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Moving Islet Cell Xenotransplantation to the Clinic

Wayne Hawthorne

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Abstract

The ultimate goal to provide a cure for all patients suffering from type-1-diabetes has remained out of the patients reach despite major advances in technologies. There has however, for a number of decades been a concerted effort to use various forms of porcine β-cells as a replacement transplant alternative to cadaveric human donors. This has seen major advances in the last decade with significant development of multi-transgenic donor pigs that now can potentially be used for xenotransplantation. This has been achieved with cellular transplants leading the way using porcine islet cell transplants as a form of β cell replacement in pre-clinical studies to treat diabetic non-human primates in various guises. These uniquely modified islet cells have the potential to offer an unlimited source of insulin-producing cells once we have solved all of the issues required to prevent loss of the xenotransplant. This chapter provides an in depth overview as to how the most recent advances have been achieved in regards to the genetic modification of donor pigs to provide protection from hyperacute rejection, instant blood mediated inflammatory reaction, xenoantibody and cellular responses to provide long-term functional islet cell xenotransplants to be able to move islet cell xenotransplantation to the clinic.

Keywords: clustered regularly interspaced short palindromic repeats, diabetes mellitus, graft rejection, immunosuppression, islets of langerhans, primates, swine, transplantation, hyperacute rejection, instant blood-mediated inflammatory reaction, islet cell transplantation, neonatal islet cell clusters, thrombosis, Type-1-diabetes, transcription activator-like effector nucleases, xenotransplantation, zinc finger nucleases

1. Introduction

The major landmark in the development of a treatment for type-1-diabetes (T1D) occurred almost a century ago, with conventional treatment still utilising exogenous insulin therapy.

Clearly for the majority of this time the main stay for its use was that of porcine insulin until it was usurped by recombinant human insulin in the early 1980s [1]. Despite the development of these newer insulin's and their modified treatment regimens still used to this day, insulin's role only remains in the prevention of elevations of blood sugar levels which ultimately give rise to ketoacidosis and ultimately death, where it is not adequately controlled [2]. Even with the advent of the insulin pump (IP) and integrated control with a Glucose Management System (GMS) the fact remains that the life-saving benefits of exogenous insulin therapy are still inadequate to prevent the serious long-term secondary complications of T1D. Sadly, patients with T1D still suffer significantly from cardiac disease, nephropathy, retinopathy and micro vascular disease. Even with the use of insulin pumps, it still has not reduced the severe hypoglycemic episodes resulting in coma and even death of these patients due to the significant lack of biofeedback and blood sugar re-balancing by glucagon secretion as produced by islet cells [3, 4].

The current transplant treatments being offered to patients suffering from T1D, both whole organ pancreas and pancreatic islet cell allotransplantation remain the Gold standard in treatment but are only available to treat small numbers, more or less just subsets of patients with T1D. In the case of simultaneous pancreas and kidney (SPK) transplantation where the whole pancreas is transplanted in combination with the kidney to treat patients with T1D and renal failure these patients do incredibly well, but this transplant is a major surgical procedure that is not an option for all patients suffering from T1D [5, 6]. Likewise islet cell transplantation remains an option only for the small subset of patients suffering severe hypoglycemic unawareness [7, 8]. These patients have seen great benefits from their transplants such as protection from hypoglycemia and from the progression of the severe secondary complications. Despite the benefits pancreas and islet allotransplantation provide, we remain unable to offer these treatments universally to all patients with T1D due to the significant shortage of donor organs and the need for continuous immunosuppression to prevent graft rejection [9, 10].

The major reason we may never be able to offer widespread application of such transplants is the sad and unfulfilled ability to provide what is an extremely valuable resource in that of human organ donor supply, where a gap remains unlikely ever to be filled with a disproportionate gap in the supply as compared to the demand for transplants. To be able to offer an alternative source of tissue for clinical transplantation we must be able to produce cells that are demonstrated to be safe and effective from a reproducible alternative source of β-cell replacement tissues [11]. Decades of concerted effort in the xenotransplantation field has seen the emergence of porcine islets as the most plausible source of tissues to provide a safe, effective, reliable and renewable source of islet cell tissues [12]. However, in order for xeno-islet-transplantation to move to clinical therapy we must clearly provide a safe and stable porcine donor source that avoids the many existing barriers of xenotransplantation, including the necessity for a suitable and effective immunosuppressive regimen [13]. To be able to do this, those of us in the xenotransplantation field have concentrated on utilizing the pig as the donor source due to it being an easily housed and bred animal that has been farmed for centuries. It is also of a large

enough size that organs from pigs can quite readily size match humans for transplanta-tion. Additionally, for such a large animal they are also rather unique, having a relatively short gestational cycle being less than four months (3 months 3 weeks 3 days) and they produce large litters (generally 10 but can have up to 20 in a single litter). This places them in a rather fortuitous position for us to be able to utilize them as a reproducible tis-sue source for generation of donors for tissues and cells, following appropriate genetic manipulation. Genetic manipulation has been necessary in order to be able to transplant pig tissues across the xenogeneic barriers into humans and this has taken multiple trans-genes to achieve [11, 14].

