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MINIREVIEWS

Use of genetically-engineered pig donors in islet transplantation

Rita Bottino, Massimo Trucco

Rita Bottino, Massimo Trucco, Institute of Cellular Therapeutics, Allegheny-Singer Research Institute, Allegheny Health Network, Pittsburgh, PA 15212-4772, United States

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Correspondence to: Dr. Rita Bottino, Institute of Cellular Therapeutics, Allegheny-Singer Research Institute, Allegheny Health Network, 320 East North Avenue, Pittsburgh, PA 15212-4772, United States. rbottino@wpahs.org
Telephone: +1-412-3596395
Fax: +1-412-3596987

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease wherein the pancreas does not produce enough insulin due to islet beta cell destruction. Despite improvements in delivering exogenous insulin to T1D patients, pancreas or islet transplantation remains the best way to regulate their glycaemia. Results from experimental islet transplantation have improved dramatically in the last 15 years, to the point where it can be comparable to pancreas transplantation, but without the accompanying morbidity associated with this procedure. As with other transplants, the limiting factor in islet allotransplantation is the relatively small number of organs made available by deceased human donors throughout the world. A strong case can be made for islet xenotransplantation to fill the gap between supply and demand; however, transplantation across species presents challenges that are unique to that setting. In the search for the most suitable animal for human xenotransplantation, the pig has many advantages that make it the likely animal of choice. Potentially one of the most beneficial advantages is the ability to genetically engineer porcine donors to be more compatible with human recipients. Several genetic manipulations have already proven useful in relation to hyperacute rejection and inflammation (instant blood mediated inflammatory reaction), with the potential of even further advancement in the near future.

Key words: Genetic-engineering; Diabetes; Pig; Islets; Xenotransplantation

Core tip: Type 1 diabetes is widespread and debilitating. Islet allotransplantation from deceased human donors can reverse diabetes but there are too few donors to provide much help for more than a few recipients. Xenotransplantation of pig islets, readily obtainable in large quantities, can bridge this gap. Genetic manipulation of pigs in order to render their tissue more compatible with human recipients can improve graft function and would be necessary for clinical trials. Experience within the pig-to-nonhuman primate model help to determine the most beneficial enhancements,

while technology evolves to provide improved techniques for multiple genetic manipulations.

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INTRODUCTION

Organ and tissue transplantation have come a long way since the mid-twentieth century, which saw the world's first successful human organ transplantations[1]. Improvements in the ability to mediate rejection through the use of more advanced pharmaceuticals, the experience gained by surgeons, the refinements of surgical techniques and advancements in general technology have had a positive effect on outcomes over the last 50 years. Human organs such as liver, heart, kidney and pancreas are routinely transplanted to treat serious or even fatal diseases. Pancreas transplantation has been successfully used to treat type 1 diabetes (T1D), however, it remains a technically challenging procedure. Islet transplantation is an experimental yet therapeutic alternative to whole pancreas transplantation for the treatment of T1D. The Edmonton protocol, reported in 2000, ushered in a new era of islet allotransplantation as a cure for diabetes. Enthusiasm was high after all 7 patients of the study remained off-insulin for 1 year[2]. Follow-up at 5 years showed that while insulin independence was difficult to maintain, the procedure was still beneficial and potentially life saving due to the ability of transplanted islets to provide protection from severe hypoglycemia[3]. With steady improvements and refinements, islet allotransplantation has now reached, at least in some experienced centers, successful rates of insulin independence and duration of graft function that are not much different from pancreas transplantation. Importantly, these benefits are derived without the accompanying morbidity associated with the more complex whole organ transplantation procedure[4,5].

Due to the limited number of deceased human donor organs available for transplantation, however, and particularly of pancreata that meet donor qualifications for islet transplantation, only an extremely limited number of the most severely diabetic patients can hope to benefit from islet allotransplantation. Alternative sources must be found to bridge the gap between supply and demand. Stem cell research has shown encouraging results and may prove to be an effective therapy one day, however, it is yet far from therapeutic applications[6,7]. Until that day, the xenotransplantation of porcine islets to replace human islets should receive serious consideration as an alternative therapy.

