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Xenotransplantation: The next generation of engineered animals

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Introduction

All forms of transplantation except autografts and transplants between genetically identical twins involve crossing genetically encoded barriers. In allotransplantation we try to lower the barriers by a variety of means: we match the major histocompatibility genes and the blood groups of donor-recipient pairs and perform crossmatches to minimise the effects of prior sensitisation to any HLA antigens which may be mismatched. We prefer related over unrelated donors, because the former pick up some matches not only for what we know but inadvertently for unknown but nevertheless relevant genetic differences. Despite these efforts we still have to overcome the effects of other unmatched, untested or unknown genetic differences by immunosuppressing the recipient; this is not always successful and always comes at a price.

Of course xenotransplantation is no exception and what is more the genetically encoded barriers are even higher. However, because a pig is a pig and not a human we can directly attack the genetic barriers – we can genetically modify the pig and so provide a genetic solution to a genetic problem and even go a little further.

We generally think of potential genetic modifications under headings based on the technology employed: gene knockouts – to remove particular pig characteristics, archetypically the αGal epitope; and transgenesis – to add human characteristics to the pig tissue e.g. the human complement regulator DAF. However to be comprehensive we need to broaden that view and one approach is to think of genetic modifications as either prophylactic or therapeutic. This allows us to take advantage of the enormous power of genetic engineering and to think about making changes which go beyond simply trying to make the pig donor tissue a little more like a human allograft i.e. beyond prophylactic modifications. Some therapeutic genetic modifications may even overcome problems which still are an issue in allotransplantation e.g. local expression of immunosuppressive molecules within xenografts may minimise the need for systemic immunosuppression.

What to remove?

The αGal epitope

The αGal epitope is a terminal carbohydrate residue present on both N- and O-linked carbohydrates and on some glycolipids of all animals up to New World primates, but is notably absent from Old World primates including humans. It is similar to human blood group B and like B its absence in humans is associated with the development of anti-αGal antibodies. αGal is widely but not universally present throughout pig tissues, it is present in high concentration

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on pig endothelium and the immediate reaction of human anti-αGal with these epitopes results in hyperacute rejection. There is general agreement that Gal KO is an important prophylactic genetic modification for xenotransplantation of vascularised organs. However, while αGal is present on foetal and neonatal islet tissue, it is absent from adult pig islets[1,2]. As a consequence it is arguable that it is not necessary to delete/inactivate α1,3 –galactosyltransferase (Gal KO) to facilitate pancreatic islet xenotransplantation from adult pigs, indeed the early highly successful study from Hering[3] showed that this modification is not essential. Despite this success and perhaps even more interestingly, the similar success of Larsen[4] using neonatal islets, we have argued that there is nothing to be lost and possibly longer term gains from using Gal KO pigs for islet cell xenotransplants[5]. Gal expression decreases as pig islets mature[6], hence the immature 'expandable' component (if any) in adult pig islet xenografts may be specifically targeted, along with the acinar baggage that also expresses Gal. Thus, we take the view that the Gal KO pig is the basic "standard platform" pig on which other genetic modifications can be assembled.

Other pig antigens

There has been much discussion of the importance of non-Gal antibodies in various patterns of rejection and indeed some take the view that this is a priority area for research[7]. It is clear that humans and other higher primates do have preformed antibodies against other, non-Gal, porcine antigens. The level of these antibodies and possibly the range of specificities vary between individuals. In some cases they are present in sufficient amounts to cause hyperacute rejection of vascularised organs from Gal KO pigs. The specificities of some of these have been defined but there is no indication that knocking out another one (or at most two) would remove the problem. The level of difficulty rises exponentially as one adds further knocked out genes. From a practical point of view adding even one more to Gal KO would severely limit what can be done by the traditional transgenic approach.

