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Porcine Islet Xenotransplantation for the Treatment of Type 1 Diabetes

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1. Introduction

Type 1 diabetes is a disease that typically occurs in childhood and adolescence and has been estimated to account for 5% to 10% of all diagnosed cases of diabetes. It is caused by the destruction of beta cells in the islets of pancreas resulting in insulin deficiency that eventually leads to high glucose levels in the blood. If not properly treated, this condition can lead to long-term secondary complications of diabetes such as kidney disease, heart disease, and blindness. Individuals with type 1 diabetes require insulin injections for survival, but insulin injection never achieves perfect regulation of glucose in the blood and secondary complications of diabetes still develop. An attractive alternative treatment for type 1 diabetes is the replacement of islets by transplantation. Islet transplantation offers a physiological means of delivering insulin thus has the potential to control better the levels of glucose in the blood. With an islet transplant, the beta cells that have been destroyed are replaced by new beta cells in the islets, which are able to sense the changes in blood glucose levels.

The very first attempts at islet transplantation occurred prior to the discovery of insulin in 1922 (Banting & Best, 1922) when Von Mering and Minkowski demonstrated in 1889 that the pancreas was responsible for the regulation of blood glucose as removal of the pancreas made the dogs diabetic *(*von Mering, 1889*)*. Minkowski subsequently attempted to reverse diabetes in the diabetic dogs by auto-transplanting fragments of pancreas under the skin but his attempt failed (Minkowski, 1892). Subsequent advances in rodent models established the foundation for techniques used in current day islet transplantation. In 1989, the Islet Transplant Group at the University of Alberta carried out Canada's first islet transplant *(*Warnock et al., 1989*)*. Long-term insulin independence was achieved when a combination of freshly isolated and cryopreserved islets were used (Warnock et al., 1992). Up to 1999, of the 267 islet allografts transplanted worldwide, only 12.4% have resulted in insulin independence for periods of more than 1 week, and only 8.2% have done so for periods of more than 1 year (Brendel et al., 1999). Despite these sobering long-term results, compared with intensive exogenous insulin therapy, islet transplantation provided superior metabolic control, prevented hypoglycemic events and held the potential to decrease secondary complications of diabetes (Alejandro et al., 1997) - a substantial impetus to encourage continued support of the field. Islet transplantation, however, faces a number of challenges, including a shortage of suitable human donors for transplantation and the required long-

term use of harmful immunosuppressive drugs in order to prevent rejection of the graft. For these reasons, islet transplantation is currently reserved for patients with brittle diabetes. However, as improved anti-rejection regimens are being developed, islet transplantation is becoming a viable option for more patients with type 1 diabetes. This only serves to widen the gap between the supply and demand of islets for transplantation. A potential strategy to overcome this challenge is the transplantation of xenogeneic tissue, or tissue from a different species, as an alternative source of islets. The first recorded attempt at using xenotransplantation to treat type 1 diabetes was performed by Watson-Williams and Harsant in 1893, when they treated a young boy in diabetic ketoacidosis by implanting fragments of a sheep's pancreas subcutaneously. Although there was a temporary improvement in the young boy's glycosuria, he ultimately died shortly thereafter (Williams, 1894). Since this time a number of xenogeneic sources of insulin producing cells have been explored including porcine (Korbutt et al., 1996), bovine (Marchetti et al., 1995), rabbit (Lacy et al., 1989) and fish Brockman bodies (Wright et al., 1992). Arguably, the most attractive alternative source of islets for human transplantation are porcine islets (Figure 1).
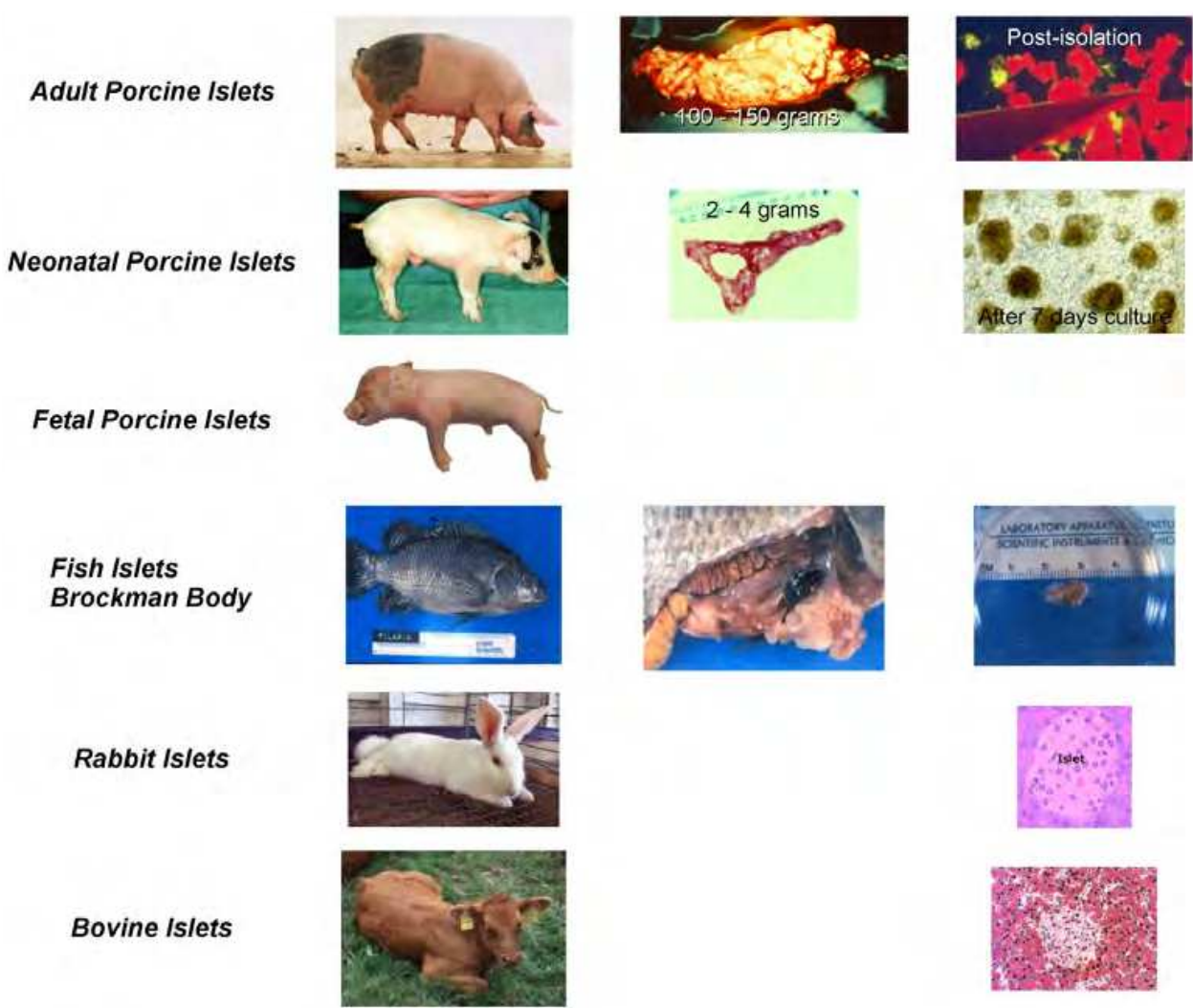

Fig. 1. Xenogeneic sources of insulin producing tissue. Each of these sources have been examined, however, the most promising source for clinical islet transplantation is porcine islets.