SWINE SOURCES OF ISLETS FOR TRANSPLANTATION

Porcine insulin is very close to human insulin with only one amino acid of difference, and for many years, until recombinant human insulin became available, it was used in clinical practice to treat diabetes[8]. In many other ways, the pig is a suitable source of islets for xenotransplantation into human recipients. In the last decade, several groups from around the world have shown success in restoring insulin independence for a period > 6 mo in diabetic nonhuman primate recipients of pig islets, a breakthrough achievement in xenotransplantation[9-17]. While the results of pig-to-nonhuman primate models cannot fully predict the pig-to-human results due to dissimilarities among the different donors and recipient species, they are necessary steps to reach clinical application[18,19]. These results provide evidence that porcine islets would very likely function to restore glycemic control in humans.

The pig is already a potentially interesting donor for tissues and organs (in particular heart, kidney, lung and liver) due to the anatomical and size compatibility with human recipients. Many tissues (*e.g*., cardiac valves) are already broadly utilized with human patients[20].

While non-vascularized or decellularized tissues such as the valves are easier to implant than organs or islets, as they do not entail the complexity of dealing with xenorejection, the willingness of the public to accept porcine cardiac valves helps lay the groundwork for acceptance of additional medical uses for the pig.

Practical considerations also make the pig a likely candidate for medical use. While nonhuman primates are the closest animals to humans from an evolutionary standpoint and, therefore, would be immunologically and physiologically more adaptive to human needs, their use in clinical xenotransplantation would not likely be accepted. Apes are endangered species, thus raising ethical concerns. They have litters of one or two offspring (like humans) and their growth requires years to reach full size, making it difficult to achieve a sufficient number of potential donors to satisfy demand. Many other nonhuman primate species are small in size, with organs unsuitable for transplantation in adult humans. Perhaps the most relevant concern is the possibility, which cannot be considered negligible, that they may transmit diseases to humans more easily than those carried through other animals such as pigs. The Acquired Immune Deficiency Syndrome epidemic is too recent to be forgotten. Human immunodeficiency virus, originated with simian immunodeficiency virus, and made the leap from chimpanzees to humans. On the other hand, far from being endangered, pigs are bred by the millions as commodities for human consumption, thus mitigating many concerns about their usage. They breed and mature quickly and produce large litters allowing for plentiful donors. The phylogenetic distance

between humans and swine dates back approximately 100 million years, making the potential for disease transmission to humans less likely than in nearer related species[21]. One concern with the use of pig tissue is the possible transfer of porcine endogenous retroviruses (PERVs), which are dormant in the pigs themselves but might be reactivated with the transfer of porcine tissue into human recipients. A 1997 study showed that PERV could infect human cells *in vitro*, which temporarily halted research into xenografts[22]. However, there is no clinical evidence in which the retrovirus has been transmitted or reactivated in live human subjects in the many years since humans have been receiving pig products. While initial caution was justified, it is now believed that the original fears associated with PERV were overstated and that any potential transmission in a clinical setting appears manageable[23,24]. To limit concerns about transfer of additional donor disease, pigs could easily be sourced from pathogen-free herds. Also importantly, pigs are of the correct size and physiology to allow for successful organ transplantation in humans and it, therefore, makes sense to maximize efficiency with the use of the same animal for both organs and tissues such as islets.

In transplantation, the advantage of using animal sources is also apparent in the ability to elect organ harvesting, avoiding brain death and ischemia in the donor, and the stressful consequences of life-support. A strong body of evidence suggests that the pathological consequences of brain death in the donor reduce graft survival in allotransplantation[25,26]. More specifically, islet cells are sensitive to oxidative stress consequent to ischemic injury, which can be deleterious in the transplantation setting, and can be avoided completely with the use of animals such as pigs as donors, available on an elective basis.

