First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – Chapter 2b: Genetically Modified Source Pigs

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Abbreviations: α Gal, galactose- α 1,3-galactose; Cas, CRISPR-associated system; CRISPR, clustered regularly interspaced short palindromic repeat; DSB, double strand break; GM, genetically modified; GTKO = α 1,3-galactosyltransferase gene-knockout; h, human; IBMIR, instant blood-mediated inflammatory reaction; Neu5Gc, N-glycolylneuraminic acid; RGN, RNA-guided endonuclease; TALEN, transcription activator-like effector nuclease; tracrRNA, target-independent trans-activating crRNA; TFPI, tissue factor pathway inhibitor; ZFN, zinc finger nuclease

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Abstract

Genetic modification of the source pig offers the opportunity to improve the engraftment and survival of islet xenografts. The type of modification can be tailored to the transplant setting; for example, intraportal islet xenografts have been shown to benefit from the expression of anticoagulant and anti-inflammatory transgenes, whereas cytoprotective transgenes are probably more relevant for encapsulated islets. The rapid development of pig genetic engineering, particularly with the introduction of genome editing techniques such as CRISPR-Cas, has accelerated the generation of new pig lines with multiple modifications. With preclinical testing in progress, it is an opportune time to consider any implications of genetic modification for the conditions for undertaking clinical trials. Obviously, the stringent requirements to fulfill designated pathogen-free status that are applied to wild-type pigs will apply equally to genetically modified (GM) source pigs. In addition, it is important from a safety perspective that the genetic modifications are characterized at the molecular level (e.g. integration site, absence of off-target mutations), the phenotypic level (e.g. durability and stability of transgene expression), and the functional level (e.g. protection of islets *in vitro* or *in vivo*, absence of detrimental effects on insulin secretion). The assessment of clinical trial protocols using GM pig islets will need to be done on a case-by-case basis, taking into account a range of factors including the particular genetic modification(s) and the site and method of delivery.

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Introduction

Source pigs are necessarily a key consideration in the formulation and revision of guidelines for clinical trials in islet xenotransplantation. Chapter 2 of the first IXA porcine islet consensus statement, published in *Xenotransplantation* in 2009 (and updated in this issue), focused on the conditions required for source pigs to fulfill designated pathogen-free status [1]. However, the scope of the initial document did not extend to the use of genetically modified (GM) pigs as ‘donors’.

Our task in this section is to: 1) review the rationale for genetic manipulation of the donor pig, and present evidence for the efficacy of existing modifications; 2) summarize the rapidly advancing technology for introducing multiple genetic modifications into the pig; 3) address the question of genotypic and phenotypic stability, i.e., the need for genetic ‘quality control’; 4) describe assays to assess the effects of genetic engineering on islet function; and 5) discuss the potential safety implications of using GM donors.

Breeding and maintaining GM animals *per se* is subject to regulation; however, since regulatory requirements will likely vary from case to case and from country to country, a discussion of this issue is beyond the scope of this chapter.

Genetic modification of the donor pig to improve islet engraftment

The primate response to porcine islet xenografts depends on several factors, including the type of islets (adult, neonatal, fetal), the mode of delivery (‘naked’, microencapsulated, macroencapsulated), and the transplant site (intraportal, intraperitoneal, subcutaneous, intramuscular, bone marrow etc.). This in turn will determine what type of genetic modification may be applicable.

In current clinical allotransplantation practice (with intraportal delivery of naked islets), engraftment can be compromised by the instant blood-mediated inflammatory reaction (IBMIR), which mediates early destruction of a significant islet mass through activation of complement and coagulation and infiltration by innate immune cells [2,3]. IBMIR is likely to be exacerbated in recipients of intraportal porcine islet xenografts by the action of pre-existing anti-pig antibodies [4,5].

An obvious genetic approach to improve engraftment is to modify the islet surface to eliminate xenoantigens and/or to express regulators of complement, coagulation and platelet activation. Deletion of *GGTA1*, the gene encoding $\alpha 1,3$ -galactosyltransferase, abolishes expression of the carbohydrate xenoantigen galactose- $\alpha 1,3$ -galactose (α Gal) and reduces the incidence of hyperacute rejection of solid organ xenografts in the nonhuman primate model [6]. This modification, termed GTKO, improved the engraftment and survival of neonatal pig islets transplanted intraportally in macaques, with evidence of reduced intrahepatic inflammation [7]. Interestingly, GTKO had no apparent impact on the early loss of adult pig islets transplanted into cynomolgus monkeys [8]. This lack of effect may be explained by the decline in α Gal expression by pig islets as they mature [9]. Similarly, transgenic expression of the human complement regulator CD46 (hCD46) did not reduce early loss of adult pig islets, although it did promote long-term xenograft survival [8]. Nevertheless, the combination of GTKO with expression of a human complement-regulatory protein(s) is now generally regarded as the best platform on which to build further genetic modifications for both neonatal and adult islet xenotransplantation [10,11], just as it is for solid organ xenotransplantation [12]. A recent pig-to-baboon study showed that thrombosis induced by neonatal wild-type islets, while refractory to treatment with recombinant antithrombin, was dramatically reduced when GTKO/hCD55/hCD59 pigs were used as donors and the recipients were immunosuppressed [11]. Thus, the combination of GTKO with one or more complement inhibitor transgenes appears to improve xeno islet transplant outcomes.

