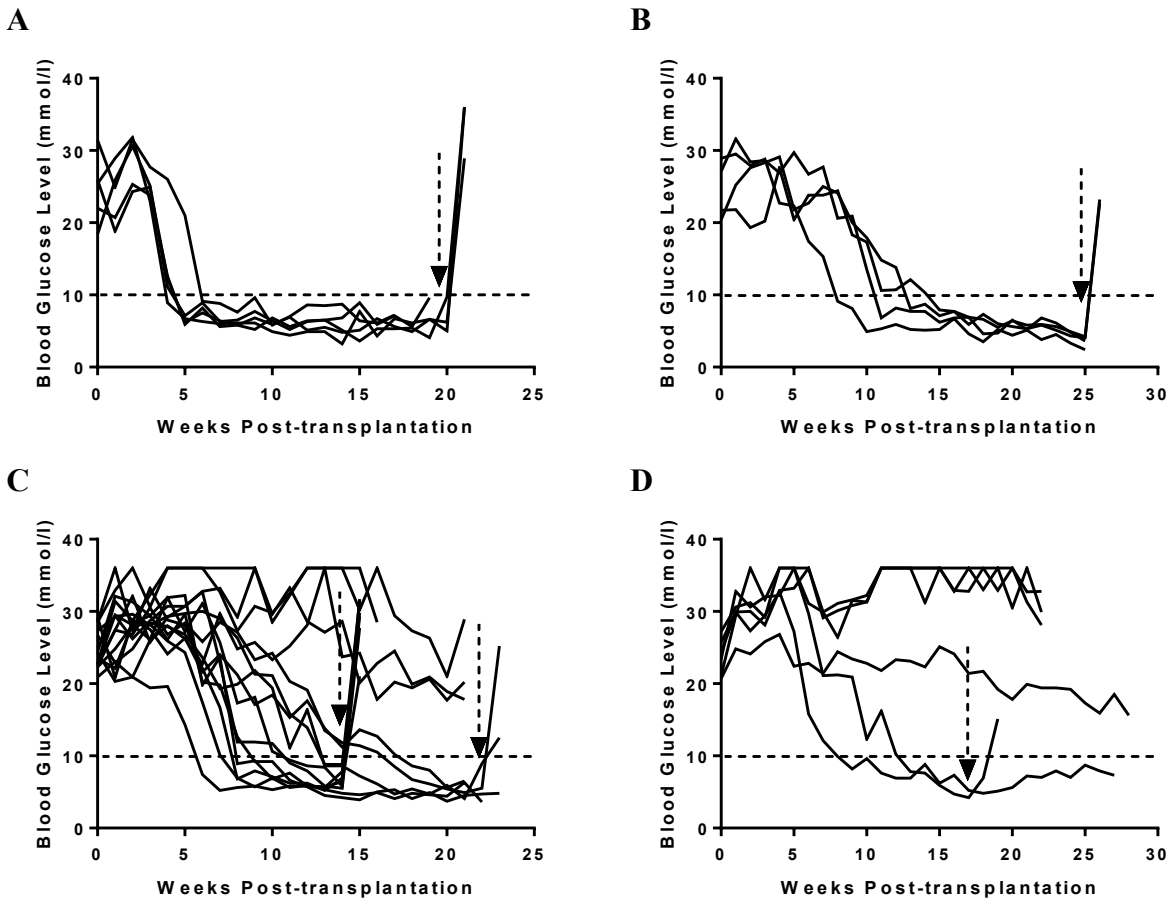

Figure 5-3. Blood glucose levels of the mice that received neonatal pig islets. **A:** WT (n=5), **B:** GalTKO (n=4), **C:** GalTKO/hCD46 (n=15) and **D:** GalTKO/hCD46/hCD39 (n=6) pig islets. The arrows facing down indicate the time when survival nephrectomy was performed to remove the kidney bearing the islet graft. Blood glucose levels below the dash line (10 mmol/l) represent normoglycemia and above the dash line represent hyperglycaemia.

of GalTKO/hCD46/hCD39 pig islet recipients were significantly ($p<0.01$) higher than those of GalTKO islet recipients. In addition, the percentage of mice that remained diabetic until the end of the study in recipients of WT and GalTKO islets was 0%, 24% in recipients of GalTKO/hCD46, and significantly ($p<0.01$) higher (~68%) in recipients of GalTKO/hCD46/hCD39 pig islets (Figure 5-4B).

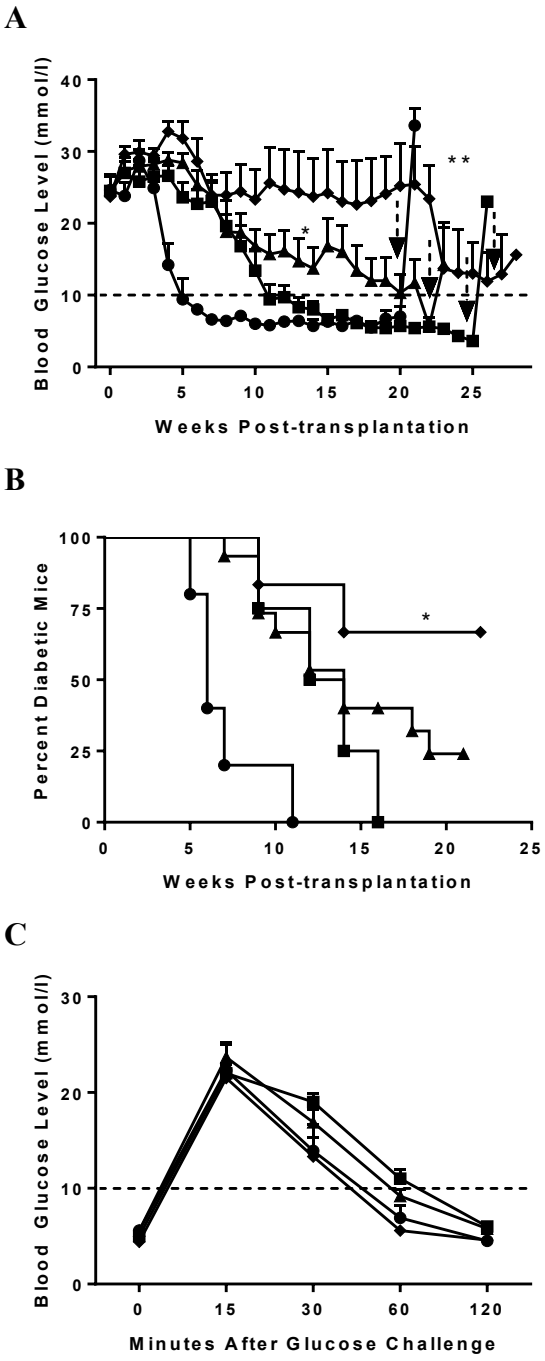

Figure 5-4. Metabolic follow up of mice transplanted with WT or GMP islets. **A:** Average blood glucose levels of NSG mouse recipients of GMP or WT pig islets. Black circle, square, triangle and diamond symbols represent blood glucose levels of mice that received WT (n=5), GalTKO (n=4), GalTKO/hCD46 (n=15) and GalTKO/hCD46/hCD39 (n=6) pig islets, respectively. The arrows facing down indicate the time when survival nephrectomy was performed to remove the

kidney bearing the islet graft. Blood glucose levels below the dash line (10 mmol/l) represent normoglycemia and above the dash line represent hyperglycaemia. **B:** Proportions of the mice that remained hyperglycemic after transplantation. **C:** Results of intraperitoneal glucose tolerance test of the mice that achieved normoglycemia after transplantation with GalTKO (square), GalTKO/hCD46 (triangle) or GalTKO/hCD46/hCD39 (diamond) pig islets. Asterisk indicates p value less than 0.05. Double asterisks indicate p value less than 0.01.

The IPGTT results showed that mouse recipients of GMP or WT pig islets that maintained normal blood glucose levels after transplantation had good response to glucose challenge (Figure 5-4C). For the two mice that received GalTKO/hCD46/hCD39 neonatal pig islets and maintained normal blood glucose levels, their response peaked at 15 minutes (20.5 and 22.4 mmol/l) after the intraperitoneal injection of glucose, then the glucose levels continued to decline to the initial state at 120 minutes post-glucose challenge (Figure 5-4C). The mice that received WT, GalTKO, or GalTKO/hCD46 pig islets showed similar pattern as what was observed in recipients of GalTKO/hCD46/hCD39 (Figure 5-4C). The blood glucose levels of these mice reached maximum at 15 minutes after glucose injection and varied from 15.2 to 29.6 mmol/l (Mean±SEM = 22.9 ± 1.1 mmol/l) but were not significantly different at all-time points of measurement. Collectively these results indicate stable function of the islet transplant in all mice with normal blood glucose levels irrespective of the type of islets. At the end of the study, these mice became diabetic within 2 days after survival nephrectomy (Figure 5-3A to 5-3D), indicating that the islet transplant maintained normal blood glucose levels.

Variance in endocrine cell mass was observed in GMP islet xenografts. We characterized by immunohistochemistry the presence of insulin-, glucagon- and CK-7-positive cells in the islet grafts. All mouse recipients of WT neonatal pig islets had massive amounts of insulin-positive cells

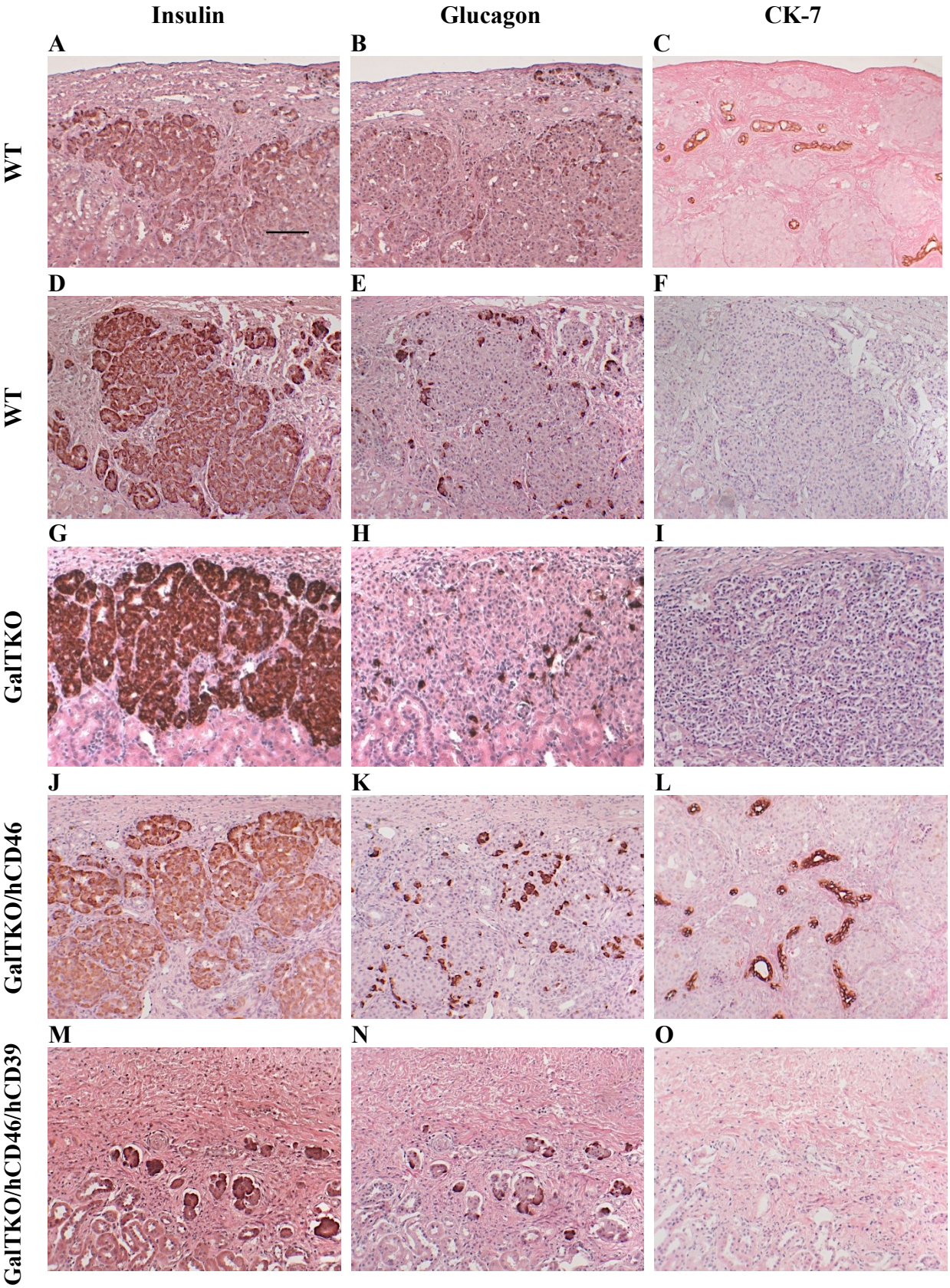
Insulin
Glucagon
CK-7
A
B
C
WT
D
E
F
WT
G
H
I
GalTKO
J
K
L
GalTKO/hCD46
M
N
O
GalTKO/hCD46/hCD39