Another advantage that has emerged as direct consequence of cutting edge scientific achievements is the ability to modify the pig genome by knocking out or fostering expression of transgenes finalized to fill gaps between species, making their tissues more compatible to the recipients. The advantages of increased compatibility between donor and recipient would be hugely beneficial, ranging from the need for less islets (therefore less donors) to the possibility of less severe immune suppression necessary to block rejection.

HYPERACUTE REJECTION: ALPHA 1,3-GALACTOSE

One of the major achievements in genetic engineering of pig tissues thus far has been the knocking out of the carbohydrate alpha 1,3 galactose (Gal). This is critical to xenotransplantation because Gal plays an essential role in triggering massive and immediate graft destruction (defined as hyperacute rejection) when pig tissues are transplanted into nonhuman primates as would also occur in humans[27].

All animal species including pigs express Gal on the surface of their cells in a mode similar to that of the carbohydrates (and relative anti-sera) involved in blood group compatibility. Humans and Old World monkeys, however, have lost the ability to synthesize Gal due to genetic inactivation of the enzyme alpha 1,3-galactosyltransferase[28]. Upon exposure to bacteria that expresses Gal shortly after birth, humans (and old world monkeys) produce anti-Gal antibodies. Consequently these natural antibodies remain in the blood circulation where they activate complement-mediated destruction of any cell/tissue that expresses Gal[29]. Graft destruction occurs within minutes when tissue that expresses Gal is exposed to human plasma.

It became clear, therefore, that lack of Gal expression in any animal intended for human transplantation would be one of the main achievements necessary to prevent hyperacute rejection.

To this aim, studies conducted to sequence DNA transcripts encoding the alpha 1,3-galactosyltransferase enzyme in various animal species allowed scientists to identify the two key mutations associated with lack of Gal expression in old world monkeys, apes and humans[30].

By reproducing the same mutation that occurred naturally during evolution, it was then possible to create a pig cell line not expressing Gal, and pigs were generated by nuclear transfer and cloning in which the enzyme alpha 1,3-galactosyltransferase was knocked out (GTKO pigs)[31]. This represented a major milestone in the advancement of the xenotransplantation field.

In regard to islet transplantation, however, Gal is not thought to play such a major role as it does in whole organ transplantation, due to Gal being expressed only minimally on islet cells in adult pig tissue[32]. This finding can explain the success in islet transplantation achieved by several groups, using Gal expressing adult pig islets transplanted into immunosuppressed nonhuman primate recipients[10,11,17]. In contrast, there is a higher expression of Gal on pig islets at birth and throughout the neonatal period than with adult islets[32]. Therefore, with a growing interest to use neonatal islet-like cell clusters rather than adult islets, the knocking out of Gal will remain relevant for islet xenotransplantation as well as for organ transplantation.

The first experiments conducted to prove the lack of hyperacute rejection confirmed the expected findings in regard to Gal, however, to some disappointment, acute rejection of the graft still occurred within days after transplant, suggesting that additional factors remain to be corrected to allow higher compatibility[33].

HYPERACUTE REJECTION: NON-GAL

In vitro studies have shown that when pig tissues and islets are exposed to human serum, antibodies bind to the islets even when using GTKO donors, suggesting that more antigens are recognized by pre-existing antibodies[34]. Two non-Gal antigens have

received particular attention: N-glycolylneuraminic acid (NeuGc) also known as Hanganutziu-Deicher, and β1,4 N-acetylgalactosaminyltransferase (B4GALNT2)[35-37].

Similarly to Gal, NeuGc is not expressed in humans (nor in New World monkeys) but it is in most other species. The pig-to-nonhuman primate model of transplantation (where nonhuman primates are Old World monkey) is not affected, but lack of expression of NeuGc and consequent antibody production in humans will be relevant in the clinical setting. Pigs that express neither Gal nor NeuGc have recently been generated, and it is likely that this genetic background will constitute a better donor for potential human use[38].