Xenotransplantation leads all other technologies in the race to provide a viable source of transplantable tissues to treat T1D due to the incredible advances in technologies provid-ing the ability to manipulate the donor tissues or cells prior to transplantation. Over the last two decades we have seen incredible advances and changes in the technologies available for such, even since the first genetically manipulated transgenic pigs were produced express-ing the human complement regulator CD59 [15], we have seen more dramatic advances due to the rapid adoption of extremely cutting edge technologies which are further discussed below. This is even reflected in the more traditional cloning technologies where zinc finger nucleases (ZFNs) [16, 17] have been used to develop a number of new pig lines. But by far the most effective and potentially further productive multiple transgenic combinations has been the adoption of targeted gene knockouts using transcription activator-like effector nucleases (TALENs) where there have been amazing advances in the production of pigs with multiple genetic knockouts [18]. The more recent advances have slingshot the production of transgenic pigs forward many years by the use of type II clustered regularly interspaced short palin-dromic repeats (CRISPR)/Cas9 system [19–21]. These various strategies of genetic modifica-tion have 'humanized' the transgenic pig to be more readily usable in the clinical setting. Most notably, these approaches have aimed at either antigen reduction or human transgene expression [22].

This chapter provides an in depth overview as to how the most recent advances have been made in regards to the genetic modification of donor pigs to provide protection from hyper-acute rejection, IBMIR, Xeno antibody and cellular responses to provide long-term functional islet cell xenotransplants to be able to move islet cell xenotransplantation to the clinic.

2. Overcoming the Islet-specific barriers inhibiting islet xenotransplantation

Islet cell xenotransplantation has been plagued by not only xenotransplant barriers but also islet specific barriers, which have inhibited its success until they have recently been overcome. The most significant barrier in xenotransplantation has been seen at the time of first point of contact with the recipient's blood stream and thus the cellular and anti-body targeting cofactors. Primary graft loss can occur almost immediately following the first direct recipient blood contact with the graft. This is due, principally, to hyperacute rejection

(HAR). HAR occurs when the xenoreactive natural pre-formed antibodies to the oligosaccharide galactose α1-3 galactose (αGal) of the recipient recognize the xenoantigen on the graft endothelium, and the resulting complement-mediated immune response leads to the triggering of the cascade of humoral and cellular events. These preexisting antigens on the cell surface of the donor tissue are rapidly recognized by the recipient's antibodies after revascularization which initiates the complement coagulation cascade, leading to thrombosis and edema and also the triggering of cellular inflammation that destroys the graft as quickly as within minutes to hours.

More readily observed in whole vascularized organs where HAR is characterized by immediate vascular engorgement and discoloration of the organ as can be seen in **Figure 1** of a kidney transplant one-hour post-transplantation whilst undergoing HAR.

Islets do not have large blood vessels or a significant vascular endothelium for this to occur in the same way. However, islets are infused into the vasculature of the liver usually via the portal vein. Following their transplantation into the liver's sinusoids, they trigger an almost immediate and profound micro and macro-vascular thrombotic change within the liver. This ultimately leads to larger vessel thrombosis and potentially thrombosis of the liver that they have been transplanted into [23].

Also at the time of transplantation islet cells are exposed to other barriers such as islet specific inflammation (instant blood mediated inflammatory reaction) referred to as IBMIR [24]. IBMIR was first demonstrated by William Bennet a PhD student in the Swedish group of Korsgren and Groth where he exposed human islets to freshly collected human ABO-compatible non-anticoagulated blood which he placed together into surface coated-heparinized polyvinyl chloride tubing loops to observe the resultant reactions and then subsequently also performed intraportal transplantation of porcine islets into pigs to observe and report on the advent of IBMIR [24].

Others have subsequently explored this process in depth using both human and xeno-islets. What they discovered was that allo-islets coalesced and ended up embedded in clots, where
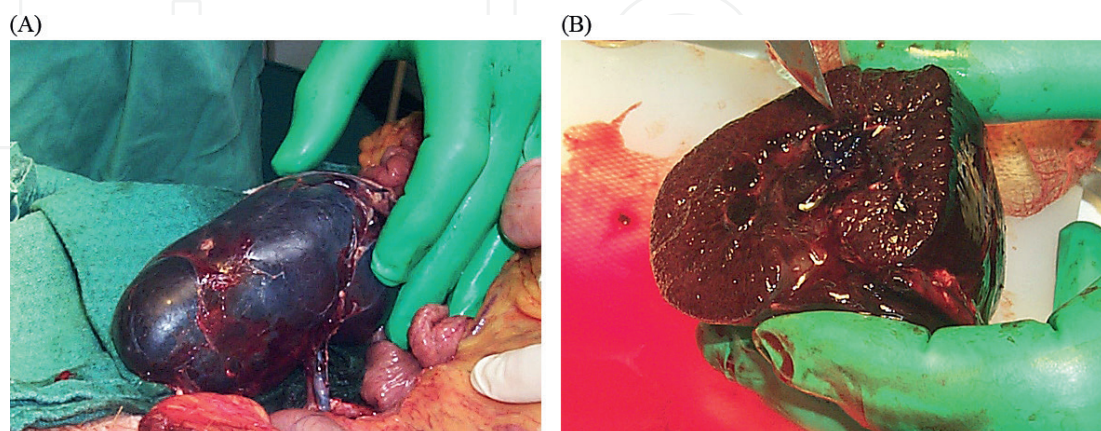