Figure 5-5. *In vivo* characterization of WT and GMP neonatal pig islet grafts from the mice that achieved normoglycemia post-transplantation. **A, B, C:** representative images of WT pig islet graft stained for insulin, glucagon and CK-7. **D, E, F:** representative images of WT islet graft stained for insulin, glucagon, and CK-7, the latter was not present in this graft. **G, H, I:** representative images of insulin, glucagon and CK-7 stained GalTKO islet graft. CK-7 positive cells were absent in all four islet grafts. **J, K, L:** representative images of insulin, glucagon and CK-7 stained GalTKO/hCD46 islet graft. **M, N, O:** images of insulin, glucagon and CK-7^{-} stained GalTKO/hCD46/hCD39 islet graft from the mouse that had decreased blood glucose level post-transplantation. Scale bar represents 100μm.

in their islet grafts (Figure 5-5A, 5-5D), which were surrounded by sporadic glucagon-positive cells (Figure 5-5B, 5-5E). In addition, CK-7-positive cells (brown tubular structure) were identified in two of the five islet grafts (Figure 5-5C); while the other three islet grafts did not contain CK7-positive cells (Figure 5-5F). The islet grafts from GalTKO mouse recipients showed similar results compared to the WT islet grafts (Figure 5-5G, 5-5H) but none of the grafts from all four recipients showed CK-7 positive stained cells (Figure 5-5I). GalTKO/hCD46 neonatal pig islet grafts also showed massive amounts of insulin- and glucagon-positive cells (Figure 5-5J, 5-5K). CK-7 positive cells were identified in seven islet grafts (Figure 5-5L). Insulin- and glucagon-positive stained cells were also observed in the islet grafts of GalTKO/hCD46/hCD39 mouse recipients (Figure 5-5M, 5-5N), which appeared to be significantly less compared to WT and the other two GMP islet grafts. In addition, CK-7 positive cells were not detected in any of these grafts (Figure 5-5O).

Islet grafts from three mice that received GalTKO/hCD46 and remained hyperglycemic at the end of the study contained variable amounts of insulin- and glucagon-positive cells. Islet graft from one mouse contained massive amount of insulin (Figure 5-6A) but no glucagon-positive cells

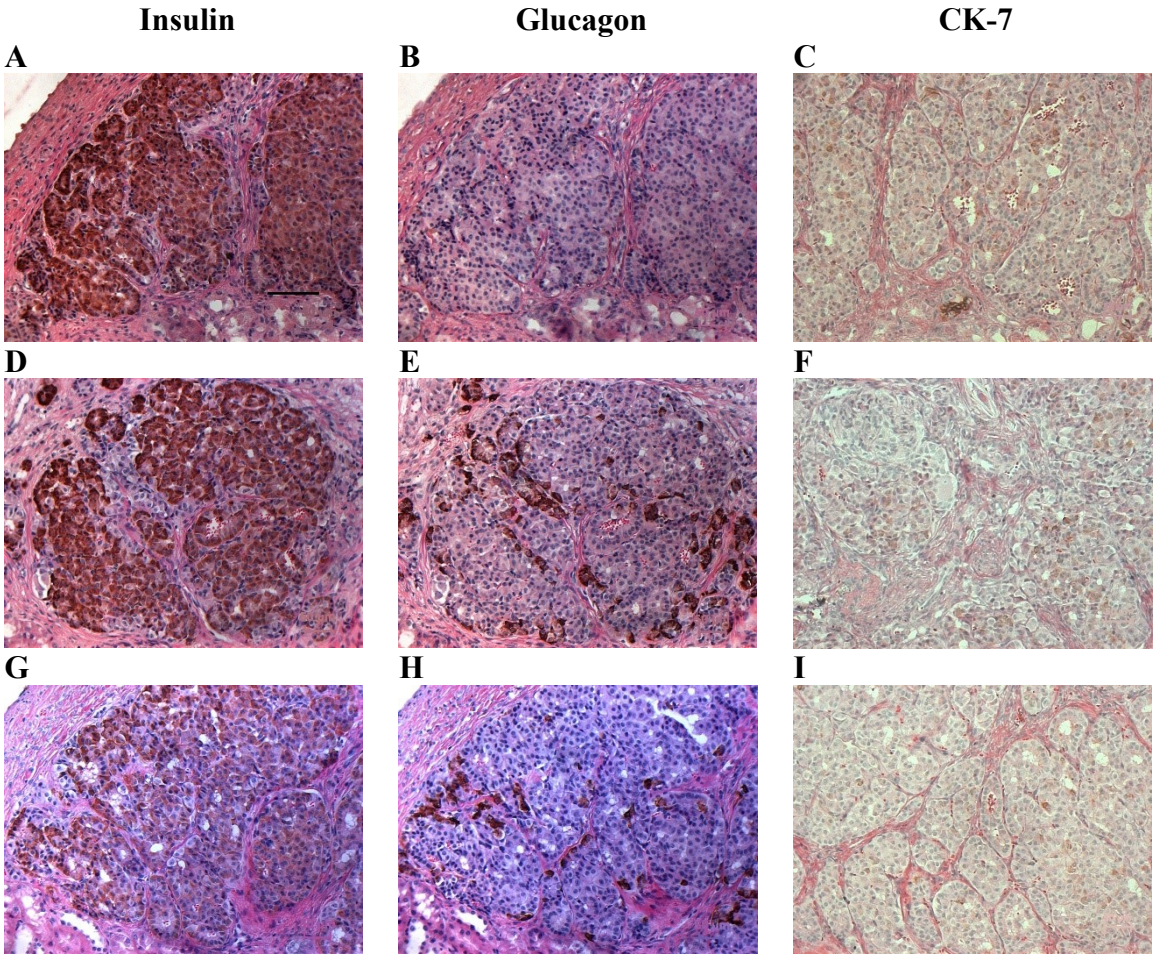

Figure 5-6. *In vivo* characterization of GalTKO/hCD46 neonatal pig islet grafts from the mice that did not achieve normoglycemia post-transplantation. **A, D, G:** images of insulin stained islet grafts from three mice. **B, E, H:** images of glucagon stained islet grafts from three mice. **C, F, I:** images of CK-7 stained islet grafts. Scale bar represents 100µm.

(Figure 5-6B) detected in this graft. The other islet graft contained massive amount of insulin (Figure 5-6D) and glucagon-positive cells (Figure 5-6E) while the remaining islet graft contained less insulin- and glucagon-positive cells (Figure 6G, 6H). These grafts contained very little (Figure 5-6C) to none CK-7 positive cells (Figure 5-6F, 5-6I).

5.3 DISCUSSION

In this study, we characterized the islets from 5- to 7-day-old GalTKO, GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pigs and compared the results to islets from WT pigs. Analysis of Affymetrix Porcine Gene Expression Assay showed that *FOS* and *GGT1* were the most common genes that were altered in GMP islets compared to WT pig islets. *FOS* was up-regulated and *GGT1* gene was down-regulated in the three GMP islets. Since islets from neonatal pigs undergo remodelling during development, it is possible that up-regulation of *FOS* gene in GMP islets may be an indication of naturally occurring cell death that aids in tissue remodelling [295], which appeared to be higher in GMP islets compared to WT pig islets at 7 days of culture. It is conceivable based on the ability of the GMP islets to reverse hyperglycemia in mice post-transplantation that GMP islets may be a bit slower in their development compared to WT pig islets. In addition, *EGR1* gene was up-regulated in both GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pig islets. EGR1 protein regulates the transcription of numerous target genes, and thereby plays an important role in regulating the response to growth factors, DNA damage, and ischemia (i.e., cell survival, proliferation and cell death) [296-298]. It is possible that GalTKO/hCD46 and GalTKO/hCD46/hCD39 pig islets will be more susceptible to damage induced by the pancreas procurement, shipment and/or islet isolation procedure. EGRI mediates responses to ischemia and hypoxia; regulates the expression of proteins such as IL-1β and CXCL2 that are involved in inflammatory processes and development of tissue damage after ischemia [297]. As a counter regulatory effect, we also observed up-regulation of *ZFP36* gene in GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pig islets compared to WT pig islets at 7 days of culture. ZFP36 protein destabilizes several cytoplasmic AU-rich element (ARE)-containing mRNA transcripts by promoting their poly(A) tail removal or deadenylation, and hence provide a mechanism for

attenuating protein synthesis and plays a role in anti-inflammatory responses [299-302]. ZFP36 also promotes ARE-mediated mRNA decay of hypoxia-inducible factor HIF1A mRNA during the response of endothelial cells to hypoxia [303].

As expected, the *GGT1* gene was down-regulated in GMP islets compared to WT pig islets and this was confirmed by the absence of αGal in GMP islets but not in WT pig islets (Figure S1). We also observed that *CAND1* gene was downregulated in both GalTKO and GalTKO/hCD46 neonatal pig islets at 7 days of culture. It is possible that downregulation of this gene may be an indication that differentiation of islet cells during the *in vitro* culture may be slower in these two types of GMP islets compared to WT pig islets reflecting a slight delay in their function post-transplantation. *ECI2* gene was also downregulated in both GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pig islets at 7 days of culture. ECI2 is a key mitochondrial enzyme involved in beta-oxidation of unsaturated fatty acids. The utilization of fatty acids as an energy source is characteristic of nearly all free-living organisms. The series of enzyme-catalyzed reactions required to degrade fatty acids is evolutionarily conserved and is accomplished primarily through beta-oxidation pathway [304]. *ATP6V1C2* gene, which was previously reported to be present in human placenta and kidney but not in the pancreas [305] was detected in pig islets and was found to be down-regulated in both GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pig islets. This gene encodes for a multi-subunit vacuolar-type proton pump that is essential for acidification of diverse intracellular compartments in all eukaryotic cells [304, 306, 307].

In terms of the KEGG pathways, GalTKO neonatal pig islets showed seven KEGG pathways that have been altered; among them were pathways in cancer, PI3K-Akt signalling, focal adhesion, ECM-receptor interaction, small cell lung cancer pathway, toxoplasmosis and PPAR signalling pathway. In GalTKO islets, the top 3 altered genes in the cancer pathway were *FOS*,

LAMB2 and *LAMA4*. The potential role of *FOS* had been previously discussed above. *LAMB2* and *LAMA4* encode laminin subunit beta 2 and laminin subunit alpha 4, respectively. Laminins, as extracellular matrix glycoproteins, are the major non-collagenous constituents of basement membranes. The alteration in laminins may result in the interruption of cell adhesion, differentiation, migration, and signalling [308]. In addition to *LAMB2* and *LAMA4*, *ITGB5* was among the top 3 altered genes involved PI3K-Akt signalling pathway, focal adhesion and ECM-receptor interaction. *ITGB5* encodes for integrin beta-5, transmembrane receptor involved in vascular endothelial growth factor signalling [309], programmed cell death [310] and cell migration [311]. *LAMC1* was also identified as the top 3 genes that had been altered in small cell lung cancer pathway and toxoplasmosis together with *LAMB2* and *LAMA4*. *LAMC1* encodes for another type of laminin, the laminin subunit gamma-1, which is another type of extracellular matrix glycoprotein forming the basement membrane. *SORBS1*, *PCK2* and *ACOX2* are among the top 3 altered genes involved in the PPAR signalling pathway. *SORBS1* encodes for Sorbin and SH3 domain-containing protein 1 (also named CAP/Ponsin protein), which is an adaptor protein that regulate cell adhesion, growth factor signalling and cytoskeletal formation. There is evidence showing that the overexpression of this protein may disrupt cell-matrix contact morphology [312]. CAP/Ponsin may also enable insulin-induced phosphorylation and affect glucose uptake [313]. *PCK2* encodes for phosphoenolpyruvate carboxykinase 2, mitochondrial (PCK2, or PEPCK-M). As a critical enzyme of gluconeogenesis, PCK2 converts oxaloacetate to phosphoenolpyruvate in the presence of guanosine triphosphate [314]. Through the recycling of guanosine triphosphate in pancreatic beta cells, PCK2 regulates glucose stimulated insulin secretion [315]. Hence, down-regulation of *PCK2* in pig islets may affect glucose metabolism. *ACOX2* encodes for acyl-CoA oxidase 2, which is a key protein in fatty acid metabolism and bile acid synthesis [316]. Taken

together, most of the altered genes in GalTKO islets were involved in cell proliferation, extracellular matrix formation and energy metabolism. Similar changes in cancer pathways and cell adhesion were also observed in GalTKO/hCD46 islets, however, these KEGG pathways were not significantly different compared to that observed in WT pig islets.