Porcine Endogenous Retrovirus (PERV)

Genetic engineering has been suggested as a method of removing the threat posed by PERV. Sequences could be deleted so as to make the pig donor non-infectious. Non-transmitter pig strains, such as David Sachs' dd line[8], already exist and can be generated by selective breeding. To mix the metaphors; while the fat lady is still to sing, it seems increasingly unlikely that PERV will present the difficulties that Chicken Little predicted.

What to add?

Complement regulators

"In the beginning….." when the addition of human complement regulators to porcine tissue was first proposed, the rationale was based on the notion of "homologous restriction"[9]. Indeed CD59 was initially called homologous restriction factor or HRF. The term refers to the relative or complete inactivity of the regulatory factors of one species (the donor) against the target complement components of the recipient species. Thus, there was evidence, mostly from in vitro experiments, that was interpreted as showing that the complement regulators of pigs were inefficient or inactive against human complement. This provided the rationale for making pigs transgenic for a number of human complement regulators: Decay Accelerating Factor (DAF, CD55), Membrane Co-factor Protein (MCP, CD46) and the eponymous Homologous Restriction Factor (HRF, CD59). This strategy was remarkably effective, so effective in fact that it became apparent that it was not simply the prophylactic addition of an effective regulator, but that a therapeutic or pharmacological effect was involved. Expression of human CD55 or CD46 with or without CD59 completely abrogated hyperacute rejection of vascularised organs in the pig-to-primate model despite the presence of high titre anti-αGal antibody. This effect was much greater than could have been expected from simply circumventing homologous

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restriction, as allografts across the ABO or positive crossmatch barriers are regularly hyperacutely rejected despite there being no species based limitation on the effect of the human complement regulators. Thus, it was apparent that the effect was in fact therapeutic and when expressed at supraphysiological levels the complement regulators were able to prevent complement activation even when activated by a full blown antibody-antigen interaction. Subsequently, Paul Morgan[10,11] showed that pig complement regulators were perfectly effective against human complement.

It is now clear from the point of view of preventing HAR that expression of one or more complement regulators in supraphysiological amounts is another critical component of the "optimal pig". Whether this is CD55 alone or in combination with CD59, or CD46, is not resolved and in fact the important issue may be the quantity expressed rather than which are employed. However, one should not forget the work of Morgan, there may be disadvantages in making a pig express human complement regulators as these are all receptors for human tropic viruses such as echovirus[12,13] (CD55 and CD59) and measles (CD46)[14–16]. There is therefore the theoretical possibility that expression of these molecules in pigs may make them susceptible to infection with these agents. Therefore, one might consider using a highly expressed pig CD55 or CD46 rather than a human regulator.

Two other points should also be made: first, as it is impossible to delete all the targets of non-Gal antibodies, some of their effects may be mitigated by transgenically expressed complement regulators; and second, they may also serve to reduce the effects of induced antibody which is a major feature of acute vascular xenograft rejection. Thus transgenically expressed complement regulators can be seen as an important component of the immunosuppression required to maintain a xenograft.

Regulators of Coagulation and Thrombosis

It is now abundantly clear that thrombosis is a central feature of xenograft rejection, whether it is as part of hyperacute and acute vascular rejection of kidneys and hearts or of the instant blood mediated inflammatory response (IBMIR) following intraportal islet cell transplantation. While the thrombosis associated with these have quite different pathogeneses, the solution is likely to be the same.

The pathogenesis of thrombosis in the rejection of vascularised xenografts involves very similar considerations to those discussed in relation to complement. In this case there are clearly demonstrated molecular incompatibilities resulting in homologous restriction of the endothelial regulators of the coagulation cascade[17]. However the primary driver of thrombosis is immune mediated endothelial injury i.e. rejection, which converts the normally anticoagulant endothelial surface to a highly procoagulant state[18]. This process involves local loss of the coagulation regulators. Molecular incompatibilities may contribute by allowing thrombosis to be amplified and broadcast beyond the immediate site of injury. The resulting pathology varies from full blown consumptive coagulopathy to limited microvascular thrombosis, depending primarily on the severity of the rejection process[19]. Two examples of molecular incompatibility in the thromboregulatory machinery are (i) the markedly reduced capacity of the pig thrombomodulin – human thrombin complex to activate human Protein C[20], and (ii) porcine von Willebrand factor (vWF), which unlike its human equivalent binds and activates human platelets in the absence of sheer stress[21,22]. The pathogenesis of thrombosis induced by islets is quite different. These present a highly thrombogenic exterior surface which is normally tissue effaced and consequently completely lacks any thromboregulatory machinery but expresses Tissue Factor. When islets contact the portal blood the result is instantaneous activation of coagulation – IBMIR[23].