Figure 1. (A) Hyperacute rejection of a kidney graft 1 h post revascularisation. (B) Note the extremely engorged appearance of the graft with vascular thrombosis, engorgement and edema of the pulp of the graft.

they were infiltrated specifically with leukocytes and their cellular morphology was disrupted. Xeno-islets tend to also be infiltrated with neutrophils and the process occurring in a more aggressive and destructive manner [25, 26]. The macro-loop system setup provides a useful tool to explore the mechanisms that occur in IBMIR in both allo and xeno islet transplantation. The setup of the loop system can be seen in **Figure 2** where porcine islets are mixed with 7 ml of freshly collected human ABO compatible blood and incubated for an hour.

Fortuitously this process can be abrogated with the addition of anticoagulants such as heparin, which are added to the islet milieu in combination with a soluble complement receptor, completely preventing or delaying this process from occurring. More specifically it can prevent the initial triggering of the inflammation and as such reduce both initial islet loss and subsequent specific immune responses [25, 26]. However, in the Xenotransplantation setting it appears that the same protection may not be offered unless islets are specifically developed to avoid the IBMIR process.

In addition to these immune activated processes, pancreata and islets are also extremely affected by hypoxia that occurs with the organ donation process and removal of the organ from its blood supply. More significantly the loss of islet cell vascularization and the depravation of oxygen to the islets during the islet isolation process cause significant damage and loss of the islets [27].

We therefore have a number of unique possibilities, as we are able to develop transgenic pigs as donors where we can address these various problems and prevent them from occurring. With the genetic engineering of porcine donors we can modify them in such a way that they are more compatible with the human recipients they will be transplanted into. Multiple genetic manipulations have already proven useful specifically in relation to hyperacute rejection, IBMIR, hypoxia, innate immune responses and even T cells with the potential of even further advancement in the near future [28].
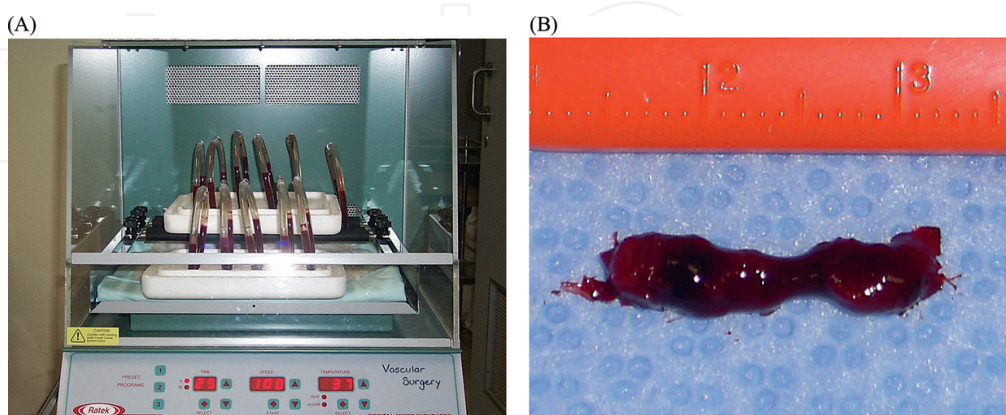

Figure 2. (A) An orbital mixer incubator with the PVC tubing loops containing porcine islets mixed with freshly collected non-anticoagulated human ABO-compatible blood. (B) The resultant clot after one hour of incubation of wild type porcine islets mixed with freshly collected non-anticoagulated human ABO-compatible blood.

2.1. Overcoming hyperacute rejection by genetic modification

The most impacting of the barriers, HAR, caused by the existence of the pre-formed anti-bodies to αGal prevent direct xenotransplantation as they are expressed at high levels on pig cells and in particular on islet cells, but the rate differs depending on the age of the pigs, seen at their highest levels on neonatal islet cells [29, 30]. In an eloquent study Rayat et al. examined αGal expression on various porcine islet cell preparations and correlated this with the proportion of cytokeratin 7 (CK7)-positive ductal cells. In vitro and in vivo expression of αGal and CK7 was significantly ($p < 0.05$) higher in less mature neonatal islet cells compared with matured neonatal islet and adult porcine islet cells, while the reverse was observed in the proportion of beta cells [31]. These pre-formed antibodies to the oligosaccharide galactose α1-3 galactose are not on human tissue and thus HAR does not occur in the allo setting [32].

