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Program and Abstracts

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Transregio Collaborative Research Centre 127 of the German Research Society (DFG) and the 16th Minisymposium Xenotransplantation of the German Working Group Xenotransplantation (DAX)

Editor: Joachim Denner

Address for correspondence:
Dr. Joachim Denner
Robert Koch-Institut
Nordufer 20
D-13353 Berlin
Germany
DennerJ@rki.de

Program

Wednesday, October 30, 2013

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Comment about the Ethics Meeting of the Academy of Catholic Theology (Z1, October 4–6, 2013, Munich, Germany)
Bruno Reichart, Speaker of the German transregio collaborative research centre xenotransplantation, Munich, Germany

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The complement system and the principles of xeno-reactivity: Hurdles and challenges to overcome the humoral immune response in xenotransplantation Wilhelm Schwaeble, University of Leicester, United Kingdom.

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Xenotransplantation of trangenic porcine islets expressing immunomodulatory molecules
Lelia Wolf van Bürck, Ludwig Maximillians University, Munich, Germany

Transplanting allo-islets without immunosuppression
Barbara Ludwig, University Hospital Carl Gustav Carus, Dresden, Germany

Special lecture
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Zohar Gendler, Beta-O_2 Technologies Ltd., Israel

Analysis and reduction of ischemia/reperfusion injury in a porcine model of kidney transplantation
Wolf-Rüdiger Ramackers, Medizinische Hochschule Hannover, Hannover, Germany

Coagulation and endothelial cell activation in xenotransplansplantation
Wolf-Rüdiger Ramackers, Medizinische Hochschule Hannover, Hannover, Germany

Reduction of xeno-antigens in porcine pulmonary heart valves by decellularization and glycolytic enzymatic treatment
Andres Hilfiker, Medizinische Hochschule Hannover, Hannover, Germany

Ex-vivo testing and preclinical heterotopic thoracic cardiac xenotransplantation of multi-transgeneic organs.
Jan-Michael Abicht, Ludwig Maximillians University, Munich, Germany

Production of multi-transgenic pigs tailored for xenotransplantation by using zinc-finger nucleases and Sleeping Beauty transposons
Björn Petersen, Institute of Farm Animal Genetics, Friedrich Loeffler Institute, Mariensee, Germany

Effective combination of xenoprotective transgenes
Konrad Fischer, Technische Universität München, Freising, Germany

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Andrea Bähr, Ludwig Maximillians University, Munich, Germany

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Posters and additional abstracts

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Katja Findeisen et al.

After decellularization of porcine heart valves: no non-Gal antigeneic epitopes detectable by non conditioned human sera
Robert Ramm et al.

Production of high expressing A20/DAF- transgenic pigs on a GGTA1-KO background
Hellen E. Ahrens et al.

Generation of transgenic pigs carrying siRNA vector directed against human Tissue Factor expression
Hellen E. Ahrens et al.

Cloning and characterization of replication-competent ecotropic porcine endogenous retroviruses (PERV-C) in the genome of pigs used and intended for clinical pig-to-human xenotransplantation
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Abstracts

Discordant cellular and organ transplantation from bench to bedside

Bruno Reichart
Speaker, German Transregio Collaborative Research Centre Xenotransplantation, Ludwig-Maximilians University, Munich, Germany

It is more than 1 year since our Consortium commenced. The following abstracts provide an account of what has been achieved so far and document our understanding of xenotransplantation and some of our future strategies:

1. Since (microencapsulated) islets are the first in the clinic, our basic research focuses on immediate (innate) rejection reactions and means of counteracting them. Once transplanted and working, porcine and primate islets may respond differently, for example to glucose challenges.
2. We will gain preclinical experience regarding the safety of genetically modified islets as quickly as possible.
3. We are currently in dialogue with German authorities (Paul-Ehrlich-Institution as representative of the European Medicines Agency) to decide how best to formulate a successful clinical application within European standards.
4. Are there any ethical objections from within German society? A recent 3-day meeting, held at the Munich Catholic Academy explored the issues, including opinions from the Jewish and Islamic communities. In brief, the outcome revealed no major objections as long as xenogeneic procedures are safe and effective. All talks will be published in German, with abstracts in English. There will also be an English review of the meeting possibly published in the journal Xenotransplantation.
5. Successful preclinical organ (heart, kidney) xenotransplantation is still difficult to achieve. Heterotopic thoracic (working) heart transplantation has been established with consistent results. It seems that hyperacute and delayed humoral rejection reactions are manageable; but thrombotic microangiopathy currently presents the main obstacle. Porcine hearts that express human thrombomodulin will be used next, together with complement antibodies and/or co-stimulation blockade. Human thrombomodulin has provided promising results in the laboratory (cell based).
6. Porcine heart valves will be tested using single and multiple antigen knock out.

In conclusion, the German Xenotransplantation Consortium supported by the German Research Foundation (DFG) is on track for a successful (first?) period. There seems to be broad support from a majority of German cardiovascular surgeons, and this is spreading to those in other parts of Europe [1].

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Modulation of immune responses to porcine xenoantigen by targeting stimulatory and inhibitory signalling pathways

Reinhard Schwinzer
Transplant Laboratory, Department of General-, Visceral-, and Transplantation Surgery, Hannover Medical School, Hannover, Germany

Genetic modification of pigs (e.g. transgenic expression of human complement regulatory molecules or inactivation of α1,3 galactosyltransferase) [1] enabled the development of promising strategies to overcome hyperacute rejection after pig-to-primate xenotransplantation. However, cellular rejection still remains a hurdle for successful xenograft survival. Cellular rejection of porcine cells in xenotransplantation models is mediated by monocytes/macrophages, natural killer (NK) cells, and T cells.

Research in our laboratory focuses on receptor-ligand interactions regulating the responses of human cells to porcine tissues and thus could be targets for immunomodulation. NK cell activation is tightly regulated by different inhibitory and activating receptors and their ligands. Transgenic pigs overexpressing HLA-E have been generated [2]. In vitro experiments revealed that cells from these pigs are only partially protected from NK cell-mediated lysis. This might be due to the fact that the inhibitory receptor for HLA–E (CD94/NKG2A) is not expressed on all human NK cells. We are currently exploring the concept of downregulating NK cell activity by enhancing CD161/KLRB1-mediated signalling. CD161 is a C-type lectin-like receptor which is expressed on the great majority of human NK cells and can transmit inhibitory signals after binding of its ligand LLT1. The data obtained so far suggest, that porcine cells genetically engineered to overexpress human LLT1 posses reduced potential to activate human NK cells.

Activation of T cells by antigen presenting cells (APC) requires interactions between T cell receptor (TCR) and MHC/peptide ("signal one" of T cell activation) as well as interactions between costimulatory receptors and their ligands ("signal two" of T cell activation). T cells receiving "signal one" alone without "signal two" are not activated but achieve a state of unresponsiveness (anergy). In vitro and in vivo data suggest that effective inhibition of human T cell activation can be obtained by blocking the costimulatory CD28-CD80/CD86 interaction using monoclonal antibodies (mAb) or an antagonistic CTLA-4.Ig fusion protein [3]. Furthermore, blocking of TCR-MHC and CD154(CD40L)-CD40 interactions by mAb to porcine MHC class-II or CD40 significantly reduced human T cell activation. New approaches for the modulation of anti-pig immune responses may result from the finding that enhancement of inhibitory signals diminishes T cell activation. We have recently shown that overexpression of the human inhibitory ligand PD-L1 on porcine APC markedly decreased their capacity to activate human T cells and promotes regulatory T cells [4, 5]. Current studies suggest that enhanced inhibitory signalling does not only downregulate T cell reactivity but also dampens induced antibody responses to porcine xenoantigen. Thus, disruption of stimulatory receptor-ligand interactions (e.g. by blocking antibodies or "knock-out/down" technologies) combined with transgenic overexpression of inhibitory ligands in porcine cells and tissues could be an effective approach to downregulate human anti-pig immunity.