2. Porcine islets as a potential solution to the shortage of human islets

Pigs have been the focus of islet xenotransplantation for a number of reasons – they are inexpensive, readily available, breed quickly and produce large litters. Their islets display many morphologic and structural similarities to human islets, and respond to glucose levels in the same physiologic range (Cardona et al., 2007). Porcine insulin differs from human insulin by only one amino acid, and as such has been used clinically to treat patients with type 1 diabetes for many years (Dufrane & Gianello, 2009). Pig donors have many advantages with respect to transplantation: i) they are not exposed to compromising conditions such as comorbidity, brain death and cold ischemic injury as many deceased human donors are; ii) they may be housed in pathogen-free facilities which may allow for an on-demand source of islets with limited risk of pathogen transmission; iii) they may be genetically modified in order to change the expression of proteins, which may ultimately allow for the procurement of less immunogenic tissue (Hering & Walawalker, 2009; Ricordi et al., 1990; Korbutt et al., 1996).

2.1 The optimal age of the donor pig

Three main age groups of donor pigs have been investigated to date - adult, fetal, and neonatal – however, the optimal age is still being debated. Adult pigs are a potential source of tissue, as the isolated islets function well both *in vitro* and *in vivo* immediately upon isolation, and the yield is substantial. Ricordi et al. demonstrated that approximately 255,000 islets could be isolated per adult pig pancreas, using a technique modified from the human islet isolation procedure. The final preparation was 85-90% pure and reversed hyperglycemia in nude mice (Ricordi et al., 1990). Adult pig islets, however, do have their disadvantages. They are difficult to isolate and maintain in culture, are fragile, and are more susceptible to ischemic and hypoxic damage than neonatal porcine islets. In addition, they lack growth potential (Smith & Mandel, 2000), which limits their ability to recover from any damage upon transplantation. Adult islets may also be relatively more immunogenic upon transplantation, which may only increase the need for immunosuppression (Bloch et al., 1999). In order to be suitable for clinical transplantation, the pigs must be maintained in a pathogen-free environment until they are of an appropriate age for donation. This can be very costly and logistically very difficult, which can limit the applicability of adult pigs as islet donors.

The processing of fetal pig pancreata yield porcine fetal islet-like clusters (FICC), an immature group of cells that is capable of producing insulin. In 1988, a simplified procedure for the procurement of FICC was developed by Korsgren et al., which is a simple procedure that does not require the ductal infusion of collagenase or ficoll gradient separation of islets that is seen in adult islet isolation. Media changes every second day purifies the islets, but the functional ability of the FICC is still poor due to their immaturity. They can be maintained in culture for up to 30 days, and are capable of proliferating, however, *in vivo* reversal of hyperglycemia in animals can take months (Korsgren et al., 1991). In addition, the yield of FICC per pancreas is low, and an estimated 100 porcine donors would be necessary to transplant one 70-kg patient (Korsgren et al., 1991).

A functionally mature islet source with the isolation ease seen in fetal pig donors can be found in neonatal porcine donors. Korbutt et al. developed a protocol in 1996 for the isolation of neonatal porcine islets (NPI), which is easy to perform, with a consistent yield of approximately 50,000 islets per pancreas. The preparation consists of 35% fully

differentiated islets, and approximately 57% endocrine precursor cells (Korbutt et al., 1996). These precursor cells allow the islets to differentiate and divide in the post-transplantation period (Binett et al., 2001; Hering & Walawalker, 2009; Korbutt et al., 1996; Rayat et al., 1999). NPI have been shown to reverse diabetes in both small and large animal models, including non-human primate (NHP) models, after a delay of up to 8 weeks due to their immaturity (Arefanian et al., 2007; Arefanian et al., 2010; Cardona et al., 2006; Kobayashi et al., 2005; Korbutt et al., 1996; Rayat & Gill, 2005; Ramji et al., 2010). They are also believed to be more resistant to hypoxic injury and less immunogenic than adult islets (Bloch et al., 1999). In addition, neonatal pigs require fewer facilities and resources in order to house them compared with adult pigs.

There are two major disadvantages of using NPI as a source for transplantation. First, a yield of 50,000 NPI per pancreas translates to the need for approximately 70 donor piglets per patient, which is much greater than adult pig donors (Korbutt et al., 1996). Second, NPI express antigens on their surface that can predispose the tissue to rejection upon transplantation. Although the most studied of these antigens is galactose α(1,3) galactose (Gal), it is not likely the only xeno-antigen responsible for rejection (Rayat et al., 1998).

A concern regarding the use of porcine tissue in humans is the possible transmission of xenosis, in particular, the transmission of porcine endogenous retrovirus (PERV). PERV is present in all pigs, as is a retrovirus encoded in their germline, and is therefore a potential source of xenosis. To date, however, there has been no evidence of transmission of this virus to humans or non-human primates (Cardona et al., 2007; Hering et al., 2006; Rood et al., 2007; Valdes-Gonzales et al., 2005).

2.2 Immunological responses to porcine islets

After the transplantation of xenogeneic tissue, there are 3 major pathways of rejection that can destroy the graft in a rapid manner – the innate immune system, hyperacute rejection mediated by pre-formed natural antibodies and complement, and acute cell mediated rejection.

2.2.1 Innate immune system – instant blood mediated inflammatory reaction (IBMIR)

The innate immune system in the form of IBMIR is the major pathway of the first mechanism of graft loss. When islets are injected into the portal vein of the recipient, there are a number of factors that activate both the coagulation cascade and platelets (van der Windt et al., 2007). The intrinsic pathway of coagulation is activated due to the remnants of collagen in the graft, which is not normally in contact with blood. In addition, tissue factor (TF), which is expressed on both α and β cells, as well as on contaminating ductal structures, activates the extrinsic pathway of coagulation. Due to molecular incompatibilities between the xenogeneic tissue and the recipient, normal feedback mechanisms, such as porcine membrane TF pathway inhibitor, which limit coagulation, do not occur (van der Windt et al., 2007). Platelets are also activated through the presence of thrombin, collagen, and von Willebrand factor bound collagen. These pathways lead to thrombus formation and subsequent ischemia and necrosis of the graft. The complement system is also activated and this is followed by infiltration of CD11+ polymorphonuclear cells and macrophages, which lead to enzymatic digestion and phagocytosis of the islets, as well as the release of cytokines that can induce apoptosis (Nilsson, 2008; Van der Windt et al., 2007). As a result, significant tissue damage occurs, and often the majority of the graft is destroyed within 24 hrs. The

damaged islets expel their insulin, and animals are at risk of becoming hypoglycemic. Although it has been demonstrated in primate models that insulin independence can be achieved even after such a significant loss of tissue, this is likely because a very large islet mass was transplanted initially. IBMIR is not limited to xenotransplantation, but the reaction is often more pronounced in this circumstance (van der Windt et al., 2007). Many strategies are being developed to attenuate this destructive reaction, including the addition of heparin in the islet preparation as seen in current clinical practice (Cabric et al., 2007), the administration of low-molecular weight dextran sulphate, and various other compounds (Johansson et al., 2006; van der Windt et al., 2007).