Attempts to prevent HAR, for example by depletion of anti-pig Abs in recipients, were not very successful and it was not until genetic modification of donor pigs that any form of protection was provided. Numerous studies have subsequently utilized an array of genetic modifications in the genetically modified donor pigs for protection. But by far the most dramatic of impacts was that seen when the first genetic modification occurred with the deletion of the xenoantigen αGal known as the Gal Transferase Knockout (GTKO) [33–35], which still remains the pig of choice as the genetic background for application of further genetic modifications. There has been quite profound data showing that following the genetic manipulation of pigs to have transplantation of neonatal porcine GTKO islets into diabetic rhesus macaques, the neonatal porcine GTKO islets had significantly decreased susceptibility of the xenografts to innate immunity mediated by complement and preformed xenoantibody, and increased survival and function when compared to wild type islets [36]. We also saw the same abrogative effects in baboons transplanted with porcine neonatal islet cell clusters (NICC) from pigs with GTKO background when compared to wild type NICC [37]. The profound effects of transplanting wild type NICC into the baboon liver produced an almost immediate production of micro-thrombi surrounding the αGal-positive wild type NICC. This clot contained fibrin, RBC, and leukocytic infiltrate from as early as 1-hour post-transplant. Even using a combination of anticoagulation consisting of heparin and recombinant human antithrombin NICC's underwent HAR. NICC were surrounded by large numbers of platelets, monocytes and neutrophils and areas staining positively for complement C3c in surrounding clot. Neutrophils were seen infiltrating NICC that stained positive for IgG deposition, leading to early destruction [37]. As can be seen in **Figure 3A** the Wild type NICC were trapped in clots, whereas can be seen in **Figure 3B** the GTKO background NICC were not thrombosed at all at any stage post transplant being able to survive long-term for more than a year post transplant.

In a very short space of time technology has leapt forward and we have established a new era in molecular biology with the advent of novel and extremely cutting edge technologies, which make great changes to the way we genetically modify pigs. The most amazing leap forward came with a new tool based on a bacterial CRISPR and CRISPR-associated (Cas), in
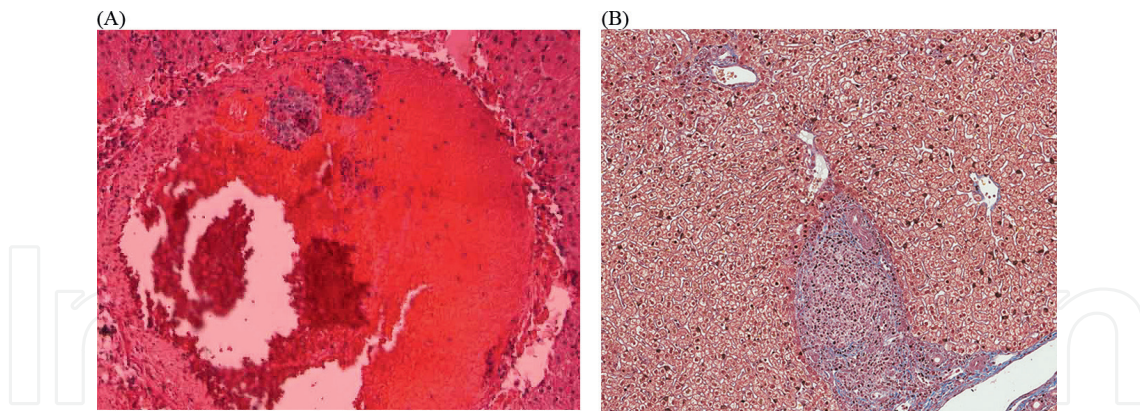
Figure 3. (A) A photomicrograph from a histology slide of a biopsy of a baboon liver transplanted with wild type NICC, note the two NICCs contained in a large clot consisting of RCC, platelets, fibrin and infiltrating cells that almost obstruct the entire venule it is sitting in. Section is at 100× magnification and stain is H&E. (B) A photomicrograph from a histology slide of a biopsy from a baboon liver transplanted with NICC from GTKO background neonatal pig, the islet is intact and well revascularized with minimal infiltration at 1 week post transplant, note the open portal venule with no sign of micro or macrovascular thrombosis. Section is at 200× magnification and stain is MSB.

particular the associated protein-9 nuclease (Cas9) from *Streptococcus pyogenes* which has led to a revolution in technology, causing considerable excitement in the entire science world let alone in xenotransplantation. In particular the genes have great potential to be exploited in the system for RNA-programmable genome editing [38]. Now a number of groups have been able to use the CRISPR/Cas9 system to efficiently perform biallelic knockout of the α-1,3-galactosyltransferase gene in porcine blastocysts derived from the somatic cell nuclear transfer of αGal epitope-negative cells which also lacked the αGal epitope on their surface [39].

There have also been a number of other quite recent publications in which it has been shown that researchers have targeted various combinations to delete α(1,3)galactosyl transferase (GGTA1), the gene for the enzyme cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH) resulting in the generation of pigs that do not express NeuGc [20]. We have also seen the use of the slightly older but still cutting edge technology that is still very effective, TALENs which has been designed to target exon 6 of porcine GGTA1 gene resulting in the production of GGTA1-null miniature pigs [40, 41]. Miyagawa and colleagues were the first to generate the α1,3 galactosyltransferase and cytidine monophospho-N-acetylneuraminic acid hydroxylase gene double-knockout pigs using this system [40]. ZFNs and somatic cell nuclear transfer have also been used for the generation of GGTA1 biallelic (double) knockout pigs [17, 42].