In GalTKO/hCD46/hCD39 pig islets, the pathways that had been altered included TNF signalling pathway, salmonella infection pathway, chemokine signalling pathway, legionellosis signalling, rheumatoid arthritis pathway and NF-kappa B signalling pathway (Table 1). *FOS*, *EDN1* and *PTGS2* were the top 3 altered genes involved in TNF signalling pathway. *EDN1* encodes endothelin 1 (ET-1), which is a potent vasoconstrictor produced by endothelial cells. Abnormal expression of *EDN1* may trigger tumorigenesis (provided by RefSeq 2015). *PTGS2* encodes for prostaglandin-endoperoxide synthase 2 (also known as cyclooxygenase-2, COX-2), which converts arachidonic acid to prostaglandin H2. The up-regulation of PTGS2 may indicate an inflammatory state. *FOS* together with *CXCL8* and *CXCL2* were the top 3 altered genes in salmonella infection signalling in GalTKO/hCD46/hCD39 islets. CXCL8 and CXCL2 encode for IL-8 and macrophage inflammatory protein 2-alpha, respectively, both of which are chemoattractant for neutrophils [317]. *CXCL8* and *CXCL2* were also among the top 3 altered genes in chemokine signalling pathway and legionellosis pathway. *CXCL12* was the top altered gene in chemokine signalling pathway, rheumatoid arthritis signalling and NF-kappa B signalling pathway. *CXCL12* encodes for stromal cell-derived factor 1, which is a strong chemoattractant for lymphocytes. There is also evidence showing that CXCL12 signalling together with CXCL7 signalling contribute to the metastasis of pancreatic cancer [318]. Together, genes involved in the altered KEGG pathways in GalTKO/hCD46/hCD39 islets indicate a pro-inflammatory condition in these islets.

We also evaluated the function of islets cultured for 7 days after transplantation into diabetic NSG mice. We observed a delay in the reversal of hyperglycemia in recipients of GMP islets compared to mouse recipients of WT pig islets. In this study, the islets were not isolated immediately after pancreas procurement, which may have affected our results. Pancreases were transported in University of Wisconsin solution at 1.7 to 4.4 °C and islet isolation was performed at approximately 24 hours after pancreas procurement. It is conceivable that this delay in islet isolation may have altered islet function. To eliminate the potential bias, the shipping process was mocked in WT pig pancreases and we think that difference in the ability of GMP and WT pig islets to reverse hyperglycemia, may be due to other factors such as cellular composition. For example, between the islets from GalTKO/hCD46 neonatal pigs, further analysis revealed that the three mice transplanted with these islets and remained diabetic throughout the study period received islets from one pig (pig #1). The other six mice that maintained normoglycemia throughout the study period received islets from the other pig (pig #2). Islets from these pigs showed similar pattern in insulin, glucagon and CK-7 expression prior to transplantation as examined by immunostaining (Figure 5-7). However, when the number of insulin- and glucagon-positive cells in islets from these pigs were compared prior to transplantation (Table 5-2), we found that there were more insulin- and glucagon-positive cells (Mean insulin$^+$ or glucagon$^+$/10,000 μm^2 ± SEM; 6.3±1.6, n=5, and 6.9±2.6, n=6, respectively) in pig #2 islets compared to pig #1 islets. Islets from pig #1 contained 5.0±3.2 insulin$^+$ cells/10,000 μm^2 and 4.9±3.1 glucagon$^+$ cells/10,000 μm^2. All islet grafts from pig #2 contained abundant insulin-positive cells (Figure 5J) with sporadic glucagon-positive cells (Figure 5K) and CK-7 positive cells (Figure 5L). While islet grafts from pig #1 contained variable amounts of insulin- (Figure 6A) and glucagon-positive cells (Figure 6B) with very little or no CK-7 positive cells detected in these grafts (Figure 6C). It is probable that other endocrine cells may

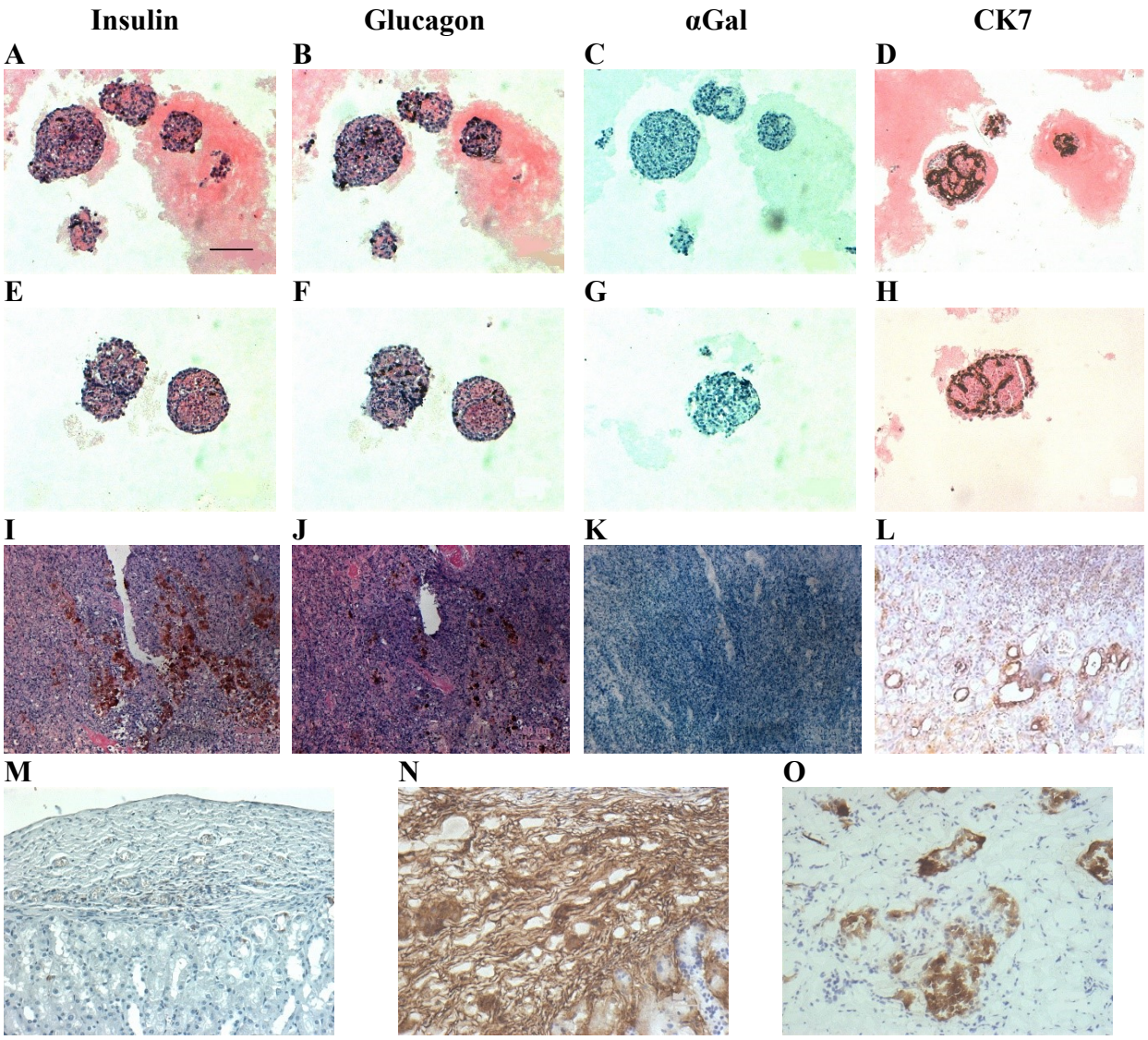

Figure 5-7. Insulin, glucagon and CK-7 positive cells were present in islets from GalTKO/hCD46 and GalTKO/hCD46/hCD39 but αGal was absent in these islets. **A, B, C, D:** Islets from GalTKO/hCD46 pig #1 at 7 days of culture prior to transplantation. **E, F, G, H:** Islets from GalTKO/hCD46 pig #2 at 7 days of culture prior to transplantation. Mice that received GalTKO/hCD46 islets from pig #1 remained hyperglycemic throughout the study period and mice that received islets from GalTKO/hCD46 pig #2 maintained normal blood glucose levels for >100 days post-transplantation. **I, J, K, L:** Islet graft from a mouse that received GalTKO/hCD46/hCD39 pig islets that achieved normal blood glucose levels but the skin at the transplant site was infected. Insulin, glucagon and CK-7 positive cells were present in the graft but αGal positive cells were not present. Mononuclear cells were also present in the graft due to

infection. **M, N, O:** GalTKO/hCD46/hCD39 islet graft from a mouse that remained diabetic throughout the study period. Human CD46 and human CD39 were present in the graft. Scale bar represents 100μm.

also differ in these grafts, but this remains to be confirmed in future investigation.

The engraftment of GalTKO/hCD46/hCD39 pig islets appears to be less successful. As with GalTKO/hCD46 pig islets, similar pattern was also observed in GalTKO/hCD46/hCD39 pig islets in terms of the effect of the source of pig islets on their function post-transplant. The two mice that maintained normal blood glucose levels throughout the study period received islets from the same pig. Unfortunately, the grafts from these mice were either infected (Figure 5-7) or not available as the mouse died. The mouse that displayed decreasing blood glucose levels was transplanted with islets from one pig (Figure 5M, 5N, 5O) and the remaining three mice that remained diabetic received islets from two different pigs. Interestingly, less beta cell mass was found in all of GalTKO/hCD46/hCD39 islet grafts from diabetic mice, which might partially explain the delay in reversing hyperglycemia in mice that received these islets. The differences in the cellular composition and successful engraftment of islets post-transplantation may have contributed partly to the differences in function of the islets post-transplantation. A thorough *in vitro* characterization of the islets in terms of cellular composition and glucose stimulated insulin secretion before transplantation would be ideal and may provide further explanation as to the differences in the ability of islets to successfully reverse the diabetic state of recipient mice. However, due to the limited available islets, we focused our study on the characterization of islets using the *in vivo* mouse model. Despite the differences in the effectiveness of islets in reversing diabetes, similar pattern in insulin secretion was observed in response to glucose challenge once recipient mice of different GMP and WT pig islets achieved stable normal blood glucose levels.

Table 5-2. Number of insulin- and glucagon-positive cells in islets from GalKO/hCD46 neonatal pigs.

GalTKO/hCD46	Number of Islets	Insulin+/1x10 μm^2 (mean ± SD)	Glucagon$^+$/1x10 μm^2 (mean ± SD)
Pig #1	5	6.3 ± 1.6	6.9 ± 2.6
Pig #2	6	5.0 ± 3.2	4.9 ± 3.1

The microarray and *in vivo* data suggest that there may be a correlation between the altered genes/pathways and function of GMP islets compared to WT pig islets. For example, some solute carrier genes were up-regulated in GMP islets such as *SLC22A3* in GalTKO islets, *SLC2A5* in GalTKO/hCD46 islets, *SLC1A1* and *SLC1A2* in GalTKO/hCD46/hCD39 islets. These genes are responsible for the uptake of organic cation, fructose, amino acid and glutamate into cells, and the up-regulation of these genes may reflect abnormal metabolism in cells from GMPs. In addition, intercellular adhesion molecules play important roles in islet development. Genes related to cell adhesion were altered, such as up-regulated *CDH13,* down-regulated *ITGB5*, and up-regulated *LAMB2* in GalTKO pig islets. These genes are responsible for cadherin, integrin and laminin expression, respectively, which together compose of extracellular matrix. Up- or down-regulation of these genes may result in mis-communication between cells, therefore impairing islet function. Also, the up-regulation in *FOS* or *EGR1* expression might affect cell proliferation, differentiation and transformation in GMP islets. These results suggest that the genetic modification might alter patterns of gene expression in pigs, and the influence of this alteration should be carefully characterized to ensure stable function of the islets.