2.2.2 Hyperacute rejection

Natural pre-formed antibodies can lead to a dramatic reaction and loss of the graft. In solid organ transplantation, discordant grafts are rejected due to antibodies to carbohydrate moieties, in particular the Gal antigen. The Gal epitope, which is the result of the enzyme α1,3-galactosyltransferase catalyzing the transfer of an α-galactosyl residue to a terminal β-galactose, is present on all cell surface glycoproteins and glycolipids of all mammals except humans, apes, and Old World Monkeys (Galili & Swanson, 1991). These animals develop antibodies due to exposure to the epitope through enteric bacteria and other pathogens. Due to this humoral response, solid organ xenografts can exhibit significant destruction, and IgG deposits are associated with the grafts by 12 hours post-transplantation. By 2 days, there is significant IgM and C3, C5, and C9 complement depositions within 2 days of transplantation.

Porcine islet grafts, however, seem to evade the hyperacute response that solid organ xenografts experience. The typical antibody and complement deposition that is seen in solid organ grafts is not observed when porcine islets are transplanted into non-human primates (Hering et al., 2006). In addition, the use of α1,3-galactosyltransferase gene-knockout (GT-KO) pigs as donors has not yet proved to reduce the post-transplant graft loss as compared to wild-type pigs (Rood et al., 2007). Only approximately 5% of adult pig islets express Gal on their surface, whereas approximately 20% of NPI express it (Rayat et al., 2003). During both *in vitro* and *in vivo* maturation models of NPI, the expression of Gal is shown to reduce significantly as the precursor cells evolve into mature β cells (Rayat et al., 2003). Regardless, it has been demonstrated that NPI expressing and not expressing Gal can be susceptible to hyperacute rejection *in vitro*, suggesting that Gal is not the only xenoantigen responsible for this phenomenon (Rayat et al., 1998). Islet xenografts are also mainly revascularized by recipient endothelial cells (Nyqyist et al., 2005). It is for these reasons the natural pre-formed anti-Gal antibodies are not considered a major factor in the loss of islet graft tissue in the early post-transplant period (Cardona et al., 2006; Hering et al., 2006; Hering & Walawalker, 2009).

2.2.3 Cell mediated rejection

Likely the most important mechanism of rejection of porcine xenografts is via T-cell mediated processes. In rodent models, acute cellular rejection appears to be mediated predominately by CD4^{+} T-cells, as CD8 knockout but not CD4 knockout mice, reject their xenografts. In addition, due to the degree of phylogenic disparity between mice and the donor pig, the bias would be against direct recognition by CD8^{+} T cells. The signals required for direct T cell activation may not occur because of incompatibilities of molecular interactions between the pig antigen presenting cells (APCs) and the mouse T cells (Rayat et

al., 2003; Koulmanda et al., 2004). In genetically modified mice, which are MHC II deficient and therefore lack an indirect response, the rejection of fetal porcine islet xenografts is delayed. The depletion of CD4+ T cells further prolongs graft survival in these mice; however rejection is not completely prevented. This suggests that a direct response must occur by some mechanism upon T cell recovery in order for an immune response to occur (Koulmanda et al., 2004). However, in NHP and human models, the direct presentation route may be more significant, as it has been demonstrated that both CD4+ and CD8+ human T-cells can respond to porcine APCs (Yamada et al., 1995).

The likely mechanism is the processing of porcine antigens by recipient APCs and presentation to recipient CD4+ T cells, which can then activate the pathways necessary to destroy the xenograft. In acute cellular rejection, macrophages, eosinophils, and T cells infiltrate the xenograft and reach a maximum infiltrate within 4-6 days (Smith & Mandel, 2000). CD8+ T cells likely contribute to this rejection as well, although the mechanism of their involvement is not clear. Their activation may be through either donor presentation (direct) or cross-presentation by the host APCs (Rayat & Gill, 2003).

2.2.4 Autoimmune recognition

Type 1 diabetes is an autoimmune disorder, and as such, the prevention of the preceding mechanisms of rejection is not relevant if the xenogeneic tissue is recognized by the preexisting autoimmune repertoire. At present, the evidence suggests that this repertoire may be partially species specific, therefore the xenogeneic tissue may escape the effects of the autoreactivity; however the extent of this specificity is yet to be determined. If donor tissue shares epitopes with the target of the autoimmune cells and antibodies, they too may be targeted and destroyed by the native disease process.

The animal model that has been developed to investigate this is the non-obese diabetic (NOD) mouse. These mice spontaneously begin to develop diabetes after 12 weeks, and 90% of female mice will be fully diabetic by 30 weeks. The mechanism of diabetes development is similar to that which is found in human type 1 diabetes patients, where autoreactive T cells infiltrate the islets and specifically attack the β cells (Anderson & Bluestone, 2005). Strategies that have been shown to be effective in chemically induced diabetic mice have not been effective in NOD mice (Arefanian et al., 2007; Koulmanda et al., 2003). For example in our experience, the short-term administration of 2 monoclonal antibodies targeted against T cell activation failed to promote survival of NPI xenografts in NOD mice despite being highly effective in B6 mice. Graft survival in the NOD mice required the administration of an additional monoclonal antibody against CD4+ T cells (Arefanian et al., 2007). Koulmanda et al. have also demonstrated that the depletion of CD4+ T cells in NOD mice allowed for the prolonged survival of adult porcine islet xenografts. Interestingly, no further survival benefit was found when CD8+ T cells were also depleted. However, further results have demonstrated that autoimmunity may not be the reason for the difficulty in inducing tolerance in NOD mice. When the NOD mice were treated with streptozotocin (STZ) prior to the onset of diabetes, autoimmunity was avoided, as subsequent islet isografts were not rejected as they are in spontaneously diabetic NOD mice. The prolongation of adult porcine islet survival was virtually identical in both the spontaneously diabetic and STZ treated NOD mice, supporting the idea that recurrent autoimmunity does not substantially contribute to the rejection of islet xenografts (Koulmanda et al., 2003).