Two transgenes specifically designed to inhibit islet-mediated coagulation and thrombosis have been tested in the adult pig-to-cynomolgus monkey intraportal islet

xenograft model [10]. Tissue factor pathway inhibitor (TFPI) regulates the extrinsic coagulation pathway, which appears to be pivotal to IBMIR, and CD39 inhibits platelet activation and inflammation. Transgenic overexpression of hCD39 in mouse islets has been shown to protect against IBMIR [13], and to protect islets from T cell-mediated injury *in vivo* [14]. When hTFPI and/or hCD39 were expressed through a beta cell-specific promoter on the GTKO/hCD46 background, there was evidence of a reduction in early islet xenograft destruction (e.g., reduced release of porcine C-peptide) and inflammation (reduced serum IL-6) [10]. However, the study was limited by the availability of the multi-transgenic pigs, and did not demonstrate a consistent improvement in long-term islet xenograft function.

It will be interesting to test whether expression of human thrombomodulin $\pm$ human endothelial protein C receptor on porcine islets further attenuates IBMIR. Thrombomodulin regulates the propagation phase of coagulation by converting thrombin's activity from procoagulant to anticoagulant; endothelial protein C receptor facilitates this process and has independent anti-inflammatory and cytoprotective properties. These transgenes have been shown to be beneficial in models of cardiac [15] and lung [16] xenotransplantation, respectively.

It is conceivable that further carbohydrate 'remodeling' may improve the engraftment of intraportal islet xenografts. Pigs express N-glycolylneuraminic acid (Neu5Gc), which is recognized as the Hanganutziu-Deicher antigen by pre-existing antibodies in humans. To our knowledge the presence of Neu5Gc on porcine islets has not been examined, nor whether its expression changes with maturity, analogous to α Gal. The *CMAH* gene responsible for Neu5Gc synthesis has been knocked out in pigs [17], although preclinical testing of the effect of this mutation would need to be performed in New World monkeys since they, like humans but unlike Old World primates, do not express Neu5Gc [18].

Thus far we have discussed only genetic modifications that affect the islet surface and are therefore more applicable to naked, but not encapsulated, islets. In contrast,

intracellular cytoprotective molecules, such as heme oxygenase-1 and A20, may be relevant to both. Adenovirus-mediated expression of human heme oxygenase-1 has been shown to protect neonatal pig islets from the effects of inflammatory cytokines and oxidative stress *in vitro* [19], and transgenic pigs expressing human heme oxygenase-1 [19,20] or hA20 [21] have been generated. Transgene expression in islets from these pigs was either absent [19] or not reported [21], so *in vivo* testing of the potential benefit of these molecules to islet xenotransplantation has not yet been performed.

Genetic modification of the donor pig to prevent islet xenograft rejection

Minimizing early injury to islet xenografts, as described in the previous section, may reduce the antigenic stimulus to the adaptive immune system. Nevertheless, the T cell-mediated cellular immune response remains a significant barrier to successful islet xenotransplantation.

Two transgenic modifications designed to inhibit costimulation of T cells have been introduced into pigs: islet-specific secretion of LEA29Y (a high-affinity variant of human CTLA4-Ig) [22] or of pig CTLA4-Ig [10]. LEA29Y-expressing neonatal pig islets were protected from rejection for 28 days when transplanted into humanized mice [22], although they have not yet been tested in the nonhuman primate model. Expression of pig CTLA4-Ig provided no consistent long-term survival benefit versus GTKO/hCD46 adult pig islets when transplanted into cynomolgus monkeys [10]. Importantly, however, analysis of the latter pigs showed that islet-specific expression of up to 3 transgenes (plus ubiquitous expression of a fourth) had no overt detrimental effects on islet function [23].

