Discordant cardiac xenotransplantation: broadening the horizons

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INTRODUCTION—WHY DISCORDANT PIG CELLS AND ORGANS?

The various Eurotransplant waiting lists include almost 15,500 patients, 1,451 of which died in 2011. The situation is particularly poor for people needing heart transplantation, with 226 of 1,277 (17.7%) candidates in that category dying in 2011 [1]. Several alternatives have been suggested to overcome the grave shortage of organs. One possible solution would be clinical xenotransplantation using non-human primates as concordant donors and triple drug immunosuppression as applied using human allotransplants [2]. However, ethical and logistical considerations preclude this. Apes are endangered species and their use is out of the question, and other non-human primates are too small and their growth too slow. In contrast, discordant species, notably pigs, offer an abundant new source of organs and cells. Their gestation time is short (~4 months), pigs have multiple offspring (10–12 per litter) and mature in 6–8 months. Pigs have been bred for food for centuries in many parts of the world, so ethical objections should be minimal. The main disadvantages arise from disparities between swine and primates resulting from ~90 million years’ evolutionary divergence, which can affect important protein–protein and other biochemical interactions.

IS THE SHUMWAY PARADIGM STILL VALID?

The great Norman Shumway used to say, ‘Xenotransplantation is the future, and always will be!’ Fortunately, this pessimistic view is no longer true. In a recent review, Ekser et al. [3] listed the longest survival times reported for xenografted porcine cells and organs. Microencapsulated pancreatic islets (Fig. 1) from wild-type animals survived >800 days [4]. Non-encapsulated islets transgenic for the human complement regulatory protein CD46 survived almost 400 days [5]. Abdominally placed heterotopic (non-working) double genetically modified (CD46 and α-Gal-KO) hearts beat for up to 236 days [6], while orthotopically placed organs were life supporting up to 57 days [7]. Transplanted CTLA4 transgenic neuronal cells to treat Parkinson disease, and wild-type decellularized corneas were also successful for hundreds of days [8, 9]. Whole kidneys from CD55 transgenic animals survived for 90 days [10], although porcine renal erythropoietin is not recognized by primate recipients and must be replaced. Liver and lung transplants do, however, stand in contrast, surviving only for 8 and 5 days [11, 12].

There are a dozen groups (seven in the USA, two each in Australia/New Zealand respectively Asia, one further in Europe) around the world pursuing xenotransplantation research. Our experience in this area started in Munich in 1998 with funding by the Bavarian Research Foundation; in 2004 the German Research Foundation took over support and the Hannover groups joined; 2012 marked the beginning of the Collaborative Research Centre and inclusion of the Dresden diabetes group. The strength of our Consortium is the mix of basic researchers, animal biotechnologists, virologists and clinicians. Our main goal is to achieve clinical experience with genetically modified porcine islets and decellularized heart valves (from α-Gal- and Hanganutziu-Deicher antigen knock out animals), kidneys and hearts.

Testing the efficacy and safety of genetically modified (gm) cells, tissues, organs and other products is an iterative process. This commences with in vitro testing of efficacy using appropriate pre-existing and purpose developed biochemical and biological laboratory procedures. Small animal experiments, valvular stability tests or solid organ perfusion follow. Work then proceeds to pre-clinical non-human primate studies. As issues are identified, the cycle starts anew. In this way, we aim to generate clinically safe gm porcine products free of any infectious risk (for further information of our Consortium see http://www.klinikum.uni-muenchen.de/SFB-TRR-127/de/index.html and http://www.dfg.de/foerderung/programme/listen/projektdetails/index.jsp?id=213602983).

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Porcine cellular and organ xenotransplantation in non-human primates has so far been technically demanding, time consuming and costly, but the rewards for human health are considerable. Transplant experiments necessarily depend on the availability of gm donor animals, which up to now have mostly been generated directly by nuclear transfer cloning, a laborious undertaking that can have an uncertain outcome. This is, however, set to change in the near future. Our Consortium has access to lines of gm pigs (Table 1) of both sexes, enabling production of donor animals carrying multiple genetic modifications by conventional breeding, allowing transplant procedures to be planned on a regular basis.