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Early islet xenograft dysfunction in humanized mice

Soeren Reinke, Barbara Ludwig, Triantafyllos Chavakis, Claudia Waskow
DFG-Center for Regenerative Therapies Dresden Cluster of Excellence, Regeneration in Hematopoiesis, Technische Universität, Dresden, Germany

Allogeneic transplantation of human islets improves metabolic control in patients that suffer from type 1 diabetes but is limited by the supply of sufficient numbers of human donor islets. Therefore, the xenogenic transplantation of porcine islets potentially represents an attractive alternative. The immediate blood-mediated inflammatory reaction (IBMIR) that results in early graft rejection is one of the major hurdles preventing xenotransplantation from clinical applications. Thus, characterizing and modulating IBMIR could be the key to realize clinical porcine islet transplantation. IBMIR is mediated by multiple components of the innate immune system and in order to assess composition and function of distinct myeloid cell types involved in the rejection process, we established a humanized mouse model to study IBMIR in vivo. We combined immune deficient mice that lack T, B, and NK cells with a mutant Kit receptor affecting the function of HSCs allowing for robust engraftment of HSCs and mature myeloid cells types over long periods of time. We could show that human myeloid cells repopulate peripheral organs such as spleen and liver – a prerequisite for the study of IBMIR in a humanized mouse model. We will now engage these mice to transplant porcine islets into the liver via the portal vein to induce immediate rejection of the graft. In this scenario presence of human myeloid cells in the liver of our new mouse model will facilitate a detailed characterization of the human IBMIR against pig islets and will also allow attempts to suppress this immune reaction by specific targeting of human leukocytes.

Xenospecific regulatory T cells generated on porcine B cells are capable in controlling xenogeneic immune responses in humanized mice

Yun-Shien Lee[1], Fatih Noyan[1], Mathias Hardtke-Wolenski[1], Ann-Kathrin Knoefel[2], Richard Taubert[1], Udo Baron[3], Michael P. Manns[1], Elmar Jaeckel[1]
[1]Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover; [2]Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Hannover; [3]Epiontis, Berlin, Germany

There is a constant shortage of human organs suitable for transplantation which has led the focus of research to the possibility of transplanting porcine organs into human. In order to overcome the immunological barriers in xenotransplantion major progresses have been made in the last decades regarding antibody-mediated and complement mediated hyperacute rejection, yet coagulation incompatibilities as well as innate and adaptive xenogeneic immune responses still remain problematic. Regarding the latter, regulatory T cells have been of special interest because of their immunosuppressive capacities. Polyspecific regulatory T cells have been shown to be able to prevent xenogeneic graft versus host disease but only under lymphopenic conditions, which is not the case after transplantion. Donor-specific regulatory T cells may therefore offer another more promising approach in cellular therapy in immunocompetent specimens. Recently, we developed a protocol for the ex vivo generation of alloantigen-specific human regulatory T cells by activation with donor-derived activated B cell blasts. These alloantigen-specific regulatory T cells were far more potent in controlling T cell mediated immune responses. Transferring this protocol into a xenogeneic setting, we succeeded in expanding porcine B cells by activation with the human CD40-ligand. Activation of polyspecific human regulatory T cells led to the generation of xenospecific regulatory T cells, which show promising immunosuppressive properties when compared to polyspecific regulatory T cells in preliminary experiments.