New techniques for rapid multigenic modification

In recent years several programmable nuclease-based technologies have been developed, providing new opportunities for genetic modification of pigs. Custom-designed nucleases produce site-specific DNA double strand breaks (DSBs), thereby triggering the endogenous DNA repair system. DSBs are repaired by two major mechanisms - non-homologous end-joining or homology-directed repair [24]. Both

repair pathways can be used to modify a specific target site (= gen(om)e editing). In the absence of a repair template, DSBs created by site-specific nucleases are repaired via error-prone non-homologous end-joining through direct ligation of the break ends, often causing small insertions or deletions (indels). This can lead to a frameshift in the coding region, resulting in loss of function of the affected gene. More precise changes (specific point mutations or targeted insertions) can be introduced by homology-directed repair, achieved by including with the nuclease a donor template (DNA or single-strand oligodeoxynucleotides) containing homology arms corresponding to the target region.

Three major classes of customizable nucleases have been generated for genome editing in pigs – zinc finger nucleases (ZFNs) [25], transcription activator-like effector nucleases (TALENs) [26], and, most recently, RNA-guided endonucleases (RGNs) [27]. One advantage of these nuclease-based systems over traditional homologous recombination methods is that they do not necessarily require somatic cell nuclear transfer, but can work via the less technically demanding method of cytoplasmic injection into zygotes. Both ZFNs and TALENs are artificial fusion proteins that contain a site-specific DNA binding domain (zinc-finger motif or truncated transcription activator-like effector domain) and a non-specific DNA cleavage domain derived from the *FokI* restriction enzyme. As *FokI* must dimerize for DNA cleavage to occur, two appropriately targeted monomers are required to form an active nuclease, improving specificity.

RGNs derived from the bacterial CRISPR-Cas system have been used for genome editing in a variety of cells and organisms. CRISPR (clustered regularly interspaced short palindromic repeats) sequences and Cas (CRISPR-associated system) proteins are the two elements of an ancient prokaryotic adaptive restriction system. CRISPRs represent a repository of short, directly repeating nucleotide sequences that alternate with small unique DNA fragments acquired from invading bacteriophages or plasmids, thus forming a type of memory system [28]. These CRISPR regions are transcribed into target-specific crRNA and target-independent trans-activating crRNA (tracrRNA), which hybridize and form a complex with Cas nuclease that recognizes and cleaves foreign genetic material matching the CRISPR-derived RNA [29]. This complex system has

been developed into an elegant genome editing tool by fusing the crRNA and the tracrRNA into a synthetic, small guide RNA, i.e., a hairpin RNA structure resembling the tracrRNA linked to a 20-bp sequence homologous to the target DNA.

RGNs have at least two advantages over ZFNs and TALENs: (i) the design and preparation is simple, as no complicated protein engineering is necessary, and (ii) it is possible to modify several genes simultaneously using different small guide RNAs, allowing for efficient multiplex genome editing [30]. In 2014, knockout pigs were generated by zygote injection of CRISPR-Cas [31], demonstrating that RGNs are a promising tool for efficient and rapid genome editing in large animals. Recently, CRISPR-Cas has been used to generate pigs with mutant *GGTA1*, *CMAH* and putative *iGb3S* genes [32] and pigs lacking functional class I MHC alleles [33].

Genetic and functional quality control

Quality control for GM pigs and their islet products includes novel considerations in addition to those for wild-type pigs, including characterization of vector DNA, number of integrated copies, and transgene insertion site(s). Methods include quantitative polymerase chain reaction (qPCR), Southern blot hybridization, DNA fluorescence in situ hybridization (FISH), inverse PCR or thermal asymmetric interlaced (TAIL)-PCR, and next generation sequencing. The potential side effects of random integration can be avoided by using recombination-mediated cassette exchange to insert expression vectors into 'safe harbors' of the pig genome, such as the *ROSA26* locus [34]. If site-directed nucleases are used for genetic modification, the risk of off-target cleavage can be minimized by selecting optimal target sites using special bioinformatics tools, such as COSMID (CRISPR Off-target Sites with Mismatches, Insertions, and Deletions) [35].

Transgene RNA quantitation is classically performed by reverse transcriptase qPCR or Northern blot hybridization of mRNA from isolated islets. At the protein level, the transgene product may be detected in pancreatic sections by immunohistochemistry, in isolated and dissociated islets by flow cytometry, and in islet protein extracts by

Western blot or by other antibody-based methods such as enzyme-linked immunosorbent assay or radioimmunoassay.