Current views on the most suitable GMP for clinical trials on xenotransplantation suggest a triple-knockout pig with the addition of 1 or more human complement regulatory genes, 1 or more

human coagulation regulatory genes, a human anti-inflammatory gene and CD47 [319]. In this study, taking the microarray data and *in vivo* data together, it may be worthwhile to reconsider this approach as we have shown that GalTKO/hCD46/hCD39 pig islet grafts showed impaired or delayed function compared to the less genetic modified pig islet grafts. Hence, it is critical to choose the genes that will be knock-out or knock-in when creating GMP, or at least the function of these pig islets needs to be confirmed either *in vitro* or in small animal models prior to pre-clinical and/or clinical trials. We conclude from this study that islets isolated from GalTKO, GalTKO/hCD46 and GalTKO/hCD46/hCD39 neonatal pigs have therapeutic effect on hyperglycemia. However, variations in the ability of GMP islets in reversing hyperglycemia in mouse recipients was observed and may be the result of the differences in gene expression patterns found in islets, which needs to be further explored.

Chapter 6 ROLE OF CADHERINS IN NEONATAL PIG ISLET DEVELOPMENT

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6.1 INTRODUCTION

Neonatal pigs are being considered as an alternative source of islets for transplantation. However, the mechanism of how cell clusters develop into mature islets is not well understood in pigs. Several changes happen during pig islet development. Morphologically, cell clusters in neonatal pig pancreas are insulin-positive and are arranged alongside or within a line[254]. In the adult pig pancreas, these randomly scattered cell clusters transition into round or oval shaped islets and are situated close to capillaries[263]. Also, different cellular composition in different cell types are observed between neonatal pig islets and adult pig islets[254]. For example, there are fewer insulin-positive beta cells in neonatal pig islets (33.7±3.8%) compared to adult pig islets (82.8±1.2%)[254]. On the contrary, the percentage of other cell types in islets such as alpha cells, delta cells and pp cells are higher in neonatal pig islets than that of adult pig islets (19.5±3.0% vs 9.7±3.2%, 8.2±1.0 vs 4.0±1.0% and 8.7±1.7% vs 4.5±1.3, respectively)[254]. Another difference is the existence of cytokeratin-7 (CK-7)-positive cells in neonatal pigs. These cells serve as islet precursor cells and continuously transform into insulin-positive cells as islets mature[320]. Despite these findings, detailed mechanisms on neonatal pig islet development are not well understood.

Adhesion molecules may play important roles in pig islet development. Cadherins, short for "calcium-dependent adhesion", are a superfamily of cell adhesion molecules that form the adherens junction between cells. Each cadherin contains a cytoplasmic domain, a transmembrane region and an extracellular Ca^{2+} ion binding component[321]. The extracellular component forms homodimers, which interact with cadherins on the adjacent cell membrane, forming part of cell junction[322]. Cadherins play important roles in development, maintenance of cell and tissue structure, cell movement[323] and tumour metastasis. For example, epithelial-cadherin (E-cadherin), also known as cadherin-1, uvomorulin or CAM 120/80 and encoded by the *CDH1* gene,

is essential in blastula formation during development of mouse and xenopus[324]. The change in E-cadherin and other cadherins, drives the epithelial-mesenchymal transition (EMT) during neural crest cell development[325]. Expressed on all epithelial cells, E-cadherin connects to the actin cytoskeleton by interacting with catenin proteins in the cytoplasm. The disruption of this interaction due to the change in E-cadherin expression, drives the EMT in tumour cells[326] and is associated with metastasis in cancer[327, 328]. Neural cadherin (N-cadherin), encoded by the *CDH2* gene, also plays an important role in development. In avian embryo development, evidence shows an E-cadherin to N-cadherin switch during neurulation[325, 329]. This "cadherin switch", as well as *de novo* expression of N-cadherin, are related to increased metastasis and/or poor prognosis in cancer[330, 331]. Vascular endothelial cadherin (VE-cadherin), also known as cadherin 5 or CD144, is encoded by the *CDH5* gene. VE-cadherin is strictly endothelial specific and forms junctions between endothelial cells, which determine the permeability of the endothelium[332, 333]. The disruption of these junctions by blocking antibody to VE-cadherin increases permeability and leads to edema and haemorrhage *in vivo*[334]. Collectively, these findings indicate the important roles of different cadherins in development.

Dissociated islet cells tend to form cell clusters *in vitro*, indicating the potential roles of adhesion molecules in the development and the maintenance of islet structure. Further, the role of cadherins in regulating aggregation of pancreatic beta-cells is shown by Dahl's study, in which the clustering of beta cells in E-cadherin knockout mice was disrupted[335]. Cadherins also play important roles in islet cell aggregation, segregation, maintenance of architecture, differentiation and insulin secretion[335-338]. A recent study showed expression of E- and N-cadherin in human islets protects beta cells from apoptosis[339]. Despite the knowledge on cadherins in islet

development and growth, little is known about cadherin expression in pig islets.

To overcome the potential immune rejection or inflammation post-transplantation, increasing numbers of genetically modified pigs have been created as advancements in gene editing tools progress. For the ultimate use of pig islets in the clinic, pig islet biology as well as the effect of genetic modification on pig islet development and differentiation, needs to be further investigated. In this study, we observed the morphology of islets from pigs of different ages at different time points in culture. We also examined the pattern of E-cadherin, N-cadherin and VE-cadherin protein expression as islets developed in culture.

6.2 RESULTS

Number of endocrine cells increased in pancreas as neonatal pig age increased. Insulin-positive cells were identified in pancreas from 1-day-old pig (Figure 6-1A). These cells were distributed diffusely in the pancreatic tissue. As cells matured, insulin-positive cells began to form clusters in pancreas from 3-day-old pig (Figure 6-1B) and 7-day-old pig (Figure 6-1C). Clusters were most prominent in pancreas from 10-day-old pig (Figure 6-1D). Glucagon-positive cells were identified in pancreas from 1-day-old pig (Figure 6-1E). However, the number of glucagon-positive cells appeared to be less in pancreas from 3-day-old (Figure 6-1F), 7-day-old (Figure 6-1G) and 10-day-old pig (Figure 6-1H). Somatostatin-positive cells were observed in pancreas from 1-, 3-, 7- and 10-day-old (Figure 6-1I, 6-1J, 6-1K, 6-1L). CK-7-positive cells were seen in pancreases from neonatal pigs of all ages. CK-7-positive cells formed tubular structures throughout the pancreases from pigs of 1-, 3-, 7- and 10-day-old (Figure 6-1M, 6-1N, 6-1O, 6-1P). The cells in these tubular structures were previously observed by our lab and were identified as islet precursor cells[254].

Expression of E-, N- and VE-cadherin was observed in pancreases from 1-, 3-, 7- and 10-day-old pigs. Expression of E-cadherin in pancreas increased as the age of pig increased. While no defined structure of islets was found in the pancreatic tissue, sporadic E-cadherin-positive cells were identified in pancreas from 1-day-old pig (Figure 6-2A). E-cadherin expression increased and demonstrated an aggregated pattern in pancreas of 3-day-old pig (Figure 6-2B) and more in pancreas from 7-day-old pig (Figure 6-2C). In pancreas of 10-day-old pig (Figure 6-2D), the aggregation of E-cadherin-positive cells formed islet-like structures. N-cadherin expression was sporadic in pancreases from pigs of 1-, 3-, 7- and 10-day-old (Figure 6-2E, 6-2F, 6-2G, 6-2H). Also,

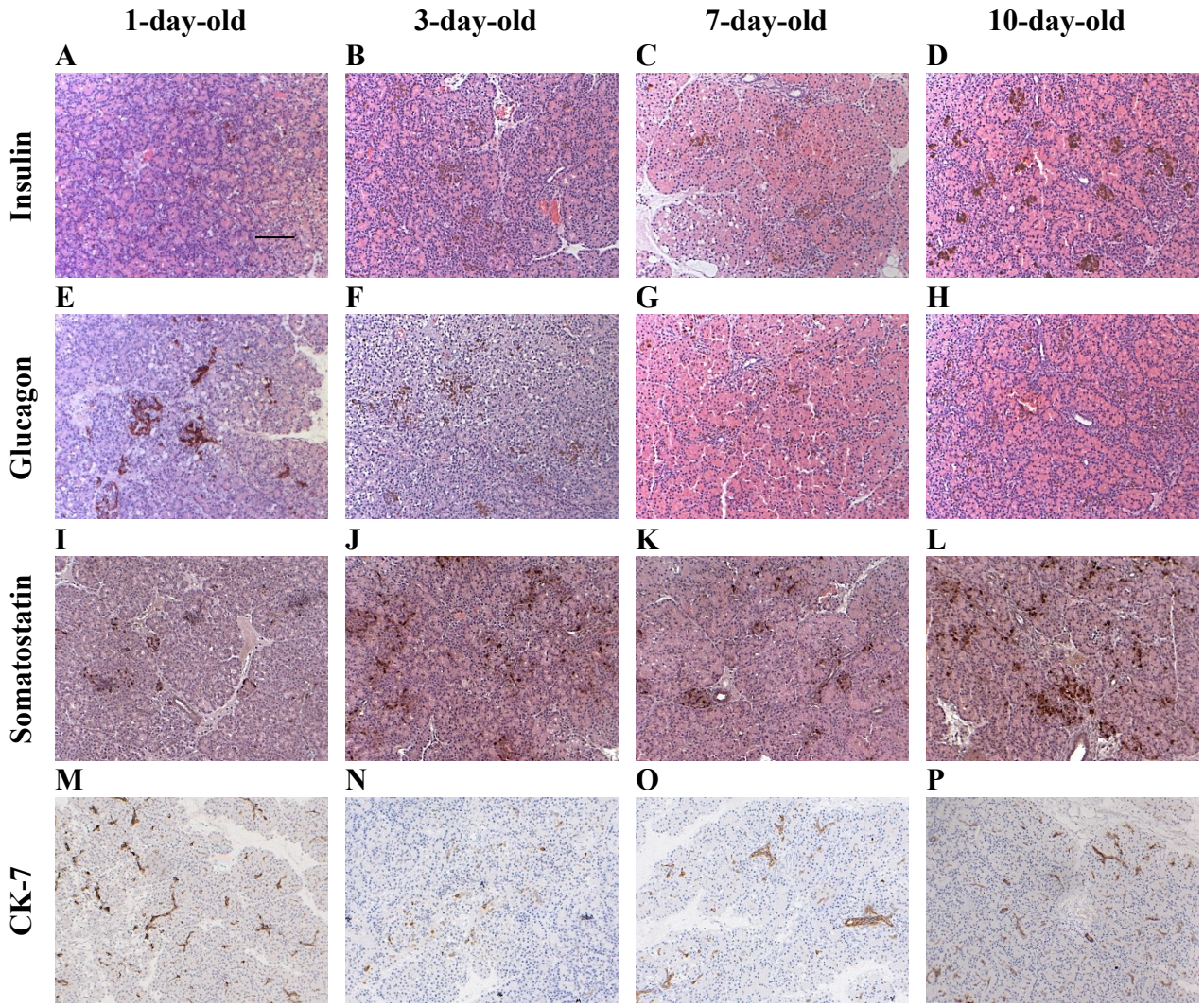

Figure 6-1. Expression of insulin, glucagon, somatostatin and cytokeratin-7 (CK-7) in neonatal pig pancreas. **A, B, C, D:** Representative pictures of insulin-positive cells in pancreases from 1-, 3-, 7-, 10-day-old pigs. **E, F, G, H:** Representative pictures of glucagon-positive cells in pancreases from 1-, 3-, 7-, 10-day-old pigs. **I, J, K, L:** Representative pictures of somatostatin stained pancreases from 1-, 3-, 7-, 10-day-old pigs. **M, N, O, P:** Representative pictures of CK-7 expression in pancreases from 1-, 3-, 7-, 10-day-old pigs. The scale bar represents 100 μm.

round to oval structures which were not insulin-positive, but rather N-cadherin positive, were identified (Figure 6-2G, 6-2H). Expression of N-cadherin suggested active innervation in