Gene expression profiling of porcine cells and tissues by microarray analysis

Antonia W. Godehardt, Ralf R. Tönjes
Division of Medical Biotechnology, Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Langen, Germany

Pig to human xenotransplantation represents an ambitious venture that requires, besides evasion of rejection mechanisms and physiological incompatibilities, the generation of pathogen-free pigs as donors for well characterized xenografts to warrant medicinal products that do comply with statutory regulatory demands [1–3]. The publication of a high quality draft sequence for the pig genome (Sus scrofa) and a series of accompanying reports for the first time offered the feasibility of whole genome expression profiling of porcine tissues and cells [4–8].
The SFB TR CRC 127 project Z2 "Microbiological Safety including Virological Safety" is based on microbial profile analysis of porcine tissues in order to prevent zoonotic events, including infection by porcine endogenous retroviruses (PERV) [9]. The project comprises the detection and characterization of potential pathogens as well as the investigation of the microbial influence on the transcriptional status of tissues and cells. Hence, specific expression patterns, e.g. up-regulation of antiviral host factors or cell cycle/apoptotic regulators, may also provide information on ongoing or precedent events that may have impact on tissues/cells quality and therefore its suitability as xenografts.
We use microarray technology for monitoring viability of tissues and cells as well as their microbial/viral status. An Agilent based, 60K DNA microarray representing 25,415 different genes of the recently published Sus scrofa genome (NCBI Sus scrofa 10.2-assembly) was generated [10]. The microarray was specified for German Landrace and Göttingen Minipig, amongst other pig species, by hybridizing complex RNA samples generated from five different pig organs and blood as well as chromosomal porcine DNA to highlight non expressed genes. Four Diagnostic PERV sequences for pro/pol (all classes of PERV), env (to differentiate between PERV-A, -B and -C) as well as 15 human transgenes such as CD59 (human complement regulatory protein), DAF (Decay accelerating factor or CD55), human A20 (hA20) and others were included. In total, the microarray displays 25,434 genes each represented by up to three different 60-mer oligonucleotides.
To reveal functionality of the microarray the transcriptional status of ST-IOWA cells freshly infected with molecularly cloned virus PERV-C (1312) [11] was monitored. Total mRNA levels at day 7, 28 and 56 post infection were compared with naive uninfected cells. All samples were tested in triplicates and the relative signal intensity of hybridized probes was compared. Special attention was given to antiviral host factors such as APOBEC and tetherin of which involvement as antiviral factors on PERV expression has been demonstrated [12–14]. Constitutively expressed housekeeping genes, i.e. porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin and cyclophilin A, respectively, were used as controls [15, 16].
The presented microarray supports the safety and quality by monitoring the transcriptional status of xenotransplants.

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Microbiological characterization of Göttingen minipigs

Joachim Denner
Robert Koch Institute, HIV and Other Retroviruses, Berlin, Germany

Xenotransplantation using pig cells, tissues or organs may be associated with transmission of porcine microorganisms which in the worst case scenario may induce zoonoses. To prevent this, a detailed analysis of the viruses, bacteria, fungi and protozoa in the donor pigs should be performed. This includes characterization of the herd as well as of each individual donor animal. Furthermore, the final transplant, e.g., the islet cell preparation, should be analyzed.
At present, the Auckland island pigs are the best characterized animals and the presence of numerous bacteria, viruses and protozoa is analyzed regularly in the animals and in islet cell preparations [1]. Preclinical [2] and clinical [3] trials transplanting islet cells from these animals did not result in transmission of porcine microorganisms including the porcine endogenous retroviruses (PERVs). PERV-A and PERV-B are present in the genome of all pigs, and they may infect human cells (for review see [4]). PERV-C are not present in all pigs, they infect only pig

cells, however recombinant PERV-A/C infect human cells and are characterized by high replication rates. Previously it was shown that the expression of PERVs was different among breeds including German landrace, Duroc, Schwäbisch-Hällisch, Large White, as well as (multi)transgenic crossbreds generated for xenotransplantation [5]. The highest expression was found in Yucatan minipigs [6]. Although PERV expression was well studied in these breeds, the prevalence of other microorganisms remains unclear.