Quite clearly the multitude of very new and extremely effective technologies have provided an array of avenues to establish a clear platform from which we can progress additional genetic manipulations required to move xeno islets to the clinic.

2.2. Overcoming IBMIR by genetic modification

IBMIR is also a well-known mechanism effecting islets and islet like cells, which can occur almost immediately or even up to hours after the islet infusion in the allo, auto and xeno-graft setting [43, 44]. Islets specifically undergo targeted innate thrombotic and inflammatory

responses, which, like HAR results in coagulation, complement activation, infiltration of immune cells and platelet adhesion [45]. Certainly one of the major players appears to be complement activation, especially via the alternative pathway, which has been shown to greatly contribute to the triggering of IBMIR in the non-human primate (NHP) islet setting [13]. Specifically the triggering of IBMIR revolves around the release of inflammatory mediators including IL-8, MCP-1 and macrophage migration inhibitory factor which is very similar to what occurs in HAR with the triggering of thrombin then promoting the activation of monocytes, neutrophils and platelets that aggregate at the point of contact with the graft. It appears the major trigger for IBMIR therefore is tissue factor, which is expressed on the surface of islets and also the fragments of acinar tissue, which surround or are sometimes attached to the islet [43, 46, 47].

More complex than originally thought, it also appears that there is a synergistic influence between the coagulation cascade and platelets which are thought to exacerbate IBMIR and as such continues the destructive cycle without direct triggering. Experimental complement activation can be controlled by cobra venom factor, coagulation by heparin or low molecular weight dextran, and platelet activation by anti-platelet agents such as Plavix [13]. This is useful as proof of principal in the experimental setting where these drugs and agents have been used, however, it is best that they are not used clinically, so alternative drugs and agents specifically targeting complement such as Compstatin, human factor H (HFH) and intravenous immunoglobulin (IVIG) are under investigation [13]. Quite clearly, as pointed out more than a decade ago, if we are to make inroads into preventing IBMIR of islets or islet like tissues we must block tissue factor or inhibit its expression to prevent the thrombotic response *in vitro* [44, 45].

Fortunately, like the problems of HAR, this barrier has been overcome by the clever genetic modification of the donor pig to avoid αGal but also express human complement regulators such as CD46/55/59 [37]. Promising results have been achieved with porcine islets from hCD46-expressing pigs, which were transplanted into diabetic cynomolgus monkeys, normoglycemia was achieved in four of five monkeys with up to 3 months follow-up [48]. By far the most impressive results to date were achieved when porcine islets from αGal-deficient pigs, protecting against the pre-existing xeno antibodies, and also transgenic for the human complement regulators CD55 and CD59 were transplanted into the immunologically taxing baboon model. The baboons received NICC and were treated with a clinically relevant immunosuppressive protocol. The islets were less susceptible to humoral injury, induced significantly less complement activation and thrombin generation limiting antibody-mediated rejection compared to wild type thus proving that xeno islets can be protected from both HAR and IBMIR in the xenotransplant setting [23].

Following the rapid uptake of the most cutting-edge technologies by the xenotransplant community we have seen dramatic advances in xenotransplantation with the CRISPR/Cas9-directed mutation and human transgene delivery system that have been used to genetically modify and develop specific pigs to prevent HAR. CRISPR/Cas9 was used to delete the GGTA1, cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) and Beta-1,4-N-Acetyl-Galactosaminyl Transferase2 (B4GALNT2) genes. As a very targeted use

of the CRISPR/Cas9 system this approach provided a significant xenoantigen reduction with the abolition of the porcine carbohydrate profiles and as such effectively prevented antibody-mediated complement dependent cytotoxicity from occurring [22].

These genetic modifications to protect against HAR and IBMIR have been mitigated by genetically disrupting the α1,3-galactosyltransferase gene and other targeted approaches to reduce xenogenicity as shown above by targeting the many various transgenes such as; GLA, HT, hGnT-III, GT-KO, CMAH, (Neu5Gc)-KO, class I MHC-KO, iGb3s-KO, β4GalNT2-KO [11, 13, 17, 22, 23, 37, 40–42]. Clearly remarkable these have made massive inroads in the direction that we need to travel to be able to move islet xenotransplantation to the clinic.

2.3. Overcoming hypoxia and inflammation by genetic modification

Ultimately the overt processes of organ donation and the islet cell isolation strip and denude the islet cells of their vasculature and deprive the islets of the oxygen and nutrients they require to sustain them. Ultimately this causes irreversible damage to the islets resulting in graft loss. Obviously the main cause of this is from hypoxia and as such is contributed to by hypoxia inducible factor (HIF)-1α and activation of its target genes that end up causing impaired islet function, apoptosis and eventually cell death [49–51]. One of the fundamental ways to potentially improve outcomes is to increase the oxygen delivery to the cells during the organ retrieval process. This has been undertaken by the use of various experimental devices such as the use of the two layer method [52, 53] and also by the experimental use of persufflation [11]. The two-layer method (TLM) utilizes a perfluorochemical (PFC) and UW solution to store the pancreas during shipping. The benefits of the use of the PFC are theoretically because it is a biologically inert liquid that acts as an oxygen-supplying media. A pancreas preserved using the TLM is oxygenated through the PFC and substrates are supplied by the UW solution. Reportedly this allows the pancreas to be better preserved using the TLM to generate adenosine triphosphate during storage, prolonging the preservation time [11].