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The solution to both scenarios is likely to be transgenic expression, again probably in supraphysiological quantities, of regulators of thrombosis. The human factors which have been transgenically expressed to date include CD39 (ecto-ADPase)[24], thrombomodulin, EPCR (endothelial protein C receptor) (this laboratory- unpublished), TFPI (tissue factor pathway inhibitor) and hirudin [25–27](a leech antithrombin). Which should be chosen? Each targets a different step and has inherent logic; CD39 targets platelets and in addition has anti-inflammatory activity through the generation of adenosine, TFPI targets tissue factor, the initiator of the extrinsic pathway of coagulation, thrombomodulin and EPCR are critical to the generation of the anticoagulant activated Protein C (APC) and hirudin is a direct inhibitor of the critical procoagulant molecule thrombin. The answer is unknown and awaits the outcome of testing. It depends in part on how many transgenes can be expressed as discussed below. Our approach is to favour CD39 and thrombomodulin because of the dual targeting of both platelets and coagulation's contribution to thrombosis. However one has to acknowledge that islets may require special consideration. Islets have abundant exposed tissue factor which would provide a clear and possibly commanding logic for TFPI. Similarly islets completely lack even porcine coagulation regulators so that expressing thrombomodulin without EPCR is likely to be suboptimal as the latter substantially amplifies APC generation by thrombomodulin.

Immunosuppressive molecules

So far the genetic modifications discussed have been aimed at removing problem molecules from the pig or providing the pig with human molecules to limit aspects of innate immunity including coagulation. The opportunity also exists to make modifications which directly target the human anti-pig adaptive immune response. The greatest source of morbidity and mortality after allotransplantation is the systemic immunosuppression required to prevent and treat rejection. It is obvious from both theoretical considerations and now from practical experience with pig-to-primate models that the total burden of immunosuppression required for a xenograft is substantially greater than for an allograft, and may in fact be at or beyond a level that could be justified clinically.

Transgenic expression of biological molecules which interfere with adaptive immunity offers the possibility of providing a graft with potent local immunosuppression without interfering with systemic immune responses and therefore the capacity of the recipient to deal with infectious agents and prevent the development of malignancies.

CTLA-4Ig and anti-CD4 have both been expressed on islet cells by transfection or transgenesis and have been able to produce prolonged allograft survival[28–31]. This approach has yet to be tried in the pig-to-primate model. Obviously only protein immunosuppressive molecules can be expressed transgenically; however the array of new biological reagents entering the clinic is impressive and are potentially able to be expressed locally in the graft. Provided the level of expression is appropriate, true local immunosuppression without systemic spill over or effects may be obtained. Antibodies require coordinated equimolar expression of both chains, which will be facilitated by new technology as discussed below.

Other targetable molecular incompatibilities

There is probably an endless list of the differences between pigs and humans that could be considered but we select just three others as examples of what might need to be considered in the future.

NK cells have an array of inhibitory receptors, with ligands including an array of MHC Class I type molecules. These receptors are restricted not just to human MHC I molecules but in many cases to polymorphic self molecules, and furthermore each NK cell has its own array of

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specificities. Thus it seems impossible to provide a pig with all the possible human MHC I specificities that might be needed to make it a universal donor. However the inhibitory receptors include one with specificity for HLA-E which is non-polymorphic and which has consequently been suggested as a candidate transgene. Unfortunately its receptor is probably present on less than half of the NK cell population. It is probably premature to consider a transgenic approach to NK cells for several reasons; first, their role in xenograft rejection may not be of sufficient importance to warrant it; second, it appears impractical given the complexity of the receptor-ligand systems and third, it is possible that their effects may be managed by the immunosuppressive agents exhibited to manage adaptive immune responses.