3. Current tolerance induction strategies

In order for T cell activation to occur, and therefore for an immune response to an antigen to occur, it must first receive two signals. The first is engagement of the antigen-specific T cell receptor and the second is a non-antigen-specific co-stimulatory signal, which is provided by an active APC. Both the direct and indirect pathways of antigen presentation, as seen primarily in NHP and rodents respectively, require this co-stimulatory interaction in order to activate T cells. It therefore seems intuitive that interference with these interactions would lead to tolerance to the foreign antigens, specifically the porcine islets. Monoclonal antibodies provide this interference and have the ability to provide long-term graft protection with only a short course of treatment, thus negating the requirement of continuous immunosuppression.
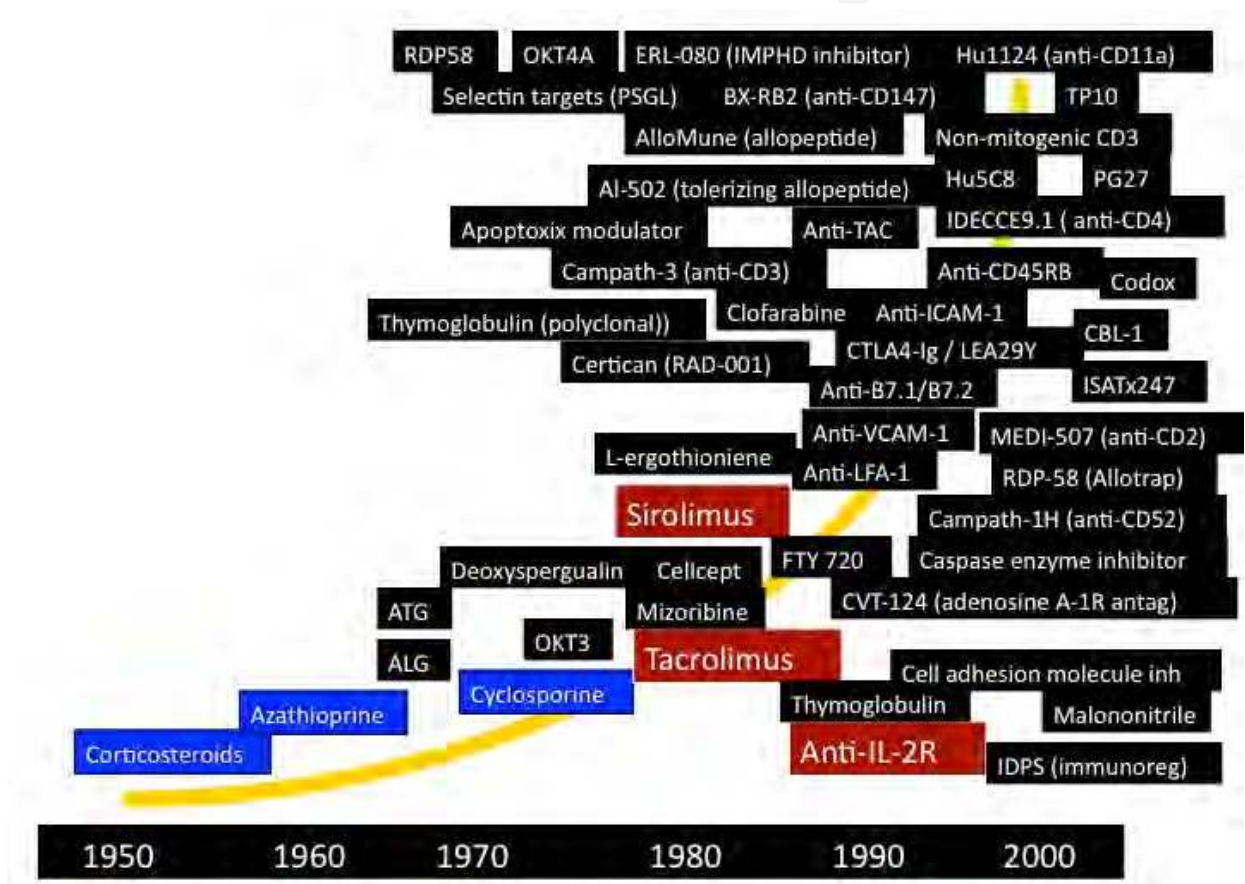

Fig. 2. The multiple strategies used over time to attempt to prevent the rejection of islet transplants.

3.1 Biologic agents - monoclonal antibodies
3.1.1 Anti-LFA-1 monoclonal antibody (mAb)

Leukocyte function antigen-1 (LFA-1) is an important adhesion molecules expressed on a variety of cells including macrophages, monocytes, and NK cells, but is most heavily expressed on T and B cells. This molecule interacts with intercellular adhesion molecules-1 (ICAM-1), which is present on vascular endothelium, lymphocytes, and macrophages. The interaction of these two molecules provides a number of functions which include: *i)* facilitating

the migration of lymphocytes to the site of inflammation; *ii)* strengthening the binding of T cells to antigen presenting cells; *iii)* providing signals necessary for the activation of T cells; and *iv)* further activating APCs to secrete cytokines and recruit other cells (Dougherty & Hogg, 1987). Due to the number and significance of these functions, interfering with this interaction can therefore lead to tolerance, which can be achieved by an anti-LFA-1 mAb.

The administration of this mAb has been shown to be effective in a number of allograft models, including cardiac, renal, and islet graft models (Nicolls et al., 2000; Poston et al., 2000; Vincenti et al., 2007). Clinically, the humanized form, Efalizumab, has been used to treat another autoimmune disorder, plaque psoriasis, with few acute side effects (Lebwohl et al., 2003). There have been 2 trials to date in clinical islet allotransplantation that have reported its efficacy (Posselt et al., 2010; Turgeon et al., 2010). In one trial, all 8 patients who received an islet transplant and were treated with an Efalizumab-based regimen achieved insulin independence; 4 of these patients achieved independence after a single islet transplant (Posselt et al, 2010). In the second study, the 4 patients that were treated with an Efalizumab based regimen achieved insulin independence after a single islet transplant (Turgeon et al., 2010). Efalizumab was ultimately removed from the market in 2009 due to 4 cases of progressive multifocal leukoencephalopathy (PML), however, none of the recipients in either of these studies demonstrated evidence of this disease (Posselt et al., 2010; Turgeon et al., 2010). In fact, these 4 cases arose from over 40,000 patients who had been treated with the medication for greater than 4 years (Turgeon et al., 2010). Although the risk-benefit profile of this medication is unfavorable for the treatment of a relatively minor condition such as psoriasis, it is perhaps better than that of the traditional immunosuppressive agents currently being used in clinical transplantation. It is therefore still being investigated as a possible agent to prevent graft rejection.

With respect to xenotransplantation, the anti-LFA-1 mAb has been shown to be effective both *in vitro* and *in vivo* (Rayat & Gill, 2005; Tredget et al., 2008). In a concordant rat to mouse model, the administration of this mAb prevented islet graft rejection in 27 of 28 mice from up to 100 days (Tredget et al., 2008). In a discordant NPI to mouse model, only 7/15 mice achieved normoglycemia with the short-term administration of anti-LFA-1 mAb, and only 6/15 maintained long-term graft survival (Rayat & Gill, 2005). Anti-LFA-1 mAb has also been shown to improve the function of adult porcine islets in mice when added to a CTLA4Ig and anti-CD154 mAb regimen (Kumagai-Braesch et al., 2007). Collectively, these studies show us that the anti-LFA-1 mAb can be efficacious in preventing islet allograft rejection, and is very promising in the prevention of xenograft rejection, however monotherapy with this medication is not sufficient.