Numerous attempts have been undertaken to improve the supply of oxygen to islets including *in situ* oxygen generation and improved revascularization of the graft and the islets themselves [54] along with possible systemic treatments of the islets during culture and transplant using already clinically approved agents such as desferrioxamine (DFO) [55]. There have been a few ways developed to improve hypoxia by the modification of the xenotransplant utilizing genetic modification of the donor pig to date.

The most specific targets investigated have been using the transgenic expression of the human A20 gene in cloned pigs. The zinc finger protein A20 is an important negative regulator of inflammation; polymorphisms in the corresponding gene, *TNFAIP3*, have been reported to be associated with numerous inflammatory diseases. The A20 gene is thought to provide protection against apoptotic and inflammatory stimuli but studies to date have been restricted to heart, skeletal muscle and porcine aortic endothelial cells (PAECs) of transgenic animals. Cultivated hA20-transgenic PAECs were protected against TNF-α-mediated apoptosis, and partially protected against CD95 (Fas)L-mediated cell death and the pig cardiomyocytes were partially protected in ischemia/reperfusion stud-

ies. This study demonstrated that human A20 expression on pig cells could be a promising molecule for protection against hypoxia in xenotransplantation studies and it may also play a role in protection against the innate immune response [56]. More specifically we can produce pigs with transgenic expression of human heme oxygenase-1 (hHO-1), an inducible protein capable of cytoprotection by scavenging reactive oxygen species and preventing apoptosis caused by cellular stress during inflammatory processes [57]. The Korean group of Curie Ahn developed a hHO-1 expressing pig and analyzed the expression and function of the transgene. Human HO-1 was expressed in tissues, including the heart, kidney, lung, pancreas, spleen and skin. Fibroblasts derived from the hHO-1 transgenic pigs were significantly resistant to both hydrogen peroxide damage and hTNF-α and cycloheximide-mediated apoptosis when compared with wild-type pig fibroblasts. Furthermore, induction of RANTES in response to hTNF-α or LPS was significantly decreased in fibroblasts obtained from the hHO-1 transgenic pigs. These findings suggest that transgenic expression of hHO-1 can protect xenografts when exposed to oxidative stresses, especially from ischemia/reperfusion injury, and/or acute rejection mediated by cytokines [57].

More recently, the CRISPR/Cas9 system was used to yield human cells devoid of manganese superoxide dismutase (MnSOD). SOD2-null cells displayed perturbations in their mitochondrial ultrastructure and preferred glycolysis as opposed to oxidative phosphorylation to generate ATP [58]. We have also seen the development of various pigs that have the addition of a potential anti-inflammatory genes such as human CD39 (hCD39), the major vascular nucleoside triphosphate diphosphohydrolase (NTPDase), converts ATP and ADP to AMP, which is further degraded to the antithrombotic and anti-inflammatory mediator adenosine. Deletion of CD39 renders mice exquisitely sensitive to vascular injury, and CD39-null cardiac xenografts show reduced survival. Conversely, upregulation of hCD39 by somatic gene transfer or administration of soluble NTPDases has major benefits in inflammation. We have previously shown its advantages with hCD39 being expressed on NICC that were transplanted into baboons where it had significant benefits in preventing IBMIR and inflammation [37].

There is now clearly abundant protection against hypoxia by the expression or over-expression of hHO-1, HA-hHO-1, hA20, XIAP, INS-XIAP and genetic manipulation to protect against inflammation targeting by hCD39, INS-CD39, hTM, ASGR1-KO, hEPCR, hTFPI, and INS-TFPI [11, 13, 37, 55–58]. The use of such systems shows great advances and promise for the potential of the CRISPR/Cas9 system in making further genetic modifications in various donor pigs in the pursuit of the ultimate pig for xenotransplantation, not just for protection against hypoxia when undergoing islet cell xenotransplantation [59].

2.4. Overcoming the innate immune responses by genetic modification

Underpinning the overt innate inflammatory response to pig grafts is an overwhelming production of cytokines that includes IL-6 [60]. Along with this immediate cytokine response,

direct antigen presentation and complement upregulation also occur. Added together this formative assault targets the donor graft once revascularization occurs. Obviously only a targeted approach to prevent this cascade of events from occurring can be undertaken by direct targeting of antigen reduction and direct complement regulation on the human-anti-porcine complement dependent cytotoxic responses. A number of approaches have been undertaken of late to target this by producing genetically modified animals created using CRISPR/Cas9-directed mutation and human transgene delivery. Pigs doubly deficient in GGTA1 and CMAH genes have been produced and have been compared to pigs of the same background that expressed a human complement regulatory protein (hCRP). A third transgenic pig type has also been made deficient in GGTA1, CMAH and B4GalNT2 gene expression. Cells from these animals were subjected to measures of human antibody binding and antibody-mediated complement-dependent cytotoxicity by flow cytometry. Human IgG and IgM antibody binding was unchanged between the double knockout and the transgenic hCRP double knockout pig. IgG and IgM binding was reduced by 49.1 and 43.2% respectively by silencing the B4GalNT2 gene. Compared to the double knockout, human anti-porcine cytotoxicity was reduced by 8% with the addition of a hCRP ($p = 0.032$); It was reduced by 21% with silencing the B4GalNT2 gene ($p = 0.012$). Quite clearly selecting such genes to target effectively mediates human antibody-mediated complement dependent cytoxicity [22].