Physiological incompatibilities—The focus for genetic modification of donor pigs has been on preventing graft loss – mainly by preventing rejection. However, the possibility exists of other molecular incompatibilities between donor and recipient that prevent or diminish the functional effectiveness of a graft. It is highly likely that xenotransplantation of a complex organ such as the liver which produces the vast bulk of the circulating proteins and responds to many others is likely to be a very difficult proposition because of molecular incompatibilities. The risks are lower for relatively simple organs such as hearts which make and respond to few complex protein molecules which is where the risk of incompatibility is highest.

Non-gal antigens—While the gene inactivation (knock out) approach has succeeded for αGal, we have noted that there is a practical limitation in the number of such modifications that can be assembled in one pig. An alternative approach is to do a functional KO by expression of a transgene encoding a glycosyltransferase that competes for substrate and so substantially reduces the expression of an epitope. This was the rationale for making α1,2-fucosyltransferase (FUT1 or H-transferase) and N-acetylglucosaminyltransferase-III (GnT-III) transgenic mice and pigs. In the former case FUT1 competes with α1,3 galactosyltransferase for its N-acetyllactosamine substrate and so inhibits the formation of the αGal epitope[32]. GnT-III probably works in an analogous manner by preventing the further development of the branching structure of complex N-linked carbohydrates and so limiting the array of terminal epitopes that can be formed[33]. Neither transgenic has proven to be particularly effective, however the approach may be revisited if a specific amenable xenoepitope is discovered.

And how to do it?

Two major technological advances have propelled xenotransplantation: the applications of transgenesis and of cloning to pigs. The former resulted in the initial DAF transgenic pigs, the latter in Gal KO pigs. Since then there has been a convergence of the technologies. The original microinjection technology that produced the DAF transgenics has been replaced by in vitro transfection of a somatic cell and subsequent cloning. This allows genetically modified pigs to be built on a previously established platform (the Gal KO pig) rather than each as individual modifications which have to be bred together.

Transgenesis and cloning were giant enabling technological leaps; however further incremental advances will ensure that these technologies serve xenotransplantation even better.

The long list of potential candidate genetic modifications to achieve an optimal pig, which will almost certainly vary depending on the transplant to be performed, suggests that the most important are various approaches to assemble multiple transgenes in one site so that they are inserted as a single heritable unit. The driving force for development of this technology is twofold. First, pigs have a generation time of about 1 year; this means that assembly of an array of genes by breeding of pigs with individual genetic modifications is massively time consuming, and until homozygosity is reached is also very inefficient because the genes randomly assort at each generation. Secondly, the inbreeding needed to achieve these

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multigenic pigs is a major risk. This was exemplified by the Gal KO pig where most groups found that their Gal KO pigs had very low fecundity because of inbreeding – and this was from breeding only a single gene to homozygosity and establishing a herd.

Several approaches to this problem are being explored: derivation of pig embryonic stem cells that can be passaged indefinitely and so subjected to multiple rounds of gene targeting, development of artificial chromosomes which can carry a large array of transgenes and be inherited as an additional chromosome, and development of novel methods of making multigenic constructs for transgenesis.