3.1.2 Anti-CD154 mAb

CD154 is a costimulatory molecule that is a member of the Tumor Necrosis Factor (TNF) family that is predominately present on T cells. It binds with CD40 on the surface of APCs and the interaction of these molecules is thought to be critical to the maturation of APCs, the promotion of antigen presentation, and the priming and proliferation of both cytotoxic and helper T cells (Seijkens et al., 2010). In NHP models of islet allotransplantation, the administration of the humanized form of anti-CD154 mAb (hu5c8) has demonstrated significant efficacy in preventing rejection (Kenyon et al., 1999a, 1999b). In three of three baboons who received islet allografts and were treated with short-term anti-CD154 mAb, delayed rejection of their grafts was observed. This rejection was reversed by the readministration of the mAb (Kenyon et al., 1999a). In rhesus macaques, six of six recipients

of islet allografts and anti-CD154 mAb induction therapy plus monthly maintenance therapy achieved and maintained insulin independence for >100 days, with no evidence of rejection in 5 of the 6 animals (Kenyon et al., 1999b). Thus in allotransplantation, the benefits of the anti-CD154 mAb are clear.

In NPI xenograft models, the administration of anti-CD154 mAb (MR-1) as a monotherapy has yielded only modest results, with approximately 40% of diabetic mice achieving normoglycemia (Rayat & Gill, 2005). However, combination therapy of anti-LFA-1 and anti-CD154 mAbs has improved survival of porcine islet xenografts. In mice transplanted with NPI, short-term treatment with both anti-LFA-1 and anti-CD154 mAbs allowed 100% of the mice to demonstrate long-term survival of the grafts (>100 days) with only 10% rejecting prior to 300 days post-transplant (Arefanian et al., 2010). These results are substantial and clearly demonstrate that simultaneous interference of adhesion and costimulatory pathways can lead to islet xenograft tolerance. Pre-clinical models with the transplantation of either NPI or adult porcine islets have demonstrated that the humanized form of anti-CD154 mAb (H106, ABI793) is very effective when combined to existing regimens, such as basiliximab, sirolimus, and FTY720 or belatacept (Cardona et al., 2006; Cardona et al., 2007; Hering et al., 2006).

The unfortunate side effect of the humanized form of anti-CD154 mAb is that it has been shown to increase the incidence of thrombo-embolic events in both human and non-human primates (Kawai et al., 2000). The administration of heparin concomitantly with the mAb did decrease this incidence, however it remains above the acceptable limit for clinical use (Kawai et al., 2000). Non-human primate models also suggest that the administration of aspirin during the treatment with anti-CD154 mAb therapy could greatly reduce the incidence of these events (van der Windt, et al., 2009). Investigation is currently underway to search for compounds that can interfere with the CD154/CD40 interaction on the surface of T cells and APCs, respectively without the increase in thrombotic events. There is promise in the use of small inhibitory molecules which have demonstrated their ability to bind to CD154 *in vitro* and effectively block its binding to CD40 (Buchwald et al., 2009; Margolles-Clark et al., 2009; Margolles-Clark et al., 2010). These small inhibitory molecules have been identified as a number of organic dyes, including Direct Red 80 (DR80) and Mordant Brown 1 (MB1), compounds traditionally used in textile industry. They have been found to bind to CD154 and block its interaction with CD40 in a dose dependent manner, with a much higher affinity than that of the anti-CD154 mAb itself. This inhibitory effect does translate to the inhibition of B and T cell proliferation *in vitro* (Buchwald et al., 2009; Margolles-Clark et al., 2009; Margolles-Clark et al., 2010; Mihalicz et al, 2011). Preliminary *in vivo* studies have demonstrated no effect as monotherapy in the prevention of NPI xenograft rejection in mice. In combination with anti-LFA-1 mAb, however, DR80 allowed 6 of 10 mice to achieve long-term normoglycemia after transplantation of NPI. However, the compounds used in this experiment are impure with the commercially available products containing only 25-40% dye content. The rate of mice maintaining normoglycemia with this regimen is expected to improve as purification of these dyes is perfected. With less impurities, there is a greater proportion of the injected compound that is biologically active, and likely less toxicity (Mihalicz et al., 2011).

3.2 Co-Transplantation with Sertoli cells

A number of anatomical immunoprivileged sites have been identified that provide immune protection to local tissues via a variety of mechanisms. These include the testes, anterior chamber of the eye, the brain, and the placenta (Cobbold et al., 2006), and transplanted

tissue in these sites have extended survival when compared with conventional sites (Streillein, 1995). (Figure 3). The testes are of particular interest, as they seem to not only prevent inflammatory reactions and therefore bystander damage, but they also contain Sertoli cells that have a number of roles. They support the germ cells that are developing within the testes and protect them from being eliminated by the immune system. They are believed to confer this immune privilege through the release of local factors that inhibit the immune response, such as TGF-β, clusterin, serine protease inhibitors, and Fas ligand, which induces apoptosis of activated T cells (De Cesaros et al., 1992). It is possible that the co-transplantation of Sertoli cells with porcine islets could protect the graft from rejection via the same physiologic factors that protect the germ cells *in vivo*. When NPI were transplanted with Sertoli cells under the kidney capsules of rats, 66% were able to survive for >90 days with only a single injection of antilymphocyte serum (Dufour et al., 2003). Unfortunately subsequent studies have failed to support these findings. Studies with non-human primates examined the co-transplantation of NPI with neonatal porcine Sertoli cells in a number of anatomical sites including the omental pouch, kidney, pancreas, and liver. After 2 months, no insulin positive cells could be seen on immunohistochemistry, concluding that the Sertoli cells had limited ability to protect the graft (Wang et al., 2005). A controversial clinical study performed in Mexico, although deemed unjustifiable at that time by the International Xenotransplant Association ethics committee (Sykes et al., 2006), demonstrated prolonged survival of NPI xenografts when transplanted with Sertoli cells in a steel wire mesh device (Valdes-Gonzalez et al. 2005). Half of the 12 adolescent recipients showed greatly reduced insulin requirements at one year post-transplantation.
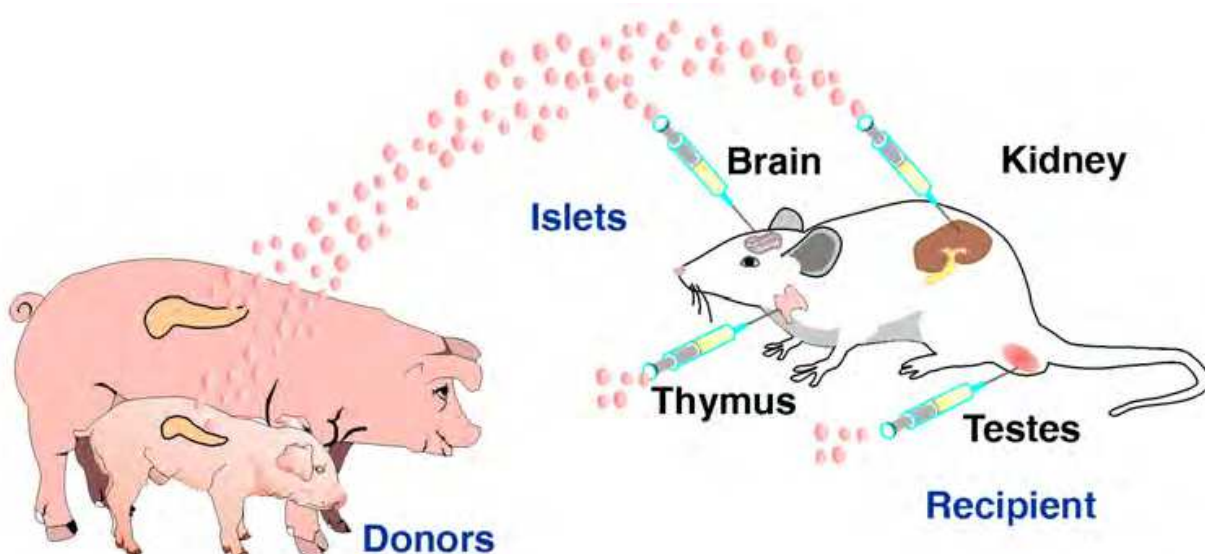

Fig. 3. Immunoprivileged sites that may be capable of conferring protection to transplanted islet xenografts.