**PORCINE PANCREAS ISLETS WILL BE FIRST IN THE CLINIC**

Porcine insulin is itself recognized and functional in primate recipients; however, without immunosuppression, unmodified porcine insulin producing cells succumb to early graft loss known as ‘immediate blood-mediated inflammatory reaction’ driven by preformed antibodies, complement and excessive coagulation. Encapsulation obviates the need for immunosuppressive drugs. Islets are surrounded with a porous biopolymer, composed mainly of alginate [14] (Fig. 1). The pores are large enough for small molecules like water, glucose, oxygen and most importantly insulin to permeate, but exclude cells and larger molecules such as antibodies. However, it is not known how long these capsules can maintain their function in vivo, since loss of integrity or occlusion of the pores would be problematic.

The New Zealand company Living Cell Technologies is a pioneer in the field, having so far treated 22 diabetic patients suffering from frequent episodes of unaware hypoglycaemia [15]. A dose finding and safety study showed improvement in some treated patients, for example reduced glycated haemoglobin levels. Most importantly, however, there was no evidence of zoonoses and no sign of activation of porcine endogenous retroviruses, a theoretical risk that has long been recognized [15]. The genetically unmodified donor animals were from a designated pathogen free (DPF) herd that originated from a feral breed from Auckland Island in the sub-Antarctic, where they had essentially no contact with other animals or humans for about 150 years, since English sailors left them for food.

A German multicentre study is now planned—this will also provide a useful opportunity to address the legal and ethical issues related to xenotransplantation. Successful completion of this study and further preclinical experiments could then lead to clinical introduction of gm islets—possibly on the basis of the microbiologically ‘safe’ New Zealand animals. In these circumstances, immunosuppressive therapy would be necessary.

Since porcine islet transplantation will be the first into the clinic, its success will be crucial for further organ procedures with kidneys and hearts. The rest of this editorial will focus on hearts.

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**Glossary**

- **α-Gal**: (α-Gal-1,3-gal): a sugar epitope present on the surface of all porcine cells; these are not present in old world monkeys such as baboons (or humans). Animals lacking α-Gal develop antibodies to it in early life [13]. α-Gal is the most important porcine xenantigen and initiates hyper-acute rejection.
- **CD46** (membrane cofactor protein), **CD55** (decay-accelerating factor (DAF)), **CD59** (membrane attack complex (MAC) inhibitor protein): complement regulator proteins that occur normally in our body; they are able to block inadvertently induced complement reactions within a fraction of a second.
- **Complement system**: the classical pathway involves a large number of plasma proteins and is initiated by an antigen/antibody reaction (in xenotransplantation, α-Gal is the main antigen). One end result is the formation of the MAC, which perforates cellular membranes leading to irreversible cellular damage.
- **Costimulation**: T- and B-cells need an antigen-specific and several non-specific, costimulatory, secondary signals to become activated. The best described costimulatory signals are CD28 (which interacts with CD80/CD86) and the CD40/CD40L systems.
- **CTLA4, LEA29Y**: CTLA4 is a protein with a receptor that down-regulates CD28/CD80, CD86 costimulation. LEA29Y is a higher affinity variant with stronger immunosuppressive activity.
- **Concordant xenotransplantation**: transplantation of cells and organs from non-human primates.
- **Discordant xenotransplantation**: transplantation of cells and organs from species with great evolutionary disparity.
- **Designated pathogen free (DPF) animal housing**: Animals live within a segregated area free of any infectious agents that may be transmitted to (primate) recipients. DPF units are shielded within a segregated area free of any infectious agents that may be transmitted to (primate) recipients.
- **Gal-KO or Gal-transferase-KO**: inactivation of the α,1,3-galactosyltransferase gene, responsible for production of α-Gal epitopes.
- **Genetically modified (gm)**: a range of procedures that include addition of human transgenes into the porcine genome, and inactivation ‘knock out’ or alteration of endogenous porcine genes.
- **Hanganutziu–Deicher antigen**: the second most important porcine xenantigen.
- **Thrombomodulin (TM)**: part of the anticoagulant pathway. One of the most powerful anticoagulants especially within the capillary system. TM reacts with human thrombin leading to activation of Protein C.
- **Transgenic (tg)**: containing an additional transgene. The term can be used loosely to include other modifications such as gene modification or inactivation.