The functional evaluation of the transgene product depends on its biological target. Examples are *ex vivo* clotting assays in human blood [13] to test genetic modifications directed towards overcoming IBMIR, or islet xenotransplantation into humanized diabetic mice to test genetic modifications addressing cellular immune rejection [22]. However, xenotransplantation of porcine islets into diabetic non-human primates remains the gold standard.

Although no major disturbances of glucose homeostasis and insulin secretion have been observed in multi-modified donor pigs for islet xenotransplantation [23], the spectrum of physiological tests (fasting blood glucose levels, insulin, C-peptide, and glucagon responses to both glucose and arginine challenge) provide a solid basis for detecting disturbances of islet function which may result from even more complex genetic modifications.

Potential safety implications of GM donors

It is anticipated that islets from GM donor pigs will create a different safety and efficacy profile, and therefore a modified regulatory path, than islets from wild-type pigs. This different path will likely be triggered regardless of whether the genetic modification was due to gene knockout or insertion. However, each unique type of GM donor and its resulting GM islet derivative will need to be managed on a case-by-case basis. Assessment will differ based on different country-specific regulatory agencies, and will depend on a variety of factors specific to the actual islet product. These factors include the genetic modification(s) present in the donor/islet, donor age (neonatal vs. adult), site of delivery (e.g., intraportal, subcutaneous, kidney capsule, peritoneum), method of delivery (e.g., encapsulated vs. naked islets), and number of islets transplanted. For example, the number of GM islets transplanted might be considerably lower than that required using wild-type islets due to the beneficial effects of transgenic modifications on islet survival and/or reduced cytotoxicity post-transplant.

Looking specifically at the nature of the genetic modification(s) in the donor islets and their impact on safety and related regulatory considerations, as described above for efficacy, it will be important from a safety perspective to have full molecular characterization of the DNA construct and the genomic integration site(s), phenotypic characterization of the donor animal (including health and viability of the pig), phenotypic characterization of transgene function and expression level, durability of the phenotype, and stability across multiple generations.

Containment and environmental considerations around the GM donor will also be important with respect to safety in regard to 1) keeping GM donors out of the food chain, 2) housing and growth of a specific GM donor in a designated pathogen free facility if certain genetic modifications result in an immunocompromised pig, and 3) excluding specific zoonotic pathogens (same as for wild-type donors/islets) according to regulatory guidelines. With these provisos, it is not anticipated that designated pathogen-free pig facilities would need to be significantly different for GM vs. wild-type donors, as they would be expected to similarly exclude the exogenous pathogens (viral or otherwise) described in detail in other sections of this consensus document.

In the USA, certain aspects of the donor pig (including containment, environment, and details of the genetic modification) are regulated by the Center for Veterinary Medicine (CVM), while the human aspects of islet xenotransplantation, including clinical trials (treatment of diabetes with GM islet products), would be regulated by another FDA agency, the Center for Biologics Evaluation and Research (CBER). Approval in each country would need to follow its own safety and efficacy guidelines.

While a number of specific genetic modifications with potential benefits for xeno-islet survival and function have been discussed here, it is premature to state which modification or combination of modifications will be essential for clinical application. If more than one genetic modification is required, the individual knockout or transgene addition needs to be well-characterized from a molecular and stability perspective, but

also the combined phenotype and synergies of the transgene combination become the primary targets for safety and efficacy testing. Regulatory agencies are starting to embrace gene stacking modalities, and their requirements will likely evolve with these rapidly advancing technologies.

Whether one or multiple genetic modifications are required for bioactive gene products, there are likely safety benefits both for health of the donor pig and the islet recipient if the transgenes are under control of an islet-specific promoter, so that the therapeutic is delivered locally at the level of the islet cell rather than systemically (10, 22, 23). In addition, as part of the molecular characterization of the GM event(s), from a safety and regulatory perspective there will be a need to demonstrate the absence of off-target events. Inactivation of essential genes or activation of deleterious genes (such as proviruses or oncogenes) has to be ruled out. The new gene editing systems described above, while highly efficient, can increase the potential for off-target events, and can generate very small indels that are difficult to track. Programs such as COSMID can reduce these CRISPR-based off-target events, and it seems likely that the development of genome analysis technology will advance as rapidly and as efficiently as the genome-editing tools.

Conclusions

Recent data from the nonhuman primate preclinical model suggest that genetic modification of the donor pig will be important, perhaps essential, for the success of clinical porcine islet xenotransplantation. What can be achieved is no longer limited by the technology; it is now possible to precisely modify the pig genome in ways that would have been inconceivable a decade ago. The use of GM donors may entail safety and regulatory considerations above and beyond those for wild-type pigs, but it should be possible to manage these issues on a case-by-case basis.

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