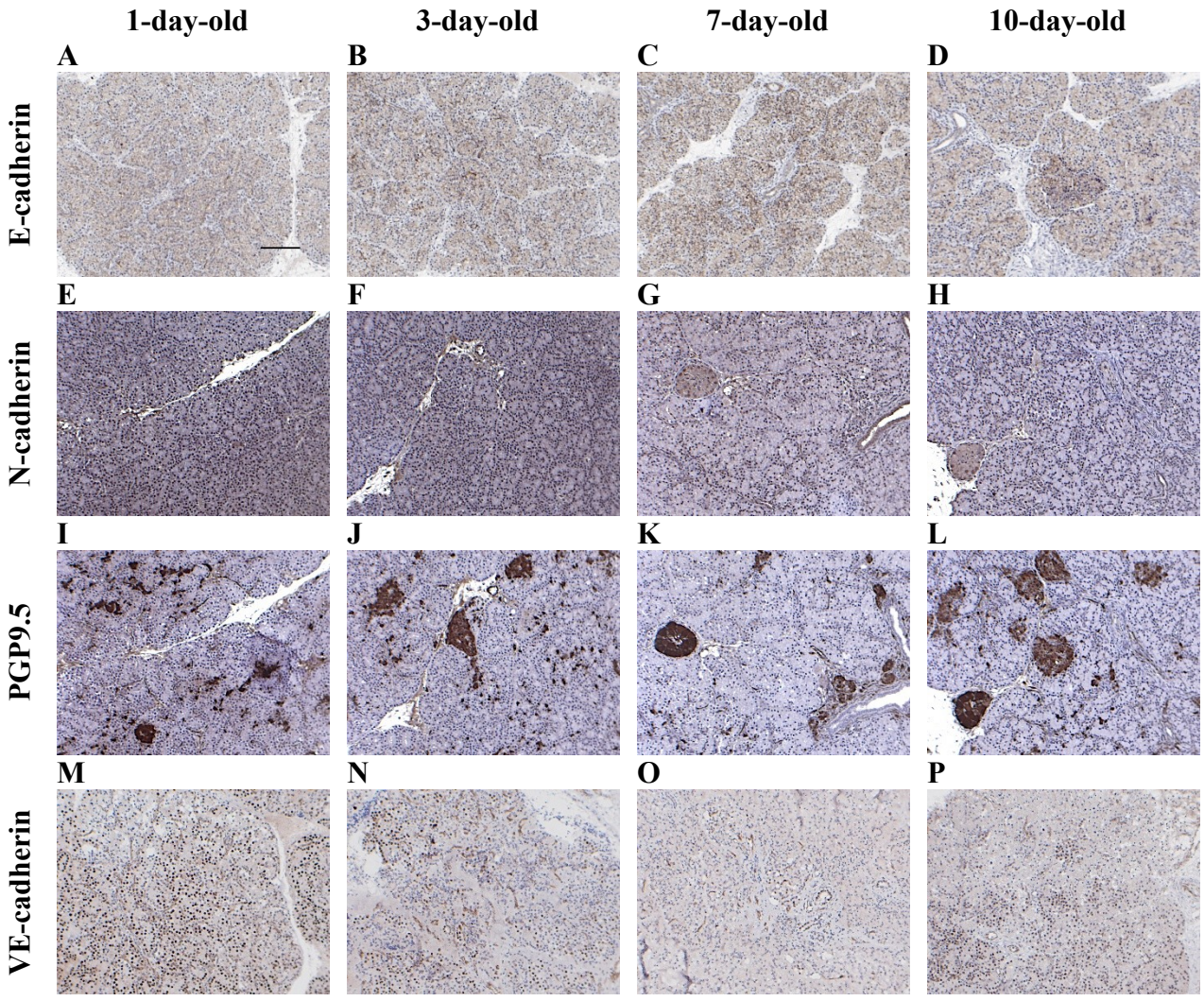

Figure 6-2. Expression of E-cadherin, N-cadherin, protein gene product 9.5(PGP9.5) and VE-cadherin in neonatal pig pancreas. **A, B, C, D:** Representative pictures of E-cadherin expression in pancreases from 1-, 3-, 7-, 10-day-old pigs. **E, F, G, H:** Representative pictures of N-cadherin expression in pancreases from 1-, 3-, 7-, 10-day-old pigs. **I, J, K, L:** Representative pictures of PGP9.5 expression in pancreases from 1-, 3-, 7-, 10-day-old pigs. **M, N, O, P:** Representative pictures of VE-cadherin expression in pancreases from 1-, 3-, 7-, 10-day-old pigs. The scale bar represents 100 μm.

pancreases from neonatal pigs of different ages. The innervation in pancreases from pigs of 1-, 3-, 7- and 10-day-old was also demonstrated by protein gene product 9.5 (PGP9.5) staining (Figure 6-

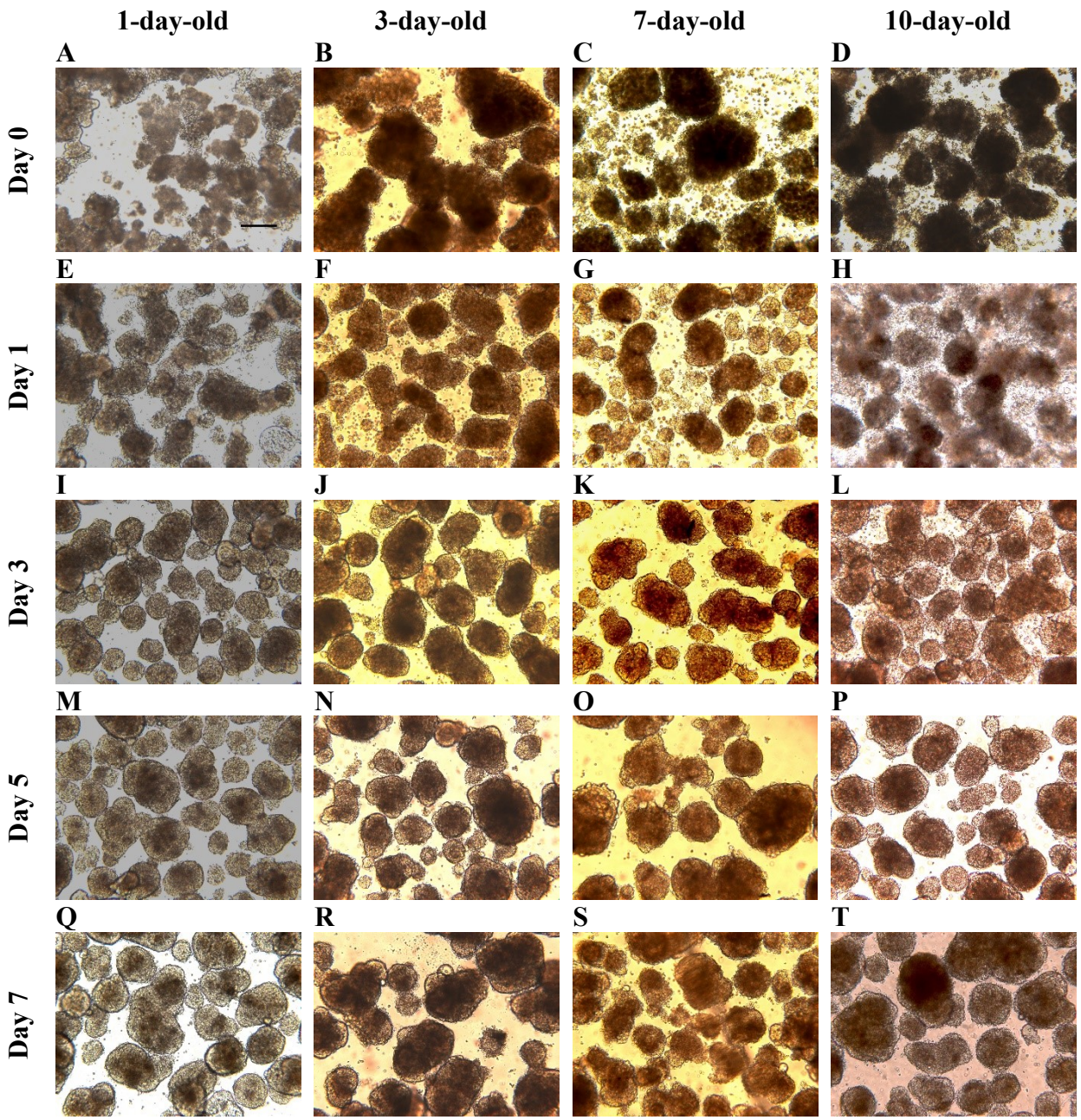

Figure 6-3. Morphology of islets at different time points of culture. **A, B, C, D:** Representative pictures of islets from 1-, 3-, 7-, 10-day-old pig on Day 0 in culture. **E, F, G, H:** Islets on Day 1 in culture. **I, J, K, L:** Islets on Day 3 in culture. **M, N, O, P:** Islets on Day 5 in culture. **Q, R, S, T:** Islets on Day 7 in culture. The scale bar represents 100 μm.

2I, 6-2J, 6-2K, 6-2L). Expression of VE-cadherin was seen in pancreases from neonatal pig of all ages. The VE-cadherin-positive cells formed vasculature-like structures throughout the pancreases from neonatal pigs of 1-, 3-, 7- and 10-day-old (Figure 6-2M, 6-2N, 6-2O, 6-2P), indicating active vasculature formation throughout the pancreas.

Neonatal pig islets formed clusters following 7 days of culture *in vitro*. Islet morphology analysis at early time points in culture revealed that islets isolated from 1-day-old pigs formed cell clusters with irregular shape (Figure 6-3A), while islets isolated from 3-, 7- and 10-day-old pigs have round or oval structures (Figure 6-3B, 6-3C, 6-3D). Single exocrine cells were also present in islet preparations in culture at early time points (Figure 6-3A, 6-3B, 6-3C, 6-3D). Cell clusters became round or oval shape with a membrane surrounding the islet cell clusters on day 1 in culture (Figure 6-3E, 6-3F, 6-3G, 6-3H). As islets from pigs of all ages developed in culture, this membrane was more evident on day 3 (Figure 6-3I, 6-3J, 6-3K, 6-3L) and day 5 (Figure 6-3M, 6-3N, 6-3O, 6-3P) in culture, while the singular exocrine cells were continuously eliminated as media was changed. On day 7 of culture, islets isolated from pigs of 1-, 3-, 7- and 10-day-old formed round or oval structures with clear membrane (Figure 6-3Q, 6-3R, 6-3S, 6-3T), and single cells were significantly less in the media.

E-cadherin and N-cadherin mRNA expression increased as neonatal pig islets developed in culture, while VE-cadherin mRNA expression showed no variable pattern. The expression of cadherin was standardized to β-actin and was shown relative to cadherin mRNA expression in islets on day 0 of culture. In 1-day-old islets, E-cadherin mRNA expression on day 1 was comparable to that on day 0 (Figure 6-4A). A 2-fold change in E-cadherin mRNA expression was

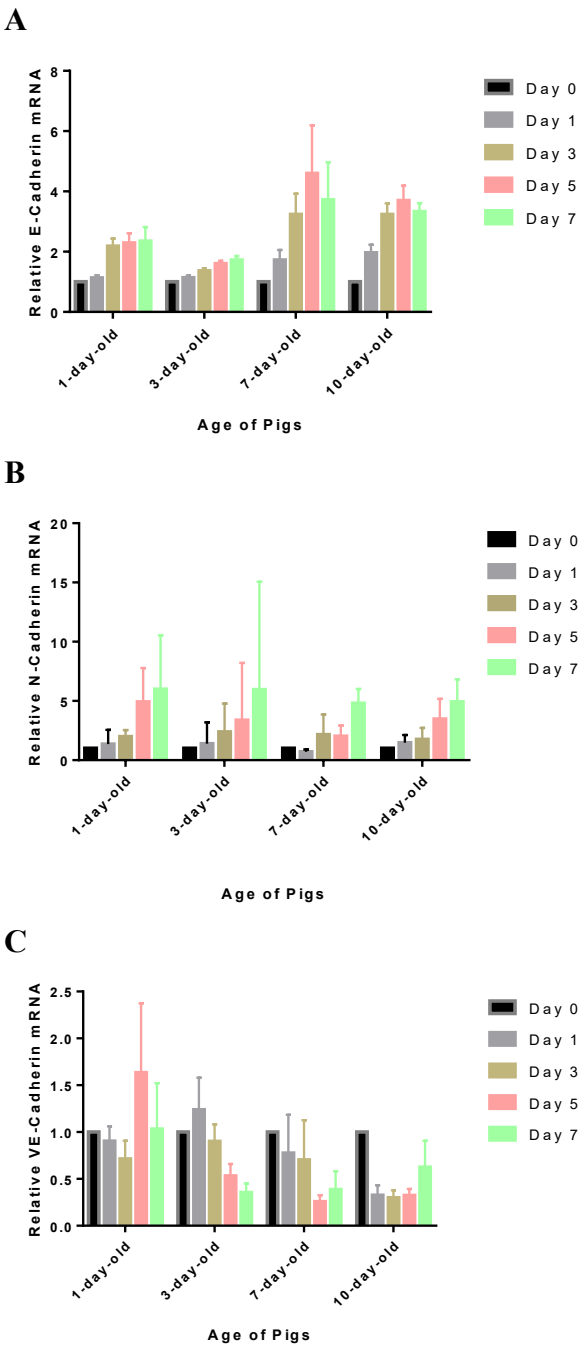

Figure 6-4. E- N- and VE-cadherin mRNA expression in NPI of different ages. **A:** E-cadherin mRNA expression in 1-, 3-, 7-, 10-day-old pig islets on day 0, 1, 3, 5, 7 in culture. **B:** N-cadherin mRNA expression in 1-, 3-, 7-, 10-day-old pig islets on day 0, 1, 3, 5, 7 in culture. **C:** VE-cadherin mRNA expression in 1-, 3-, 7-, 10-day-old pig islets on day 0, 1, 3, 5, 7 in culture.

seen on days 3, 5 and 7 in culture. In 3-day-old pig islets, E-cadherin expression continuously increased as was observed in culture. Increase in E-cadherin mRNA expression was clearly seen in islets cultured for 3, 5 and 7 days of culture from 7-day-old and 10-day-old pigs (Figure 6-4A). N-cadherin mRNA expression increased as islet matured in culture in pig islets of all ages (Figure 6-4B). VE-cadherin mRNA expression fluctuated in islets from 1-day-old pigs as it developed in culture (Figure 6-4C). In islets from 3-, 7- or 10-day-old pigs, a decreasing trend in VE-cadherin mRNA expression was seen as islets matured in culture (Figure 6-4C).