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**Figure 1.** Microencapsulated pancreatic islets are protected from immunocytes and antibodies, but nutrients and insulin permeate the membrane to keep islets alive and functional.

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**Table 1**

<table>
<thead>
<tr>
<th>Gene Modifications (inactivation)</th>
<th>Organ</th>
<th>Kidneys</th>
<th>Hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal-KO, Gal-transferase-KO</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>DAF, CD59</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>α</strong>-1,3-galactosyltransferase</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Protein C</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Complement system</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-α-Gal antibody</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Porcine cellular and organ xenotransplantation in non-human primates has so far been technically demanding, time consuming and costly, but the rewards for human health are considerable. Transplant experiments necessarily depend on the availability of gm donor animals, which up to now have mostly been generated directly by nuclear transfer cloning, a laborious undertaking that...
Table 1: The ‘genetic toolbox’ central to our strategies to minimize or abolish hyper-acute and delayed humoral rejection

<table>
<thead>
<tr>
<th>Genetic modification</th>
<th>Mechanisms</th>
</tr>
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<tbody>
<tr>
<td>α-Gal-KO</td>
<td>Deletes Gal antigen expression</td>
</tr>
<tr>
<td>h-CD46 or h-CD55 or h-CD59</td>
<td>Down-regulate the human (h) complement system</td>
</tr>
<tr>
<td>h-TM</td>
<td>Binds human thrombin, cofactor in activating Protein C (see also Fig. 2)</td>
</tr>
<tr>
<td>HO-1</td>
<td>Anti-apoptotic, cytoregulatory and anti-inflammatory</td>
</tr>
<tr>
<td>A20</td>
<td>Anti-inflammatory and anti-apoptotic</td>
</tr>
<tr>
<td>LEA 29Y</td>
<td>Blocks a T-cell costimulation pathway</td>
</tr>
<tr>
<td>HLA-E</td>
<td>Protects the graft against human natural killer cells</td>
</tr>
<tr>
<td>Multi-genetically modified pigs</td>
<td>Multi-genetically modified animals are correspondingly more complex. The genetic modifications include the gene knockouts and transgenes listed above. Obtaining these animals with appropriate levels of transgene expression requires multiple experimental iterations to optimize transgene structure and identify the best founder animals, a process that can take several years</td>
</tr>
</tbody>
</table>

α-Gal-KO + h-CD46b
α-Gal-KO + h-CD46 + h-TMb
α-Gal-KO + h-CD46 + HO-1
α-Gal-KO + h-CD55 + HO-1
α-Gal-KO + h-CD46 + LEA 29Y
α-Gal-KO + h-CD46 + HLA-E
α-Gal-KO + h-CD46 + h-TM + LEA29Yb

These genetic modifications of donor pigs prolong graft survival by different mechanisms and are combined in various multi-genetically modified pigs. α-Gal-KO, CD46, h-TM and LEA29Y gm pigs are available. Animals that also express hemoglobinase-1 and HLA-E are currently being generated and will be available in the near future.

DISCORDANT XENOGENEIC HEART TRANSPLANTATION

Opening of the aortic clamp allows primate (baboon) blood containing preformed antibodies to perfuse the porcine coronary arteries where they bind pig antigens, mainly α-Gal sugar epitopes (see glossary, α-Gal). Within fractions of a second, various complement reactions are activated leading to formation of the membrane attack complex (MAC) and destruction of graft vasculature (Fig. 2). Subsequent interstitial haemorrhage and oedema lead to graft failure, which is well documented as the hyper-acute rejection reaction.

If this can be circumvented, the next obstacle is presented by new non-Gal antibodies formed within ~3 weeks of grafting, starting the delayed humoral response. Over a similar timespan, the effects of protein incompatibilities within the coagulation system also become evident. For example, porcine thrombomodulin (TM) binds only weakly to primate thrombin, leading to levels of activated Protein C insufficient to interrupt coagulation effects, resulting in occlusive thrombotic microangiopathy in transplanted porcine organs within weeks postoperatively.