Although at first they were not thought to be involved at the time of revascularization, natural killer (NK) cells appear to also play a role in xenograft rejection. As such a number of groups have targeted HLA-G and HLA-E in an attempt to provide inhibitory receptors of human NK cells or macrophages with some effect. Weiss et al. have produced HLA-E/human beta2-micro-globulin transgenic pigs that provided a small degree of protection against xenogeneic human anti-pig natural killer cell cytotoxicity [61]. Maeda et al. demonstrated that by transfecting swine endothelial cells to express HLA-E and also HLA-G they significantly suppressed the production of pro-inflammatory cytokines from the inflammatory macrophages, which is seen to be an important target to help advance islet xenotransplantation [62]. With genetically modified pigs that have one or more of the cellular immune response inhibitors already produced for xeno-transplantation as described above including; hTRAIL, HLA-e/β2 m, pCTLA4-Ig, INS-pCTLA4-Ig, LEA29Y, hFasL, shTNFRI-Fc and CIITA-DN [11, 13, 60–62]. There would appear to be few other variable minor receptors to target, but with the major ones already targeted we are now more than readily able to move toward the clinic in regards to suppression of such responses.

2.5. Overcoming T cell responses by genetic modification

Ultimately overcoming the initial immediate barriers is of greatest need but following this we still have to provide a defense against the human T cells that recognize the pig MHC molecules (SLA). It appears that their response to pig tissue is greater than an allo-immune response as seen by the rapid infiltration of islet grafts in the loop model systems even within an hour following contact with human blood [7, 63, 64]. We have used standard clinically used immunosuppressive therapy in a pig to baboon islet transplant model and seen that the

grafts are more rapidly targeted and are eventually rejected by immune mechanisms within a month post transplantation [65]. Thus we require much heavier and directed immunosuppressive modalities to be able to prolong xenograft survival without causing untoward toxicity to the recipient [65].

As outlined previously, xenografts can become infiltrated with NK cells which are recruited by the innate inflammatory response but they can also be stimulated and recruited by upregulation of CD4$^+$ and CD8$^+$ T cells and macrophages. CD4$^+$ T cells are the predominant cell type involved in xenograft rejection with activated CD4$^+$ T cells infiltrating the rejecting pig xenograft resulting in IFNγ-mediated activation and infiltration of macrophages and NK cells [66–68]. Macrophages participate in recruitment of effector T cells as well as antigen presentation and cytokine production. Proinflammatory cytokines produced by macrophages, including TNFα and IL-6, upregulate tissue factor and can promote both inflammation and activation of coagulation [60].

We have seen in a number of studies that conventional doses of common immunosuppressive drugs such as cyclosporine, tacrolimus and glucocorticoids are not effective. However, some studies using extremely toxic and non-clinically applicable immunosuppression have shown extended graft preservation but lead to an unacceptably high susceptibility to serious infections in baboons [69]. We have seen some inroads to the success of long-term graft survival using the more novel and targeted immunosuppressive agents such as anti-CD154 Ab treatment in pig islet transplants into monkeys [70]. Using a modified Anti-CD154 based immunosuppressive regimen and islets from genetically engineered pigs on an α1, 3-galactosyltransferase gene-knockout background with ubiquitous expression of human CD46 (GTKO/CD46 pigs), and additional islet beta cell-specific expression of human tissue factor pathway inhibitor (hTFPI) and/or human CD39 and/or porcine CTLA4-lg, islets were intraportally transplanted into diabetic cynomolgus monkeys demonstrating reduced islet destruction in the first hours after transplantation. Despite encouraging effects on early islet loss, these multi-transgenic islet grafts did not demonstrate consistency in regard to long-term success, with only two of five demonstrating function beyond five months [71].

Despite several issues being raised with the potential thrombogenic side effects of anti-CD154, Cooper's group have recently published its safe and efficacious use in pig islet transplants in monkeys showing no apparent side effects in an extended series of fourteen animals over many months in which they describe the extended treatment of their monkeys with the anti-CD154 and then undertook a critical and extensive analysis of the animals tissues by microscopy looking for any microthrombotic or thromboembolic complications [70]. There are also further studies utilizing a blocking antibody against CD40 (the receptor for CD154), which is showing even more promise for pig heart, and kidney transplants in baboons with no thromboembolic complications [72, 73].