Although less is known about B4GALNT2, it is thought to play a part in the nonGal immune response after pig-to-primate xenotransplantation. Porcine B4GALNT2 was shown to cause antibody binding and complement mediated lysis in the presence of primate serum after pig-to-primate cardiac xenotransplantation using GTKO donors[37]. Preliminary data suggest that, primate antibody binding is reduced when B4GALNT2 is deleted from the donor pig.

INSTANT BLOOD MEDIATED INFLAMMATORY REACTION AND TRANSGENIC PIGS

Instant blood mediated inflammatory reaction (IBMIR) encompasses a number of pathological events that occur when islets are injected into the blood stream, which is the typical way they are transplanted into recipients[39,40].

Islets trigger blood clotting, complement activation, inflammation and ischemia, which, in turn, can damage islets and cause their lysis, with consequent release of insulin and C-peptide. These events occur even in autologous and allogeneic settings but in xenotransplantation the effect is more pronounced[34]. *In vitro*, C-peptide measurements are found to peak approximately 15-30 min after pig islet exposure to human blood, serum or plasma. *In vivo* islet transplantation studies have demonstrated that porcine insulin and C-peptide levels increase within 30 min from the time of islet infusion, causing hypoglycemia in the recipients that requires glucose infusion to keep the glycemic levels in the normal range. The impact of IBMIR and the loss of islets associated with it cannot be overstated. A sufficient number of islets must survive the peri-transplant period or long-term graft function cannot be achieved. The number of functional islets required to sustain normoglycemia is variable and depends on a number of factors. However guidelines do exist. As species incompatibility increases, so does the number of islets that must be transplanted due in no small part to the ravages associated with IBMIR[5]. The extent of IBMIR damage is not completely measurable, however, to date, pharmacological treatments have been only partially successful in modulating its impact[41,42]. Anticoagulant therapy can prevent blood clotting *in vitro* and likely prevent the formation of thrombi *in vivo*, but, preventing coagulation, at least *in vitro*, has not been shown to reduce islet cell damage, suggesting that mechanisms independent from clotting contribute to islet cell loss. Nonetheless preventing clot formation *in vivo* is necessary to prevent thrombotic consequences.

While even the slightest increase in surviving islets gives hope to graft function, survival in greater numbers is necessary to achieve reliable long-term euglycemia.

Genetically modified donor pigs can potentially overcome IBMIR and reduce early islet loss by rendering the pig tissue more compatible to humans, thus weakening or eliminating the mechanisms that cause islet damage, *i.e.*, complement activation, clotting, and inflammation. Due to the broad nature of events associated with IBMIR, multiple genetic modifications may be necessary to provide sufficient graft protection.

Human CD46 and CD55 are proteins with complement modulation properties, their expression on human tissue allows controlling and avoiding non-specific complement activation, which would lead to tissue damage. The pig has its own complement regulators, however, it appears that these are insufficient in blocking responses from other species. Human tissue factor pathway inhibitor (hTFPI) and human CD39 have been shown to provide anti-thrombotic and anti-inflammatory effects beneficial to islets *in vitro* and *in vivo* while human CD39 has been shown to decrease platelet activation and prevent clotting in transgenic mouse models[43-45].

Several groups have now demonstrated not only the necessary efficacy of pig islets transplanted into diabetic nonhuman primates, but also the benefits of genetically-engineered pig donor islets (Table 1). Graft function > 6 mo has been achieved by transplanting neonatal islet-like cells from GTKO donors[15]. Our own experiments using adult porcine islets transgenic for hCD46 demonstrated graft function for > 1 year, even while using a less intensive immunosuppressive regimen than in previous attempts using unmodified pigs and that failed to achieve similar results[12]. Despite this success, the hCD46 pig islets by themselves did not curtail early islet destruction, which led to further experimentation with multiple genetic combinations, with up to 5 unique modifications in individual donors[47]. Transgenes were selected to have impact on the mechanisms inherent to IBMIR (*i.e.*, platelet activation, coagulation, complement activation) and also to provide protection against the cellular immune response. Interestingly, some of the transgenes were selectively driven under the insulin promotor, thus, expressed on islet beta cells only (Figure 1). While, in the pig-to-nonhuman primate model, transgenic expression of hCD39 did not appear to provide the anticipated protection against IBMIR (more specifically against platelet activation), one of the diabetic monkeys that received islets from a pig transgenic for GTKO/hCD46/hTFPI/CTLA4-Ig remained insulin independent for > 1 year and, significantly, showed evidence of reduced early islet lysis[16]. Factors associated to the