Over the longer term, cellular rejection signs are similar to those observed after allogeneic procedures.

Multiple gm pigs are central to our strategies to minimize or even abolish hyper-acute and delayed humoral rejection reactions. Modifications include removal of α-Gal epitopes by genetic knockout; hyper-expression of the complement regulators CD46, CD55 and CD59 to block complement reactions initiated by secondary induced antibodies and expression of human TM to overcome the cross-specific protein incompatibility mentioned earlier, ensuring Protein C activation and anticoagulation (Fig. 2).

Table 1 provides a more complete list of the genetically modified pigs available to our Consortium. Up to now, we have used double (α-Gal-KO and CD46) and triple (α-Gal-KO, CD46 and h-TM) gm pigs in our experiments, and most recently quadruple (α-Gal-KO, CD46, h-TM and LEA29Y) gm pigs are available. Animals that also express hemoglobinase-1 and HLA-E are currently being generated and will be available in the near future.

Xenogeneic heart transplantation will clearly need additional immunosuppressive strategies. Since the time of the procedure is known in advance, the bone marrow (and therefore antibody production) is suppressed before transplantation using anti-CD20 to destroy B-cells, bortezomib in combination with cortisone to destroy plasma cells, and cyclophosphamide [16]. Extracorporeal immunoabsorption is used to remove pre-existing α-Gal and non-α-Gal antibodies and any antibodies formed postoperatively. Maintenance immunosuppression is provided by tacrolimus, mycophenolate and cortisone as for allogeneic transplants; antithymocyte globulin induction therapy is also applied.

We have so far achieved 50 days’ survival in the unique thoracic heterotopic (working) heart transplantation model developed by Barnard and Losman [17, 18] using this immunosuppressive treatment (Fig. 3A and B). Further improvements will require additional immunosuppressive treatment to overcome the delayed humoral rejection reaction, which has been difficult to treat. Total thoracic lym lymph node irradiation is an additional procedure and can be combined with costimulation blockade of the CD40/CD40L system with antibodies ([6], Fig. 2). CTLA4 (or the more potent LEA29Y) can be used to turn off another costimulating system (that of CD28 and CD80/86). Conveniently, LEA29Y may be expressed as a transgene by the donor pig (Table 1). Transgenic pigs that express LEA29Y specifically in the pancreas are available and have been used successfully in diabetic humanized immunodeficient mice by one of the projects within our Consortium [19]. Together with other modifications, cardiac specific LEA29Y is also available now.

Our main aim is to fulfill the guidelines of the Xenotransplantation ISHLT Advisory Board [20] and achieve good graft function for a minimum of 3 months in a life-supporting position in at least 60%
of our consecutive experiments. This has not been achieved so far, since it is difficult to meet within the laboratory setting. Future results of cardiac xenotransplantation must be compared with recent 6-month 60% patient survival after implantation of biventricular continuous-flow mechanical assist devices—and of course the near 90% survival of those patients without right ventricle dysfunction, coagulation disorders or difficult anatomical preconditions such as small left ventricles or significant aortic incompetence. A high antibody titre against the HLA system will be an early indication since the SLA system does not crossreact (reviewed in ref. [24]).

More recent WHO meetings have been held to discuss regulatory requirements for clinical xenotransplantation trials, in Changsha in 2008 and in Geneva in 2011 (first and second WHO global consultation on regulatory requirements for xenotransplantation clinical trials, Changsha, China, 19–21 November 2008 and Geneva, Switzerland, 17–19 October 2011, available from: http://www.who.int/transplantation/xeno/en/).

Numerous prerequisites were summarized and will be addressed within the rules and regulations of the European Medicines Agency, which in Germany is represented by the Paul-Ehrlich-Institute.
REFERENCES


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Conflict of interest: David Ayares is employee of Revivicor (Blacksburg, VA, USA), and Robert Elliott and Paul Tan are employees of Living Cell Technologies (Auckland, New Zealand).