Expression patterns of endocrine cells in islets from neonatal pigs of different ages. Insulin-positive cells were scarcely identified in islets isolated from 1-day-old pigs on day 0 in culture (Figure 6-5A). On day 0 of culture, insulin-positive cells were observed in islets isolated from 3-day-old pigs (Figure 6-5B), and insulin-positive cells were more clearly identified in islets from 7- or 10-day-old pigs (Figure 6-5C, 6-5D). On day 1 of culture, insulin-positive cells were identified regardless of the age of pigs (Figure 6-5E, 6-5F, 6-5G, 6-5H). On day 3 (Figure 6-5I, 6-5J, 6-5K, 6-5L) and day 5 (Figure 6-5M, 6-5N, 6-5O, 6-5P) of culture, round- to oval-shape islet structures were well established and more insulin-positive cells were identified in islets. On day 7 in culture, most insulin-positive cells were identified (Figure 6-5Q, 6-5R, 6-5S, 6-5T). On day 0 of culture, few glucagon-positive cells were identified in islets from 1-, 3-, 7- or 10-day-old pigs (Figure 6-6A, 6-6B, 6-6C, 6-6D), whereas glucagon-positive cells were identified in islets on day 1 of culture regardless of the pig ages (Figure 6-6E, 6-6F, 6-6G, 6-6H). As islets matured, glucagon-positive cells were identified in islets on day 3 (Figure 6-6I, 6-6J, 6-6K, 6-6L), day 5 (Figure 6-6M, 6-6N, 6-6O, 6-6P) and day 7 (Figure 6-6Q, 6-6R, 6-6S, 6-6T) in culture. Together, these results indicate the maturation of insulin-positive beta cells and glucagon-positive alpha cells in islets over

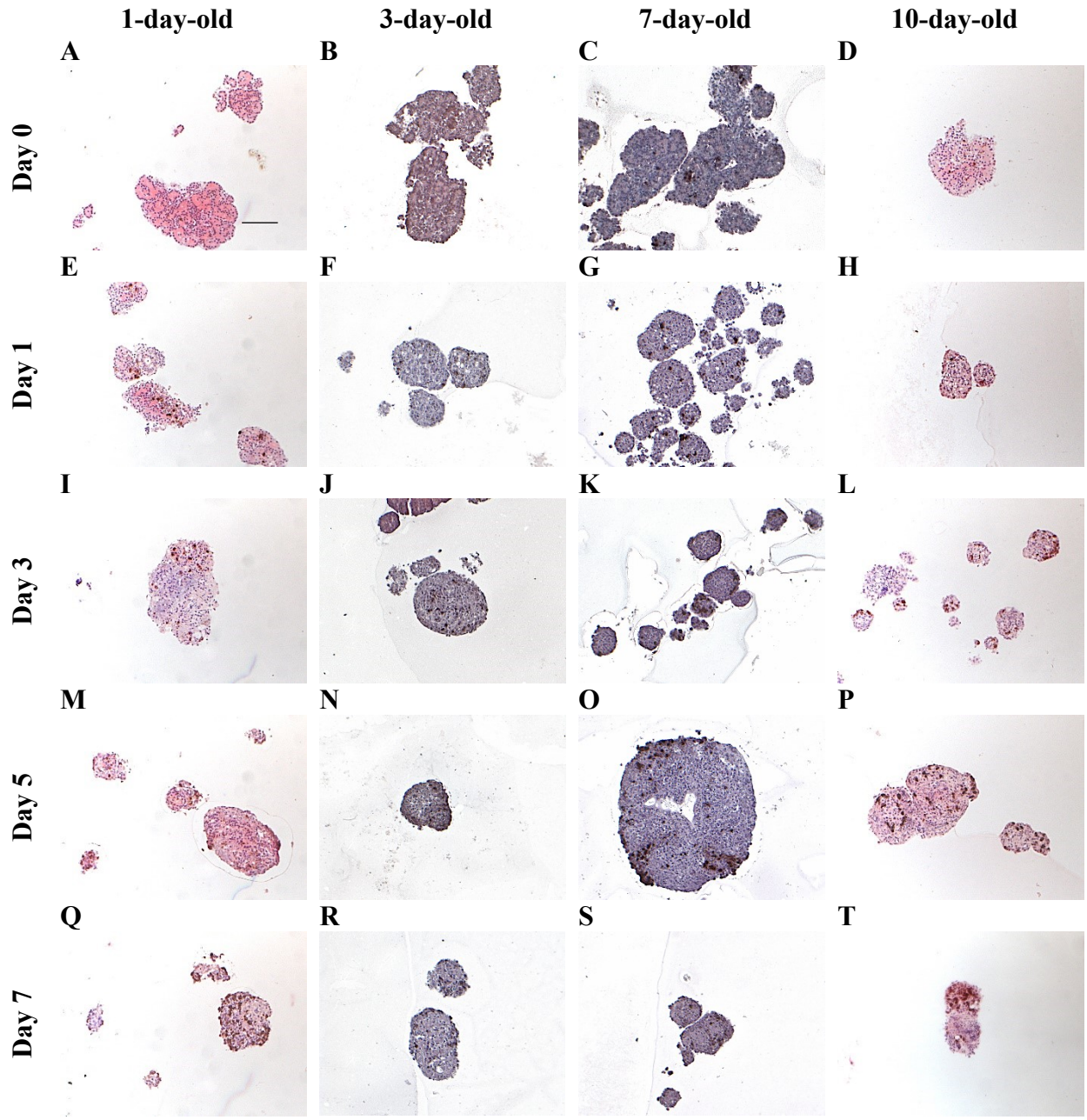

Figure 6-5. Insulin stained islets from 1-, 3-, 7- and 10-day-old pigs at day 0, 1, 3, 5, and 7 in culture. **A, B, C, D:** Representative pictures of insulin staining in islets from 1-, 3-, 7- and 10-day-old pigs at day 0 in culture. **E, F, G, H:** Representative pictures of insulin staining in islets from 1-, 3-, 7- and 10-day-old pigs at day 1 in culture. **I, J, K, L:** Representative pictures of insulin staining in islets from 1-, 3-, 7- and 10-day-old pigs at day 3 in culture. **M, N, O, P:** Representative pictures of insulin staining in islets from 1-, 3-, 7- and 10-day-old pigs at day 5 in culture. **Q, R, S, T:** Representative pictures of insulin staining in islets from 1-, 3-, 7- and 10-day-old pigs at day 7 in culture. The scale bar represents 100 μm.

the course of 7 days in culture.

Protein expression of E-cadherin, N-cadherin and VE-cadherin in neonatal pig islets dramatically increased on day 3 of culture. The expression of protein was measured by chemiluminescence. At the translational level, E-cadherin was expressed less in islets of all ages on days 0 and 1 of culture (Figure 6-7A, 6-7D, 6-7E, 6-7F, 6-7G). However, E-cadherin expression dramatically increased on day 3 of culture (p=0.065) and peaked on day 5 (p=0.002) or day 7 (p=0.003) of culture (Figure 6-7A, 6-7D) compared to that of day 0. This increase in E-cadherin expression was also seen in islets from 3-, 7- and 10-day-old pigs (Figure 6-7E, 6-7F, 6-7G). Islets from 3-day-old pigs displayed the highest E-cadherin expression (p<0.001) compared to islets from pigs of other ages, regardless of the day in culture (Figure 6-7A). Visualization of E-cadherin expression in 1-, 3-, 7- or 10-day-old pig islets were shown (Figure 6-7D, 6-7E, 6-7F, 6-7G). Similar to that of E-cadherin, N-cadherin expression was not detected in islets of all ages on days 0 and 1 of culture (Figure 6-7B), however, N-cadherin expression continuously increased from day 3 (p=0.058) to day 7 of culture (p<0.001) (Figure 6-7B, 6-7H). This increase in N-cadherin expression was also seen in islets from pigs of 3-, 7- and 10-day-old (Figure 6-7I, 6-7J, 6-7K). Regarding the influence of the age of the pigs on N-cadherin expression, islets from 1-day-old pigs showed the highest expression of N-cadherin (p<0.001), followed by islets from 3-, 7-, and 10-day-old pigs (Figure 6-7B). Visualization of N-cadherin expression in 1-, 3-, 7- or 10-day-old pig islets were shown (Figure 6-7H, 6-7I, 6-7J, 6-7K). As islets developed into cell clusters in culture, significantly higher VE-cadherin protein expression was observed at later time points (p<0.05, Figure 6-7C). In addition, VE-cadherin expression was higher in islets from 3-, 7- and 10-day-old pigs compared to islets from 1-day-old pigs (Figure 6-7C). Visualization of VE-cadherin expression in 1-, 3-, 7- or 10-day-old pig islets were shown (Figure 6-7L, 6-7M, 6-7N, 6-7O).