The co-culture of Sertoli cells and islets prior to transplantation may confer additional benefits. When co-cultured with islets, Sertoli cells have been shown to facilitate maturation, expansion, and functioning of islets. It is possible that the Sertoli cells provide similar trophic support and nutrients to the islets as they do to germ cells in the native testes through secretion of various factors. In a study by Basta et al, neonatal porcine pancreatic endocrine precursor cells were cultured with Sertoli cells conditioned media, and subsequently showed enhanced

differentiation into insulin secreting endocrine cells (Basta et al.,2004). This suggests that a secreted substance is inducing these changes and is particularly exciting in the transplantation of NPI, which contain a high proportion of these endocrine precursor cells and may be influenced by these factors in a way that adult porcine islets could not.

The addition of biologic therapy to this strategy of co-transplantation has been shown to increase its efficacy. In a recent study by our group, we confirmed that the co-transplantation of NPI and Sertoli cells in mice was not sufficient to prevent rejection. However, when anti-LFA-1, anti-CD154, or anti-CD45RB monotherapy was added, NPI graft survival increased beyond that of either treatment alone. For example, 7/7 mice who were transplanted with NPI and Sertoli cells, and were treated with a short course of anti-LFA-1 mAb achieved long-term graft survival >100 days. This is in comparison with 3/7 mice, which were transplanted with NPI alone and treated with anti-LFA-1 mAb, and 0/8 mice who were transplanted with NPI and Sertoli cells but received no mAb therapy. It appears as though the synergistic effect of the immunological protection of the Sertoli cells with the inhibition of immune cell activation by the mAb therapy is able to promote long-term porcine islet xenograft survival (Ramji et al., 2010).

Although the results of the preceding studies are exciting, there is still much to be examined in order to properly bring this to clinical studies. The mechanism of this protection is as of yet unknown, and the consequences of transplanting Sertoli cells into humans, particularly female patients, is to be determined.

3.3 Microencapsulation of NPI

Immunoisolation is another strategy to protect islets from rejection, by in a sense "hiding the islets from the recipients' immune system. By providing either a micro or macro barrier around the tissue, the ideal immunoisolation device will allow for the free passage of glucose, insulin, waste and nutrients, but excluding the passage of immune cells and antibodies responsible for the rejection process. A number of devices have been studied including cellulose membranes (Risbud & Bhondem, 2001), chitosan-polyvinyl pyrrolidone hydrogels (Risbud et al., 2000), and even steel wire meshes (Valdes-Gonzales et al., 2005). (Figure 4). Perhaps the most studied, and most promising, device for immunoisolation are alginate microcapsules.

The presence of an alginate capsule can protect the NPI from destruction by human antibody and complement *in vitro* (Rayat et al., 2000). In addition, when cultured in the presence of autologous pig serum, the microencapsulated NPI mature quickly, and are able to reverse hyperglycemia in diabetic mice at a more rapid rate than non-encapsulated NPI (Rayat et al., 2000). The proposed mechanism is by providing an extracellular support matrix to the NPI, and a possible barrier to the action of $CD4^+$ T cells. While some groups have reported that microencapsulated porcine islets can survive after xenotransplantation without the use of immunosuppressive medications (Duvivier-Kali et al., 2004; Sun et al., 1996), there is further evidence that microencapsulation alone is likely not sufficient to prevent rejection (Kobayashi et al., 2005, 2008). When microencapsulated NPI are transplanted into immune competent mice without the use of immunosuppressive medications, a progressive amount of $CD4^+$ T cells, macrophages, and B cells are seen on the surface of the capsule over time. At no time points are immune cells seen within the capsule, however, there is evidence that complement (C3 and C4) and anti-porcine IgG antibody are able to traverse the barrier. This antigen-specific response suggests that xenoantigens are
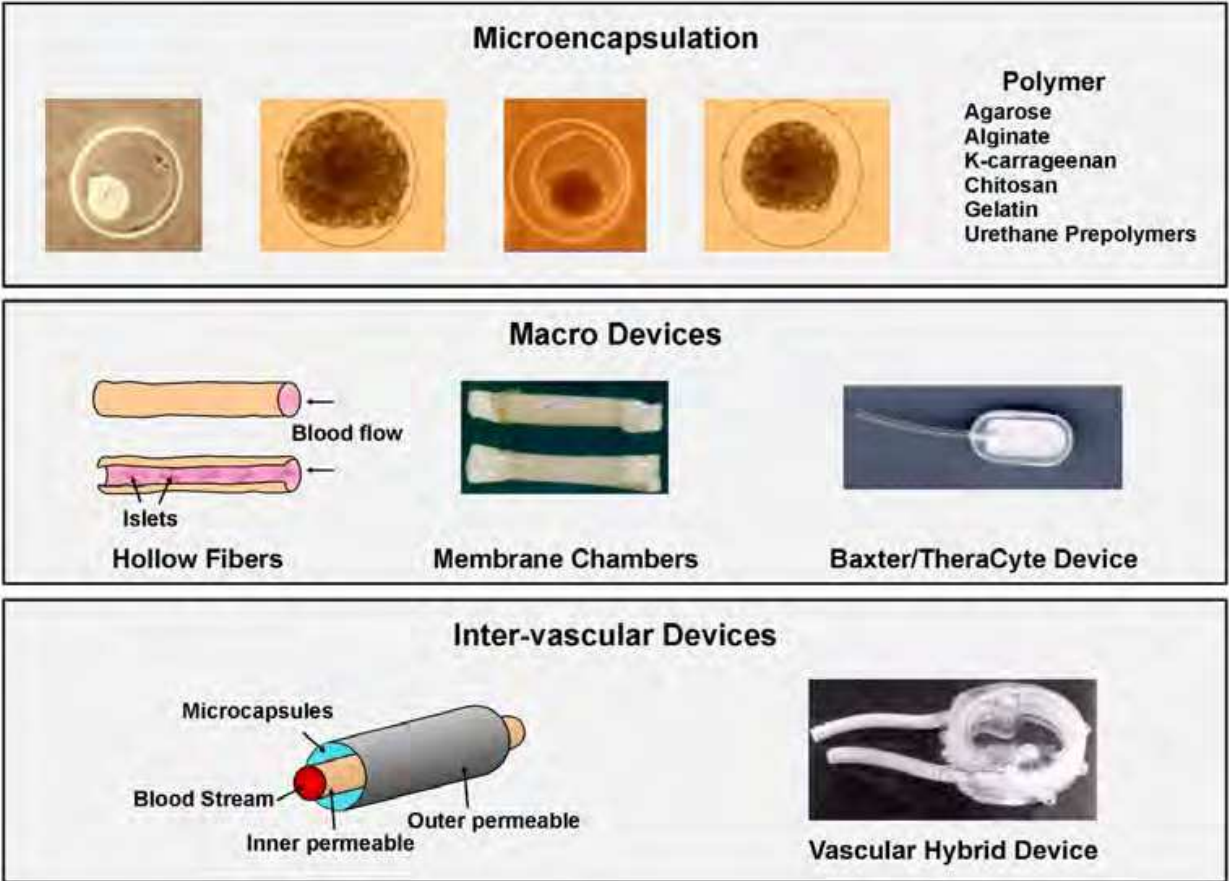
Fig. 4. Various immunoisolation devices examined for the transplantation of islets in an effort to evade the host immune system.