Problems, problems, problems……

The consequences of knocking out genes and of transgenic expression of genes are not always predictable. The first published Gal KO mice were made to test the prediction that oocyte αGal was essential to sperm adhesion[34]; it was not and the mice were fertile but mice developed cataracts[35] which was not predicted; consequently Gal KO pigs were predicted to develop cataracts, but they do not. Nevertheless making a genetic modification in mice before tackling pigs makes good sense both economically and ethically. It allows one to assess whether viable transgenics can be obtained and to look for transgene function, deleterious phenotypes, level and extent of tissue expression, interactions with other transgenes etc. Failure to generate expressing transgenics or high expressers suggests fetal lethality. We have used the H-2Kb promoter extensively and generally have had success. It is possible that this is a 'Goldilocks' promoter producing, not too much, not too little but just the right amount of expression. However, one only sees the viable offspring, and the unviable for any reason e.g. over-expression or deleterious expression at a particular developmental stage or site, are not seen without a formal study to detect them.

There may be theoretical advantages in the old microinjection approach to transgenesis which we may lose with the new relatively inexpensive and more rapid transfection/somatic cell nuclear transfer (cloning) approach. The former had a high failure rate, so many fertilised oocytes were injected and implanted and many animals were born and tested. This involved an *in utero* selection for viability etc and allowed subsequent selection for best phenotype and genetic transmissibility. Much of this selection is missing in the newer technology in which only a few clones are selected in vitro and turned into animals.

Attempts to make multigenic pigs are likely to be particularly at risk from ER stress due to overloading the endoplasmic reticulum in sensitive cell types and from interactions between gene products at various stages of fetal development. Again, modelling in mice may sound warning bells. This may influence not only the number of genes that can be co-expressed but also the combinations and the choice of promoter system. It may be necessary to use inducible promoters to obtain certain transgenics.

Horses for Courses?

There is a world of difference between a pancreatic islet graft and a vascularised graft such as a heart or kidney.

One could quickly say that the key issues for vascularised grafts are prophylactic, preventing antibody reactivity by removal of the αGal epitope and prevention of complement activation. In contrast, adult pig islets lack the αGal epitope and the main issue is therapeutic – providing an anticoagulant antidote to the Immediate Blood Mediated Inflammatory Reaction (IBMIR). Well, maybe ……..

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A pancreatic islet graft, whether from a human or a pig is a highly processed (read: prejudiced) few millilitres of tissue scraps avulsed from their natural position. Their normal blood supply is disrupted and not re-established and so they immediately begin to die. The exterior of islets is not designed to be exposed to blood and is highly phlogistic and thrombogenic; it lacks the natural regulators of both the complement and coagulation cascades. The method of transplantation, infusion into the portal circulation, immediately exposes it to just about every phlogistic mechanism available including cytokines etc. from the liver which they injure on impact, and none of which they have natural defences against. Few survive to function. Among the allografts these are the least successful, invariably fading away in a few years. Yet of all the preclinical xenografts they are the most successful and so far have been undertaken without any genetic modification at all! They will be the first xenografts to the clinic and the genetic modifier will have to argue that his craft can provide a Ferrari to succeed the Model T Ford. These considerations suggest that the array of modifications for islets may include: Gal KO (especially for neonatal islets), one or more complement regulators, anti-IBMIR genes (a selection of CD39, thrombomodulin plus EPCR, TFPI), an anti-apoptotic gene e.g. A20 or other (CD39 may double in this role) to counteract the early transplant injury including ischaemia and reperfusion injury, and, particularly in view of the need to minimize systemic immunosuppression in diabetics, one or more genes to provide local immunosuppression.

A vascularised organ is a very different proposition. Here the genetic modifier is in a strong position; in fact his attentions are essential for success. The heart or kidney is only minimally injured by the transplant procedure itself and its repositioning in the vasculature of the host is essentially similar to its position in the donor, so it is exposed to human blood in the same way. Here the first issue is how the human blood sees the pig endothelium and whether the latter's protective mechanisms are functional against human reactants. So we become concerned with a different array of genetic differences to those which would seem relevant to islets; or are they different? We might think of: Gal KO, one or more complement regulators, antithrombotic genes (a selection of CD39, thrombomodulin plus EPCR, TFPI) and one or more genes to provide local immunosuppression. Sounds familiar….so maybe a hurdler can run on the flat? Our approach is to try to build a multitalented pig and put him over a few different courses.

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