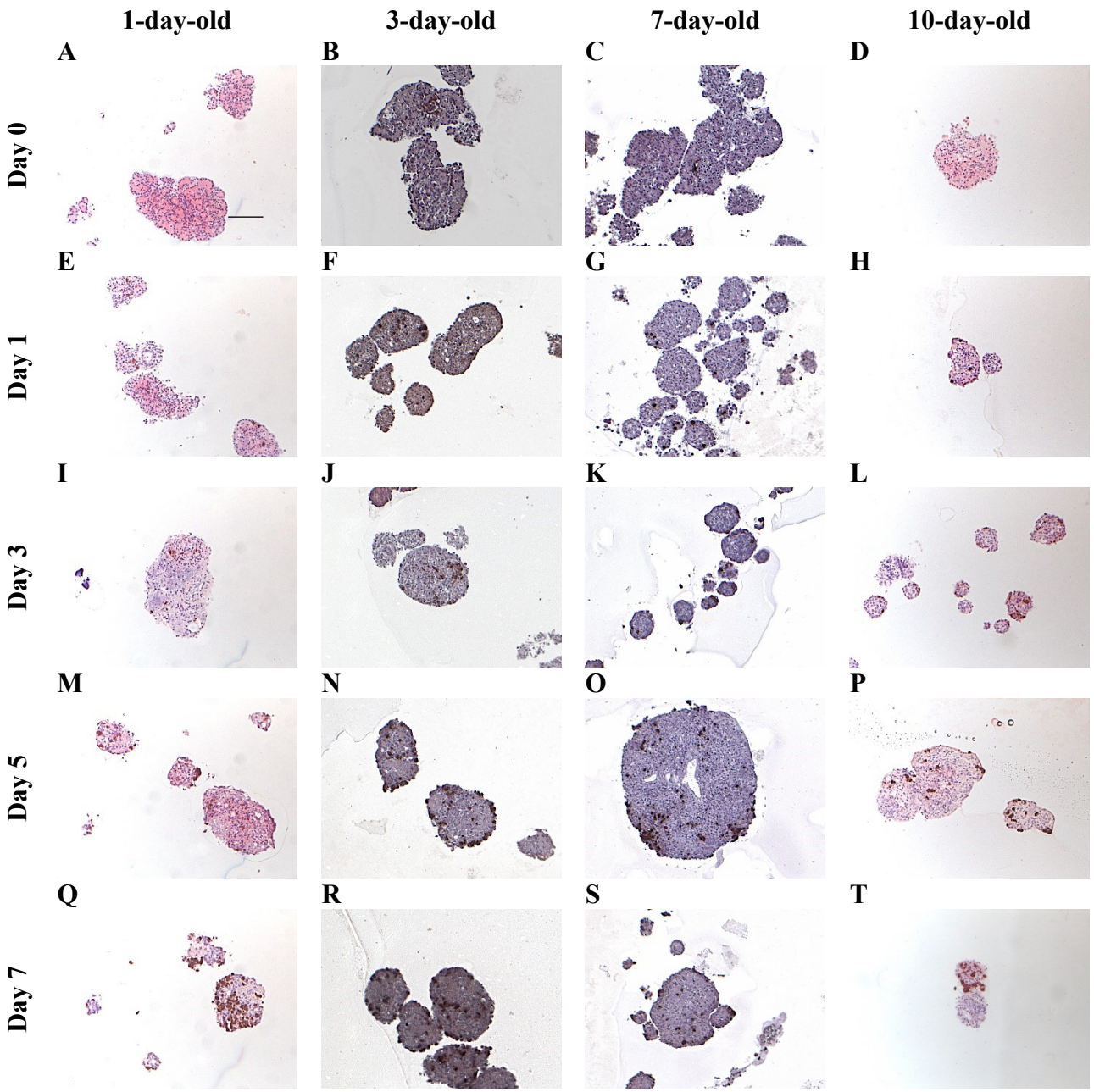

Figure 6-6. Glucagon stained islets from 1-, 3-, 7- and 10-day-old pigs at day 0, 1, 3, 5, and 7 in culture. **A, B, C, D:** Representative pictures of glucagon stained islets from 1-, 3-, 7- and 10-day-old pigs at day 0 in culture. **E, F, G, H:** Representative pictures of glucagon stained islets from 1-, 3-, 7- and 10-day-old pigs at day 1 in culture. **I, J, K, L:** Representative pictures of glucagon stained islets from 1-, 3-, 7- and 10-day-old pigs at day 3 in culture. **M, N, O, P:** Representative pictures of glucagon stained islets from 1-, 3-, 7- and 10-day-old pigs at day 5 in culture. **Q, R, S, T:** Representative pictures of glucagon stained islets from 1-, 3-, 7- and 10-day-old pigs at day 7

in culture. The scale bar represents 100 µm.

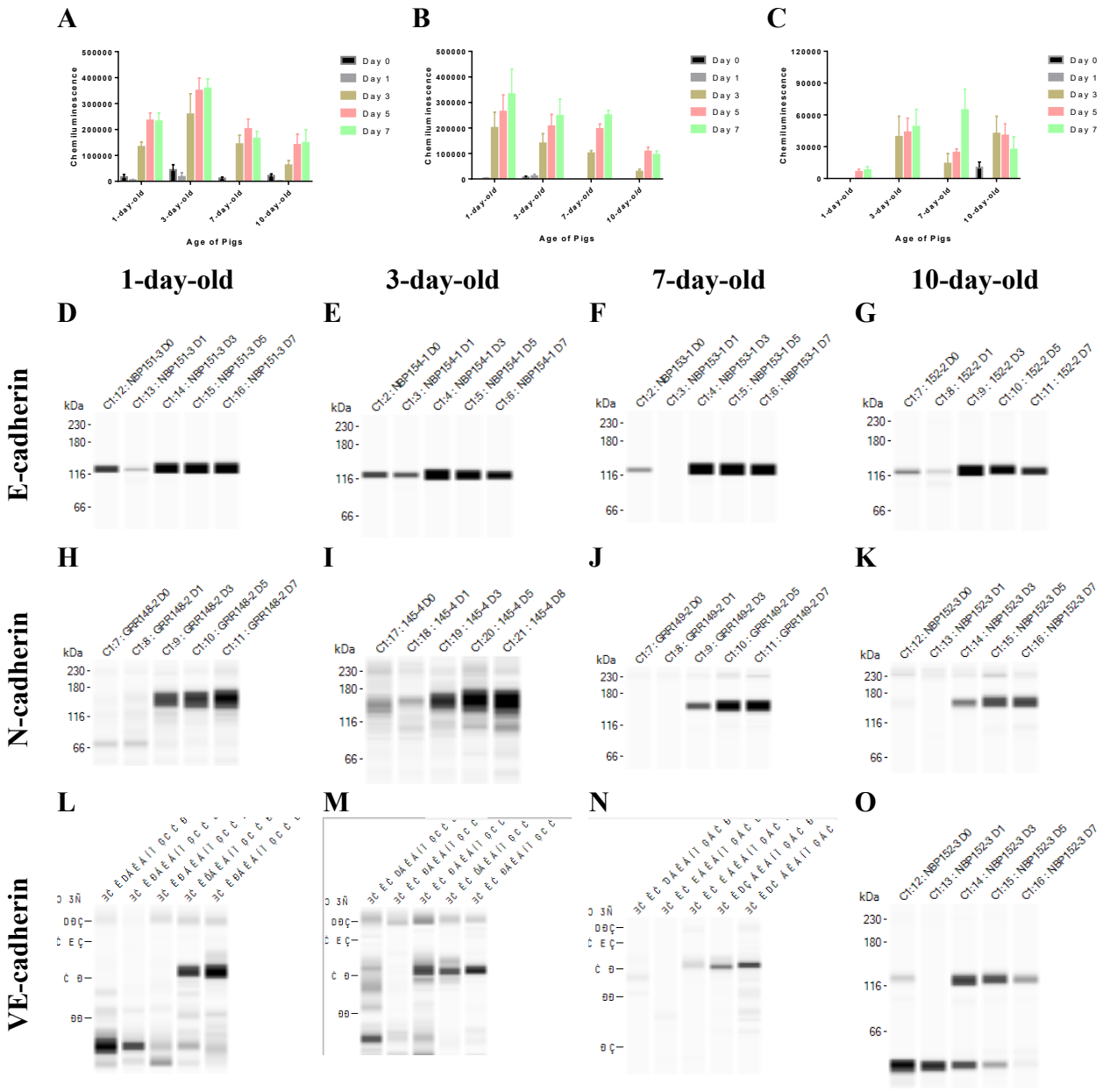

Figure 6-7. Protein expression of E-, N- and VE-cadherin in neonatal pig islets of different age during culture. **A, B, C:** Quantification of E-, N- and VE-cadherin expression by western blot in islets of 1-, 3-, 7- and 10-day-old pigs on day 0, 1, 3, 5, 7 of culture. **D, E, F, G:** Expression of E-cadherin in 1-, 3-, 7-, 10-day-old pig islets on day 0, 1, 3, 5, 7 of culture. **H, I, J, K:** Expression of N-cadherin in 1-, 3-, 7-, 10-day-old pig islets on day 0, 1, 3, 5, 7 of culture. **L, M, N, O:** Expression of VE-cadherin in 1-, 3-, 7-, 10-day-old pig islets on day 0, 1, 3, 5, 7 of culture.

6.3 DISCUSSION

The intent of this study was to investigate the pattern of several cadherins such as E-cadherin, N-cadherin and VE-cadherin as they may have a role in development and maintenance of islet structure in pig islets.

We first detected endocrine cells in the pancreases of 1-, 3-, 7- and 10-day-old neonatal pigs. As pig age increased, insulin- and glucagon-positive cells in the pancreas transitioned from a sporadic dispersion of cells to organized islet clusters. Also, the maturation of beta and/or alpha cells was indicated by increased insulin-positive cells at later time points (Day 3, 5 and 7) of culture. Somatostatin staining indicated the presence of delta cells in the pancreas. CK-7 positive ductal cells were also observed in pancreases from neonatal pigs of all ages, indicating the presence of islet precursor cells. These results were concordant with previous studies[263].

We also observed morphology of neonatal pig islets from different ages in culture. Regardless of the age of the pig, the islet structure was disrupted during the isolation process. We showed the formation of round- to oval-shape islets, as neonatal pig islets became matured and purified following 7 days of culture *in vitro*.

We further investigated cadherin patterns in pancreases from neonatal pigs of different ages. Scattered expression of E-cadherin was observed in pancreatic tissue from younger pigs, however expression began to aggregate in islet-like structures in older pig pancreas, suggesting that E-cadherin might play a role in islet development and structure. On the transcriptional level, E-cadherin mRNA expression increased as neonatal pig islets matured in culture. On the translational level, E-cadherin protein expression was low at early time points of culture, but a dramatic increase in islets was observed on day 3 of culture, suggesting active cell interaction activity. This correlates

with the formation of round- to oval-shape islet structure beginning on day 3 of culture. In terms of the influence of pig age on E-cadherin expression, islets from 3-day-old pigs showed the highest E-cadherin expression at different time points in culture, compared to islets from pigs of other ages. This suggests that islets from 3-day-old pigs might have the most robust structure. Adult pig islets are fragile and difficult to isolate, which may correlate with low E-cadherin expression, however this theory needs further investigation. In islets from pigs of all ages, the increase in N-cadherin expression on day 3 suggests active innervation of islets at later time points of culture. As pig age increases, N-cadherin expression was decreased. A potential explanation for this observation might be that innervation in islets is established at early days, decreasing the importance of N-cadherin at later days. Positive VE-cadherin staining in pancreases from different ages indicated active vascularization in the post-natal development. Low VE-cadherin expression was observed in islets at early time points of culture. Increased VE-cadherin expression on day 3 and thereafter in culture indicated the formation of vasculature in islets after isolation started on day 3 in culture.

A major limitation of this study is that the role of these cadherins was not examined *in vivo*. It would be important to investigate the expression of E-cadherin, N-cadherin and VE-cadherin in an immunodeficient mouse model that received islets from pigs of different ages. Cadherin expression would then be investigated at different time points after transplantation. Also, we observed a correlation between the expression of different cadherins and islet development. However, the causative effect was not confirmed for this correlation. Further experiments blocking expression of these molecules, either temporarily by monoclonal antibodies or small interference RNA or permanently by genetic knockout, may answer this question.

Overall, the above findings revealed the patterns of E-cadherin, N-cadherin and VE-cadherin expression in pancreas and islets from neonatal pigs of 1-, 3-, 7- and 10-day-old. It appears that

islets from 3-day-old pigs might be the optimal source for islet transplantation, and these islets are recommended to be cultured for at least 3 days after isolation. These results suggest that cadherins may play a role in the development of pig islets and may be important molecules to preserve when designing genetically engineered pigs. Gene expression knockdown experiments will confirm the importance of cadherins in pig islet development as well as in the maintenance of pig islet structure and function.

Chapter 7 GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION AND CONCLUSIONS

T1DM is characterized by the destruction of insulin-producing beta cells by auto-reactive immune cells. The injection of insulin, which is effective in lowing blood glucose level, is essential in treatment of T1DM. However, patients with T1DM develop secondary complications such as cardiovascular disease, kidney failure, retinopathy as well as hypoglycaemia unawareness at the end-stage of the disease, even with intensive insulin and continuous glucose monitor system. An alternative treatment for T1DM is the transplantation of isolated islets, which provides a more physiological response to glucose changes. Islet transplantation has been more widely applicated in various countries after the proposal of Edmonton Protocol in 2000[101], and up to 50% of islet allograft recipients remains insulin independence at 5 years post-transplantation[103]. Due to the shortage of human organ donors and the potential immune rejection (therefore patients require long term use of immunosuppressants), current clinical islet transplantation is limited to selected patients with glycaemic lability[103]. The wider application of clinical islet transplantation necessitates the finding of other source of islets as well as overcoming the potential autoimmunity and alloimmunity post-transplantation.

To alleviate the shortage of human organ donors, pigs of different ages are being considered an alternative source of organ for islet transplantation with various advantages[106]. Adult pig islets have been shown to successfully reverse hyperglycemia in autograft, allograft and xenograft transplantation. Advantages of adult pig islets include the large number and size of islets, more insulin secretion[340], rapid in reversing hyperglycemia in mice and nonhuman primates[341-343], low expression of xeno-antigen such as alpha-Gal compared to neonatal[344] or fetal pig islets[340]. However, adult pig islets are susceptible to ischemic injury and delicate in culture.