likely shed from the capsule which can then interact with the recipient APCs, leading to an indirect immune response. This immune response is sufficient to kill the NPI, as none of the transplanted B6 mice achieved normoglycemia, and by day 50 post-transplantation, nearly half of the cells in the NPI were non-viable (Kobayashi et al., 2005). The *in vivo* maturation of microencapsulated NPI in immunodeficient mice did improve the survival of the grafts in subsequent transplantation into immune competent mice. This seems to be due to the maturation of intact NPI and a decrease in their immunogenicity (Kobayashi et al., 2008).

Microencapsulated islet graft survival can be prolonged when the combination of anti-LFA-1 and anti-CD154 mAbs are also administered *in vivo*. In one study, all mice that were transplanted with microencapsulated NPI and treated with these mAbs achieved normoglycemia, with >50% maintaining normoglycemia for over 100 days. This is in contrast to only 1/20 untreated mice achieving normoglycemia. The therapy appears to prevent a humoral response, which may be the result of an altered indirect antigen response. It also prevented the migration of immune cells to the microencapsulated islets possibly through interfering with the secretion of chemokines, cell motility, and ultimately T cell activation (Kobayashi et al., 2005).

Although it seems clear that microencapsulation alone is likely not sufficient to prevent the xenorejection of NPI, when combined with other strategies such as the *in vivo* maturation of NPI and the administration of mAb therapy, it does provide some beneficial effects. Microencapsulation remains a promising strategy in providing protection to NPI and

warrants further investigation. In fact, a recent study by Dufrane et al. demonstrated survival of microencapsulated adult porcine islets for 6 months post-transplantation without immunosuppression under the kidney capsule in non-diabetic primates (Dufrane et al., 2006). This same group later showed that macroencapsulated porcine islets in a monolayer cellular device can reverse diabetes in NHP for up to 6 months post-transplantation in the absence of immunosuppression (Dufrane et al., 2010). These studies established that both micro- and macroencapsulated porcine islets are able to function long-term in stringent xenogeneic models. The challenge will be to provide the appropriate additional support, possibly in the form of the aforementioned monoclonal antibodies, in order to carry survival of the islets past the 6 month mark.
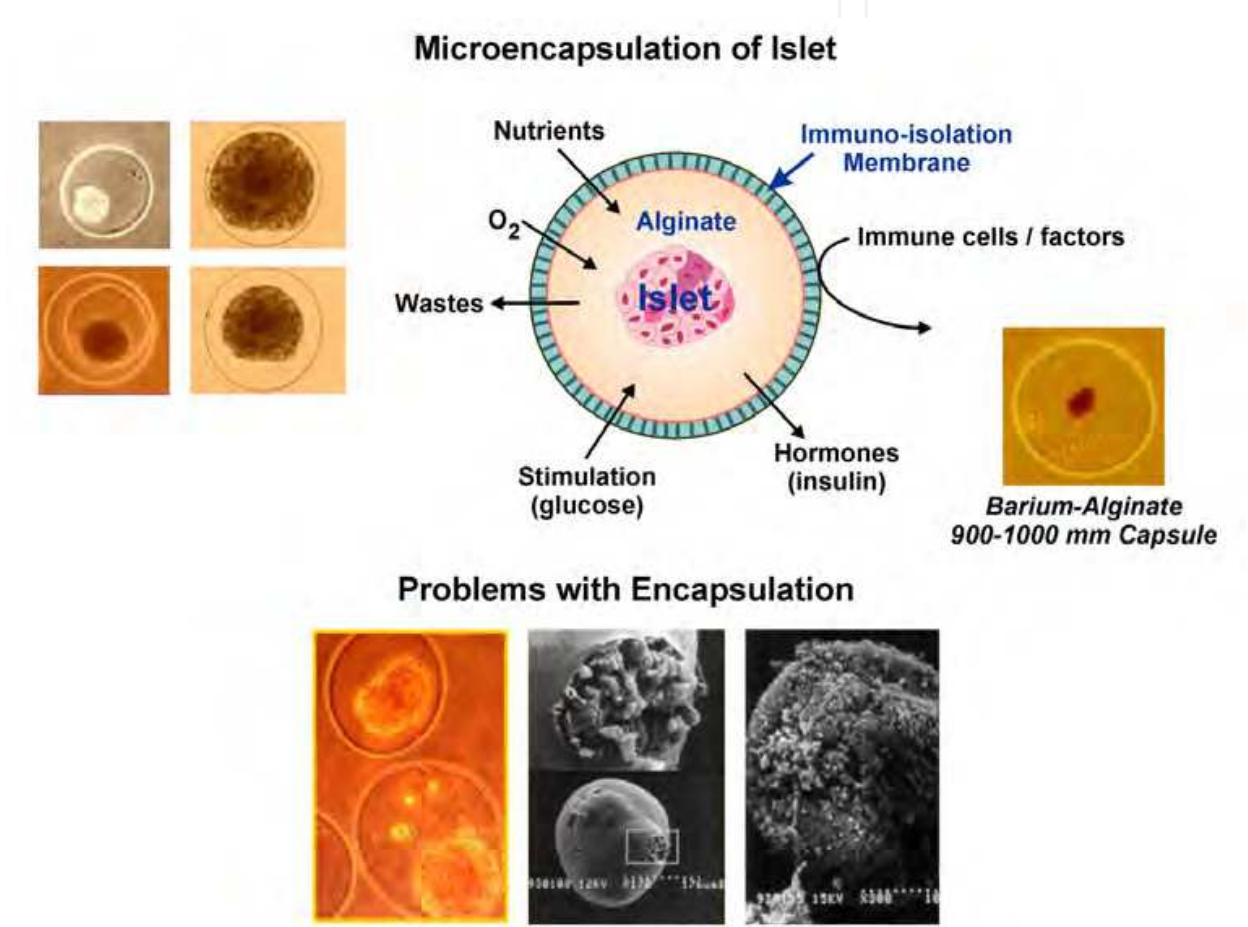

Fig. 5. Microencapsulation of islets for transplantation. The capsule allows for the passage of insulin and wastes out of the islets cells, while nutrients, including glucose are able to enter the islets. Immune cells, however, are not able to pass through the barrier, potentially protecting the islets from rejection. Problems with microencapsulation include protrusion of islets which could cause rupture of the capsule attracting the immune cells leading to graft failure.