Table 1 Genetically-engineered pig islets to diabetic NHP models

GE manipulation	Pig age	Survival (d)	Immunosuppression	Ref.
GTKO	Neonatal	249	Anti-CD154 + anti-LFA1 + CTLA-4-Ig + MMF	[15]
CD46	Adult	396	Anti-CD154 + ATG + MMF	[12]
GTKO/CD46/TFPI/CTLA4-Ig	Adult	365	Anti-CD154 + ATG + MMF	[16]
GTKO/CD55/CD59/HT	Neonatal	30	ATG + MMF + Tacrolimus	[46]

GE: Genetically-engineered; GTKO: Alpha1,3-galactosyl transferase-gene knockout; MMF: Mycophenolate mofetil; TFPI: Tissue factor pathway inhibitor; HT: H-transferase; ATG: Antithymocyte globulin.

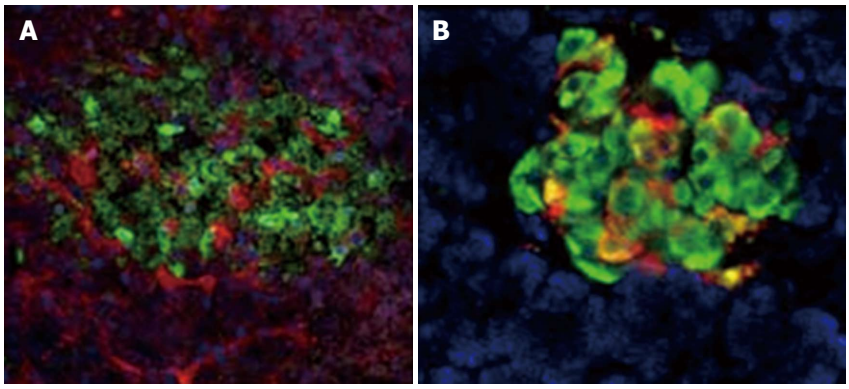

Figure 1 CD46 *vs* tissue factor pathway inhibitor. A: hCD46 transgenic expression in pig islets. Insulin is shown in green, hCD46 in red and nuclei are stained in blue. Transgene expression was ubiquitous; B: hTFPI expression in islet beta cells. Insulin is green, hTFPI in red and nuclei in blue. hTFPI is co-expressed with insulin. hTFPI: Human tissue factor pathway inhibitor.

level of transgenic expression, their modulation and biological function in transplantation settings may require further standardization as this field of study advances. While long-term success was no greater than our earlier experiments using hCD46 pig donors, the ability to mitigate early islet loss is important because it demonstrates the ability of genetically-engineered pigs to overcome IBMIR without the addition of more toxic immune suppression.