Although certain xenoantigen such as alpha-Gal is less expressed, the overall immunogenicity of adult pig islets is more than neonatal or fetal pig islets[345, 346]. Neonatal pig islets (NPI) have also been shown to have therapeutic effect on hyperglycemia in small and large animals[347]. It is commonly accepted that neonatal pigs are the optimal source of organ for islet xenotransplantation for various reasons. First, neonatal pig pancreases are less fibrous than adult pig pancreases hence it is easier to isolate NPI[345]. Second, there is evidence that NPI are more resistant to hypoxia-induced apoptosis[255]. Third, NPI have the potential to grow and differentiate after transplantation[345]. On the other hand, as the immature nature of NPI, more donor pigs are required for one transplantation and recipients necessitate more time to achieve normoglycemia[345]. Fetal pig islets share the same advantages of NPI. However, fetal pig islets secret less insulin compared to adult pig islets or NPI, and there is no success in allotransplantation or xenotransplantation in reversing hyperglycemia[345]. Based on the findings above, neonatal pig islets appear to be the optimal source of islets for xenotransplantation, while the optimal age of neonatal remains to be investigated.

As the final goal is to transplant pig islets into T1DM patients, a critical question needs to be answered: will the newly transplanted pig islets be rejected by autoreactive immune cells in the diabetic recipients? There exist huge debates on the answer to this question. On one hand, as xenogeneic islets express disparate MHC molecules on surface, the destruction by the direct recognition of islet determinants by autoreactive T cells may be less[348-350]. Therefore, pig islets may be more resistant to recurrent autoimmunity in T1DM patients. On the other hand, autoimmunity may be mediated by the recognition of MHC-peptide by antigen-presenting cells of recipients, independent of surface molecules on islets[351, 352]. This means that any exogenous islets including xenogeneic pig islets would be susceptible to recurrent autoimmune disease. In a

NOD mouse model, treatment with STZ prolongs the survival of syngeneic islets[353]. This is concordant with Koulmanda et al.'s finding of long term survival of isografts in STZ-induced diabetic NOD mice[354], which suggests that destructive autoimmunity was not developed in these mice. They also found that adult pig islet xenografts[354] or fetal pig islet xenografts were resistant to recurrent autoimmunity when anti-CD4 treatment was applied to recipients[355]. These findings suggest pig islets may be resistant to recurrent autoimmunity in T1DM patients.

Another obstacle for the clinical application of pig islets will be the potential xenorejection post-transplantation, including hyperacute rejection (HAR), IBMIR and the adaptive immune response. First, As described previously, the expression of alpha-Gal in pig islets is age-dependent. NPI express alpha-Gal, which may cause HAR when transplanted into human beings due to the preformed natural antibodies. In addition, though islets are less vascularized compared to other organs such as heart, kidney or lung, the re-vascularization post-transplantation makes islets susceptible to HAR. Second, if NPI would be transplanted into patients following the current route (portal vein) in the clinic, the IBMIR would cause damage of up to 60% of transplanted islets. Third, NPI would be susceptible to xenorejection by adaptive immune response. In order to overcome these immunological barriers to the application of NPI in the clinic, several strategies could be applied. One of the approaches is to immunologically isolate the NPI using encapsulation, which allows the perfusion of nutrients and small molecules, but stops the immune cells from damaging NPI. Another direction would be developing immunosuppressants that target recipients' immune cells with least toxicity to NPI. This necessitates comprehensive understanding of the mechanisms on how human immune system recognizes and attacks NPI. A third direction to alleviate the potential immune rejection of NPI is through the production of genetically modified pigs, which can reduce the immunogenicity of cells from pig.

The overall goal of these thesis projects is to provide a better understanding of neonatal pig islets in terms of biology and immunogenicity. In chapter 3, we investigated the immune response of PBMC from individuals with T1DM when stimulated with neonatal pig islet (NPI) cells in *vitro*. The proliferation kinetics of T1DM PBMC show a more robust response when stimulated with non-antigen-specific Concanavalin A at early day of culture (Day 3), and a lower and delayed response when stimulated with pig PBMC and the lowest response when stimulated with NPI cells (Day 6 to 7). The variation in the response may be explained by the presence of fewer antigen presenting cells in NPI cells compared with pig PBMC[254]. In a previous study by Murray *et al*., human PBMC from individuals without T1DM were co-cultured with neonatal pig splenocytes or dissociated NPI cells, and the maximum for both responses were closed and were observed on day 8 or day 9 in culture[221]. It appears that the proliferation of human PBMC from individuals with T1DM was not as robust as human PBMC from individuals without T1DM. The proliferation pattern of human PBMC from individuals with T1DM was clearly defined, and the inhibitory effect of two small inhibitory molecules, Suramin and DR80, was examined. Based on the proliferation index, the IC50 of both molecules was higher than others had found previously[224, 356]. An explanation for the inhibitory effect of these two molecules may be by the inhibition of protein kinase C, which is an enzyme involved in intracellular mitogenic signal transduction[223]. Another potential mechanism underlying this inhibition may involve the interruption of costimulatory signal through the blockade of CD40:CD150 interaction[226]. Further experiments may confirm the specific cell types and/or pathways that Suramin and DR80 target. Also, it remains unknown if this inhibition is limited to PBMC from individuals with T1DM. In addition, it is of interest to know if Suramin or DR80 would have synergistic effects with other immunosuppressants used in the clinic.

In Chapter 4, we examined the immune response of individuals with or without T1DM against NPI cells *in vivo*. Neonatal pig islets reversed hyperglycaemia in streptozotocin-induced diabetic NSG mice. Interestingly, blood glucose level minimally increased at 1- to 3-week post-reconstitution with PBMC from diabetic individuals or individuals without type 1 diabetes. In a study by Tonomura *et al.*, adult pig islets were completely rejected at 4 weeks post-reconstitution in a humanized model, accompanied with loss of detectable C-peptide, severe intra-graft infiltration of human T cells, macrophages and B cells[357]. In our study, the normoglycemia in the mice may be partially due to the presence of CK-7 positive precursor cells in the xenograft. These precursor cells continuously transform into insulin-secreting beta cells[344], maintaining normoglycemia. Immunohistochemistry analysis revealed massive insulin-positive cells in islet grafts at 1-week post-reconstitution, which became sporadic and less at 2- and at least 3-weeks post-reconstitution. Human immune cells such as $CD45^+$ immune cells, macrophages, $CD4^+$ T cells, and $CD8^+$ T cells were identified in grafts of reconstituted mice. *In vitro*, NPI induce human $CD4^+$, but not $CD8^+$ T cell proliferation[221]. Cell-mediated xenograft rejection depends on the $CD4^+$ indirect pathway[271, 272]. In our model, direct recognition of antigen on pig islet xenografts by human CD4+ T cells was less possible, as the low percentage of APC in NPI cell population. Another way that NPI would be rejected by human CD4+ T cells via the recognition of pig antigen present on human macrophages. However, the blood glucose levels of mice post-reconstitution with human PBMC remained low, indicating that there may exist miscommunication between NPI, antigen presenting cell and human effector T cells in this humanized model. In this study, we also showed the presence of $CD8^+$ T cells in islet xenografts, providing more details on the mechanisms of human immune responses against pig islet cells. Also, the presence of M2 macrophages in the islet xenograft may have a regulatory effect on the maintenance of normoglycemia[273, 274].

Collectively, we found few differences between the rejection of islet xenografts by T1DM PBMC and NT1DM PBMC.

We also compared the rejection of neonatal pig islet xenograft by immune cells from different sources. The tempo of NPI rejection was faster in NSG mice reconstituted with phylogenetically closer NOD splenocytes, compared to the mice that were reconstituted with phylogenetically disparate human PBMCs. This variation in the tempo of rejection suggest there may exist a hierarchy order in the rejection of NPI xenograft with immune cells with different phylogenetic distances, which is consistent with a previous *in vitro* study[358]. One potential explanation for this would be that the internal environment in NSG mouse may be more preferable for the phylogenetically closer NOD splenocytes than phylogenetically disparate human PBMC. This was evidenced by our primary data (data not shown) that few human B cells were identified when the phenotype of splenocytes from reconstituted mice was characterized by flow cytometry. A further staining for B cells in the islet xenografts would provide answer.

One way to reduce the potential immune rejection of NPI by human immune system is through the use of genetically modified pigs. In the field of xenotransplantation, an ideal GMP would be a triple-knockout pig with the addition of one or more human complement regulatory genes, 1 or more human coagulation regulatory genes, a human anti-inflammatory gene and CD47 (an integrin associated protein and acts as a "do not eat me signal")[359]. With advancements in gene-editing tools such as the CRISPR-cas9 system[128, 360], it has become more convenient to create pigs with multiple genetic modifications in a faster manner[219]. However, few studies have investigated whether genetic modifications would have affected the function of islets. Therefore, in Chapter 5, we characterized and compared the islets from GalTKO, GalTKO/hCD46, GalTKO/hCD46/hCD39 and wild type (WT) neonatal pigs. Islet xenografts of mice that became

normoglycemic post-transplantation with NPI contained abundant insulin and glucagon-positive cells, while varied insulin- and glucagon-positive cells were identified in the grafts of mice that remained hyperglycemic. GalTKO neonatal pig islets demonstrated the ability to reverse hyperglycemia in NSG mice. GalTKO/hCD46/hCD39 neonatal pig islets also showed a therapeutic effect, however, there were variations between islets from different batches of pigs. The engraftment of GalTKO/hCD46/hCD39 neonatal pig islets appear to be less successful than GalTKO or WT NPI. In this study, we used CK-7 as a marker for islet precursor cells. Prior to transplantation, islets from neonatal GalTKO, GalTKO/hCD46 or GalTKO/hCD46/hCD39 pigs showed positive staining for CK-7. However, the islet xenografts demonstrated variance in CK-7 staining. This unconcordance of CK-7 staining in islets before and after transplantation suggested the impaired engraftments in GMP islets. A delay in reversing hyperglycemia was observed when more genetic modifications were applied to islets. It would be more convincible if in vitro response of different GMP islets to glucose challenge would have been examined.

To explore the underlying reason of unconcordant engraftment of different GMP islets, we turned to the gene expression level in these islets. Microarray results showed that less genes were altered as more genetic modifications were introduced into the pigs. We found out a number of genes that are involved in nutrient uptake, intercellular adhesion, cell proliferation and differentiation. This suggested that the energy metabolism, cellular communication and cell growth/death in these islets may have been altered, which may partially explain the variations in engraftment and therefore the delay in reversing hyperglycemia. This project provides insight in the function of different GMP islets, which may be predicted by the altered genes involved in energy metabolism, intercellular adhesion, cell proliferation and differentiation. Any cells or organs from pigs with genetic modification should be carefully characterized before being

transplanted into human beings. This necessitates the development of technique such as next generation sequencing, which could provide a quick and concise detection of the changes in gene expression. Such that, after new genetically modified pigs are created and can be quickly justified if their functions would have been altered.

Based on the above discussion that genetic modification may influence expression of different genes and alter islet function, more understanding on basic islet biology is necessary. Specifically, the postnatal development of islets is critical in islet maturation, during which scattered islet precursor cells transform into oval or round structure. In the formation of this islet structure, intercellular adhesion molecules may play important roles in cell communication. Here in this thesis project we focused on cadherin molecules, which are calcium-dependent intercellular adhesion molecules. In Chapter 6, we aimed to examine the pattern of epithelial-cadherin (E-cadherin), neural-cadherin (N-cadherin) and vascular endothelial-cadherin (VE-cadherin) protein expression as islets developed in culture. For the first time, we showed a dramatic increase in E-cadherin, N-cadherin and VE-cadherin in islets on day 3 of culture, regardless of the ages of pigs. Day 3 was also the timepoint when islets began to form the round to oval-shaped structure. It is conceivable that there may exist a correlation between these cadherin molecules and islet morphology development. Therefore, these adhesion molecules should be carefully considered when creating new genetically modified pigs.

Islet transplantation is being considered as an attractive alternative treatment for individuals with T1DM. However, the shortage of human organ donors and requirement of immunosuppressive regimens remain obstacles for the further application of islet transplantation in the clinic. This thesis project addresses neonatal pigs as an alternative source of organ donor. The results demonstrate the inhibitory effect of DR80 and Suramin on the *in vitro* human immune

response to neonatal pig islet cells. *In vivo*, NPI rejection by human immune cells starts at 2-3 weeks post-reconstitution and was related to macrophage, $CD4^+$ and $CD8^+$ T cells. Islets from GMP have therapeutic effect on hyperglycemia. However, variations in the ability of these islet xenografts to reverse the diabetic state of recipient mice was observed and this may be due in part to the difference in patterns of their gene expression. E-cadherin, N-cadherin and VE-cadherin may play important roles in the development of pig islets and may be important molecules to preserve when designing genetic engineered pigs. Overall, this thesis study provides better understanding of neonatal pig islets as an alternative source of organ for islet transplantation.

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