Specific targeting of an immunosuppressive factor to be genetically engineered into islets could be in place of systemic delivery or if the agent was not suitable for repeated systemic treatment. A perfect candidate would appear to be costimulation blockade using anti-CD154 whose questioned thromboembolic effects would be negated by its local production at the

site of the islet. We have also shown suitability of another novel and very effective agent anti-CD2 whose systemic T cell depletion might be undesirable long term, but if produced locally would not affect the majority of the body's T cells. We have clearly shown it to be efficacious in a humanized severe combined immunodeficiency (huSCID) model using transduced NICC secreting anti-CD2 to prevent graft rejection [74]. Novel targeting by agents such as anti-CD2 would appear a very definitive route to take, as CD2 it is expressed on all T cells and sub-sets of NK cells and unlike most other T cell specific targets; it is expressed more highly on memory T cells [75].

If we are to provide comprehensive coverage of all avenues of protection we can also theoreti-cally target the genetic reduction of the expression of MHC. In fact this has been undertaken in a pig-to-baboon artery patch model. Pig arteries expressing a dominant-negative MHC II transactivator gene to reduce levels of MHC II (including on endothelial cells) had a modest effect. Targeted disruption of MHC I genes in pigs by utilizing CRISPR/Cas9 has also been achieved [19] providing an avenue forward for incorporation with the other already demon-strated to be effective transgenic manipulations to move porcine islet xenotransplantation to the clinic.

2.6. Final remarks

There appear very few remaining barriers to be overcome before xenotransplantation can move to the clinic. The recent advent of the cutting-edge molecular tools such as ZFNs, TALENs, and the CRISPR/Cas9 system have all significantly increased efficiency and precision of the production of genetically modified pigs for xenotransplantation [59]. There are a number of proof of concept studies already demonstrating long-term islet xenograft survival due to vari-ous [23, 48, 76], genetic modifications to protect against HAR and IBMIR being mitigated by genetically disrupting the α1,3-galactosyltransferase gene and other targeted approaches to reduce xenogenicity by targeting GLA, HT, hGnT-III, GT-KO, CMAH, (Neu5Gc)-KO, class I MHC-KO, iGb3s-KO, β4GalNT2-KO [11, 13, 17, 22, 23, 37, 40–42]. These have also been combined with the transgenic expression of complement regulators hCD59, hCD55/hDAF, and hCD46 [11]. There is also abundant protection against hypoxia by the expression or over-expression of hHO-1, HA-hHO-1, hA20, XIAP, INS-XIAP [11, 13] and genetic manipulation to protect against inflammation targeting by hCD39, INS-CD39, hTM, ASGR1-KO, hEPCR, hTFPI, and INS-TFPI [11, 13, 37, 55–58].

It would appear the way is clearly open for moving to the clinic since the only remaining bar-rier, the adaptive immune response, can also be surpassed by the use of new systemic immuno-suppressive therapies including a combination of local suppression by genetically modifying islets to be resistant to cellular rejection. We also have genetically modified pigs that have one or more of the cellular immune response inhibitors already produced for xenotransplantation including hTRAIL, HLA-e/β2 m, pCTLA4-Ig, INS-pCTLA4-Ig, LEA29Y, hFasL, shTNFRI-Fc and CIITA-DN [11, 13, 60–62, 71–75]. All that it would appear to be able to move to the clinic is to make the correct selection of the most appropriate combination of genetic manipulations to be able to provide the ideal multi-transgenic xenotransplant donor pig.

Acknowledgements

Abbreviations

GGTA1	α(1,3)galactosyl transferase
ASGPR	Asialoglycoprotein receptor
B4GALNT2	Beta-1,4-N-acetyl-galactosaminyl transferase2
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPR/Cas9	CRISPR-associated (Cas)
CMAH	Cytidine monophospho-N-acetylneuraminic acid hydroxylase
CK7	Cytokeratin 7
αGal	Galactose α1-3 galactose
GTKO	Gal Transferase Knockout
GMS	Glucose Management System
hCRP	Human complement regulatory protein
HFH	Human factor H
hHO-1	Human heme oxygenase-1
hCD39	Human CD39
EPCR	Human endothelial protein C receptor
HFH	Human factor H
huSCID	Humanized severe combined immunodeficiency
HAR	Hyperacute rejection
IBMIR	Instant blood mediated inflammatory reaction
IP	Insulin pump
IVIG	Intravenous immunoglobulin
INS-	Insulin producing
LMWD	Low molecular weight dextran

MnSOD	Manganese superoxide dismutase
NK	Natural killer cells
NICC	Neonatal islet cell clusters
NHP	Non-human primate
NTPDase	Nucleoside triphosphate diphosphohydrolase
PFC	Perfluorochemical
PAECs	Porcine aortic endothelial cells
SPK	Simultaneous pancreas and kidney
T1D	Type-1-diabetes
TFPI	Tissue factor pathway inhibitor
TALENs	Transcription activator-like effector nucleases
TLM	Two-layer method
GalT KO or GTKO	Xenoantigen αGal
XIAP	X-linked inhibitor of apoptosis
ZFNs	Zinc finger nucleases

Author details

Wayne Hawthorne

Address all correspondence to: wayne.hawthorne@sydney.edu.au

1 Sydney Medical School, University of Sydney, Westmead, NSW, Australia

2 Department of Surgery, Westmead Hospital, Westmead, NSW, Australia

3 Centre for Transplant and Renal Research, Westmead Institute for Medical Research, Westmead, NSW, Australia

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