GENETICALLY-ENGINEERED PIGS AND IMMUNE SUPPRESSION

Now that pre-clinical trials utilizing pig islets in diabetic NHP have shown the ability to correct diabetes for significant periods of more than a year, it becomes imperative to develop a clinically relevant immune-suppression that can prevent rejection of the xenogeneic tissue (islets). In xenotransplantation as in allotransplantation recipient immunity is always a critical factor. Once again, genetically-engineered pigs can help to provide the missing pieces of the immunological puzzle. In our own experiments, we were able to achieve graft function for > 1 year transplanting porcine islets transgenic for hCD46 into a diabetic monkey. This result, which was unprecedented at the time, was accomplished using the same procedure and immune suppression regimen (based on anti-CD154mAb costimulation blockade) that failed to produce satisfactory results using genetically unmodified pig donor islets. This clearly demonstrates the potential benefit provided by genetically-engineered pig donors in regard to recipient immunity. Our further experiments have included pigs transgenic for CTLA4Ig, which inhibits the cellular immune response. Additional pigs have been created specifically with transgenes designed for the suppression of cellular immunity either by gene expression or downregulation (Table 2).

Our successful experiments using hCD46 and 4GE transgene combinations followed the same immune suppression therapy based on anti-CD154mAb costimulation, which, due to potential thromboembolisms, will not be translatable to clinical practice. However, new anti-CD40mAb based costimulation therapy currently used in clinical trials targets the same pathway involving CD154 and has shown success in various pig to NHP organ transplantation studies without the dangers associated to the older therapy[48,49]. It is anticipated that the new therapy based on anti-CD40mAb would have similar effects on islet transplantation as well. It should also be noted that in our successful anti-CD154mAb based studies, no incidence of thrombosis were detected in any of the recipients[12,16]. Additional costimulation-blocking based therapies are already in clinical use that might prove effective in the xenotransplantation setting, especially in conjunction with transgenic donors designed to optimize tissue compatibility.

CONCLUSION

Medical science over the last 50 years has seen miracles

Table 2 Several genetic manipulations of pigs currently available with potential use for clinical islet transplantation

GE manipulation	Target	Expression	Ref.
GTKO	Humoral response	Ubiquitous	[15,16,46]
NeuGcKO	Humoral response	Ubiquitous	
B4GalNT2KO	Humoral response	Ubiquitous	
HumanCD46	Complement regulation	Ubiquitous	[12,16]
HumanCD55	Complement regulation	Ubiquitous	[46]
HumanCD59	Complement regulation	Ubiquitous	[46]
HumanTFPI	Anticoagulation	Beta cells	[16]
HumanCD39	Anticoagulation	Beta cells	
Human thrombomodulin	Anticoagulation		
Human A20 (tumor necrosis factor-alpha-induced protein 3)	Anticoagulation/anti-inflammatory/anti-apoptotic gene expression		
Human heme oxygenase-1	Anticoagulation/anti-inflammatory/anti-apoptotic gene expression		
Human signal regulatory protein α	Anticoagulation/anti-inflammatory/anti-apoptotic gene expression		
CTLA4-Ig (CD152)	Cellular response	Beta cells	[16]
HLA-E/human b2-microglobulin	Cellular response		
LEA29Y	Cellular response		
PERV siRNA	PERV activation		

GE: Genetically-engineered; GTKO: Alpha1,3-galactosyl transferase-gene knockout; TFPI: Tissue factor pathway inhibitor; PERV: Porcine endogenous retroviruse.

become the new routine with organ transplantation. We are now on the very cusp of seeing the future of diabetes control through the use of porcine islet therapy. Like the miracles before it, islet xenotransplantation has seen the impossible become possible and the doubtful become probable. We have seen the positive impact of islet allotransplantation, and the limited number of organs available. We have seen that porcine islets are capable of restoring insulin independence in nonhuman primates, and know that supply is essentially limitless. We have seen, through the introduction of genetically-engineered tissue, that graft function can be maintained for a period of up to a year. We do not doubt that future advancements will continue to bring us closer to the goal of diabetes control. Advancements in technique have been introduced recently, *e.g.*, TALENS (transcription activator-like effector nucleases) and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat-associated system) for generating pigs with multiple genetic manipulations in less time than previously possible[50,51]. This progress, together with our understanding of which manipulations may have the most beneficial effect, will help us overcome obstacles such as IBMIR, rejection and immunity until islet xenotransplantation finds itself as recognized and well-regarded as organ transplantation is today.

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