4. Future prospects for tolerance induction

The future of tolerance induction in porcine islet xenotransplantation may in the use of genetically modified pig donors such as human CD46 (hCD46) transgenic pigs (van der

Windt et al., 2009), N-acetylglucosaminyltransferase-III (GnT-III) transgenic pigs (Komoda et al., 2005), and alpha 1,3-galactosyltransferase knock-out (GT-KO) pigs (Casu et al., 2010). GnT-III transgenic pigs have been shown to have significantly down-regulated xenoantigens, both Gal and non-Gal, due to a mechanism that is not fully understood. Cynomolgus monkeys were transplanted with these islets under their kidney capsule showed slightly longer survival than wild-type pig islets in the absence of immune suppression, although these results were not significant, and question regarding the usefulness of these donors has arisen (Komoda et al., 2005). GT-KO pigs lack the enzyme alpha 1,3-galactosyltransferase, therefore the donor tissue lacks the xenoantigen Gal (Casu et al., 2005). This mutation has been shown to have minimal effect on glucose metabolism in the donor pig (Casu et al., 2005), however has shown little in the way of advantage in terms of transplant rejection in preliminary animal models (Bottino et al., 2007).

The hCD46 transgene has been introduced into porcine donors in an attempt to circumvent the IBMIR reaction. CD46 is a human complement regulatory protein, and as such is believed to assist in the avoidance of complement mediated early destruction of islets upon portal infusion. In a preclinical model using Cynomolgus monkeys, the hCD46 transgenic pigs did allow for a significantly longer period of insulin independence, even up to one year post-transplantation, as compared to wild-type pig donors, who only maintained normoglycemia up to 36 days. In addition, both sets of islets were bound with anti-pig antibodies, however, only wild-type pig islets were bound with complement. Despite this fact, early graft loss was detected in both groups, as evidenced by a release of C-peptide and hypoglycemia in the recipient (van der Windt et al., 2009). The numbers of recipients in this study was small, however the results are perhaps the most promising of any of the studies involving transgenic pigs to date.

Another future prospect for the induction of tolerance is the creation of mixed hematopoetic chimerism. Creation of this chimeric state can occur after hematopoietic stem cell transplantation, or bone marrow transplantation, which can effectively induce tolerance to the subsequent islet graft. This is done by establishing "self" tolerance to the islet graft by the donor immune cells. This has been performed in both allotransplantation and rat to mouse xenotransplantation, leading to graft acceptance and ultimately normoglycemia in the host animals. This strategy has also been shown to reduce the number of allogeneic islets required to achieve normoglycemia in NOD mice (Zhang et al., 2010). In xenotransplantation, the simultaneous or subsequent transplantation of rat bone marrow and islets to chemically-induced diabetic mice resulted in the grafts being permanently accepted and allowed the recipients to achieve normoglycemia (Zeng et al., 1992; Li et al., 1994).

The practicality of chimerism for tolerance induction in NPI transplantation is in question, however, as multiple neonatal donors will be required for one human recipient. This would require a large amount of bone marrow transplantations which may not be feasible, as there may be a limit to the number of bone marrow strains that can repopulate an animal (Chester et al., 1989). In addition the engraftment of the bone marrow tissue itself currently requires an immunosuppressive regimen, trading one potentially harmful regimen for another.

5. Cultural views of porcine islet xenotransplantation

Additional barriers to the clinical use of islet xenotransplantation as a treatment for type 1 diabetes are the ethical and cultural issues surrounding it. A number of surveys have

examined public opinion on this issue, dating back to the mid 1980's (Hagelin, 2004). Although often flawed and biased, review of these articles reveals that there is not overwhelming support for xenotransplantation, with acceptance rates of 40-50%, which can increase to 70-80% in the case of patients who may directly benefit from xenotransplantation (Deschamps et al., 2000; Hagelin, 2004; Persson et al., 2003). However, the proportion of people directly opposing it appears to be decreasing over time (Hagelin, 2004). Some studies identified factors such as a lower level of education, an older population, female gender, and being very religious as factors that decreased the likelihood of supporting xenotransplantation (Hagelin, 2004; Rios et al., 2010). In a study of Latin American health care workers, the acceptance of xenotransplantation was influenced by their specific job in the field, specifically physicians being more supportive of the idea (77%) compared with auxiliary staff being less supportive (40%) (Rios et al., 2010).

Specific to islet xenotransplantation, a recent survey of Latin-American diabetic patients revealed 79% indicated acceptance of porcine islet xenotransplantation. Seventy-five percent indicated they would accept the porcine tissue even if it only reduced their insulin requirements, temporarily prevented the progression of secondary complications, or needed to be repeated every six months. Interestingly, 40% indicated that they believe living with porcine cells could cause them psychological distress (Abalovich et al., 2010). This is in contrast to a French study from 5 years previous, where it was found that the proportion of individuals with type 1 diabetes willing to accept a transplant was much lower. Before the risks of the procedure were explained, 52% said they would agree to islet xenotransplantation. After it was explained that xenotransplantation may require more intense immunosuppression than conventional allotransplantation, leaving the patient more vulnerable to infections and malignancies, 70.5% refused. Patients also stated that insulin independence was their greatest priority, taking precedence over a reduction in complications or an increased life expectancy, therefore if this is not achieved, they were not willing to accept the risks of the procedure. Other reasons for refusal were cited as the risk of disease transmission and other risks that may not be yet identified (Deschamps et al., 2005). Although these are reasonable concerns, as the strategies to overcome these barriers are developed, it is possible these issues will be addressed by the time islet xenotransplantation becomes a viable clinical option. Therefore, although in some regions the acceptance of islet xenotransplantation may currently be limited, it is still prudent to continue the research in this area to develop a safe and acceptable treatment regimen.

6. Conclusions

Type 1 diabetes is a debilitating and costly disease, but the use of islet transplantation can reduce the burden on both the patient and the healthcare system. Due to a shortage of donor tissue, xenogeneic tissues, in particular porcine islets, are being investigated as a source of insulin producing cells. The main challenge in using these tissues is to provide a means to evade the host immune system in order to maintain a viable and functioning graft. Many methods are under investigation in order to induce tolerance, many with promising results. Due to the redundancy within the highly sophisticated immune system, the likely solution will be a combination of these strategies, which will allow us to circumvent these immune mechanisms from more than one angle at a time.

7. Acknowledgements

8. References

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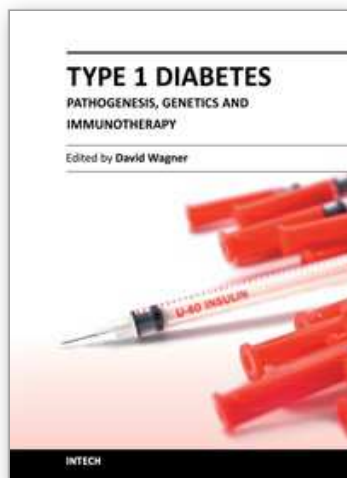
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This book is a compilation of reviews about the pathogenesis of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes. Areas including T cell development, innate immune responses, imaging of pancreata, potential viral initiators, etc. are considered.

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