In addition to the Auckland island pigs the Göttingen minipigs, which are held at Ellegaard, Denmark, represent another well-characterized pig breed. The animals are well-defined concerning their physiologic parameters, health status, and genetics since this herd was established with the purpose to supply minipigs for preclinical pharmacology and toxicology [7]. Health monitoring includes testing for a broad spectrum of viruses, bacteria, fungi and protozoa and health reports are regularly published at Ellegaard website [8]. When the prevalence and the expression of PERVs were analyzed in these animals, it was demonstrated that although PERV-A, -B and -C proviruses were found in all animals, their expression was low [9]. Unfortunately the health monitoring of the herd does not include xenozoonotic infectious agents such as cytomegalovirus, gamma-lymphotropic herpesvirus and hepatitis E [10]. Approaches to detect these viruses are under development and additional investigations are required to assess the suitability of Göttingen minipigs and other animals for xenotransplantation in terms of microbiological safety.

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Experts, policy-makers and citizens: actor constellations and xenotransplantation policies in international comparison[1]

Erich Griessler
Department of Sociology, Institute for Advanced Studies, Vienna, Austria

At the turn of the millennium, xenotransplantation was a topic with a high potential for societal dispute. The reasons were possible infection risks, necessary measures of risk management and surveillance, associated constraints of human rights as well as a number of various other ethical concerns. This situation prompted discussions about the governance of xenotransplantation on a national and international level. This paper compares international xenotransplantation policies and investigates patterns of policy-making as well as the different role of experts, policy-makers and the public therein.[2]

Although it might be assumed that xenotransplantation was discussed globally in the same way, international comparison shows cultural contingency. First, although international organizations identified xenotransplantation as a global problem requiring broad public discussion, the intensity of public debates varied strongly among countries. Second, the topical framing of xenotransplantation differed between countries. Nevertheless, the problems of infection risk and organ shortage often dominated, whereas ethics and animal welfare issues were marginalized. Finally, xenotransplantation polices varied. In most cases regulation meant the decision to provide a legal framework to continue research, some countries took a wait and see position, and a minority opted for a legal or de-facto moratorium.

When comparing ways of policy-making, it is possible to discern different types of decision-making, i.e. bureaucratic, bureaucratic-technocratic, parliamentarian-political and participatory.

The bureaucratic type was elitist and closed; it limited decision-making to the civil service and handpicked internal experts. The bureaucratic-technocratic type was a bit more inclusive, but still, the role of the civil service was particularly strong. In addition, external experts – typically natural scientists and physicians, sometimes ethicists – were involved in advisory bodies. In a few cases, stakeholders and NGOs were also included to some degree. The parliamentarian-political type also included elected politicians in decision-making. Stakeholders and NGOs were only included as informants. The participatory type involved a very broad range of actors, i.e. civil servants, experts, elected politicians, NGOs, technology assessment organisations, and the general public.

Different types of policy-making used diverse approaches towards technology assessment (TA). Whereas in bureaucratic policy-making no formal TA processes were launched, the bureaucratic-technocratic type relied on expert TA. In the parliamentarian-political type, expert TA was complemented by the involvement of elected politicians. In the participatory approach, TA andparticipatory technology assessment (pTA) were used and attempts were made to link participation to politics.

The bureaucratic type of policy-making process was informed by workshops and conferences organized by international organizations, in which civil servants participated. In the bureaucratic-technocratic type, a broader range of information was used, stemming from experts or international organizations and early movers in xenotransplantation regulation. In the parliamentarian-political approach, in addition, stakeholders and NGOs provided information. Again, the participatory type used the broadest range of information, including international organizations, scientists, NGOs, and citizens.

The different types of policy-making involved the general public quite differently. The bureaucratic type neither involved, nor informed, the public. Similarly, the bureaucratic-technocratic approach involved the public only to a small degree. Citizens were included via survey research and information about results via the Internet. Also, the parliamentarian-political approach applied survey research and information; in one country, citizen involvement also meant consultation of selected actors. The participatory type used a range of activities to involve the public (e.g. citizen juries, consensus conferences, online debates, public discussion, theater plays). In Switzerland, several mechanisms of direct and representative democracy were utilized to initiate a debate.

The four approaches also differed in respect of accountability and openness. The bureaucratic, the bureaucratic-technocratic, and the parliamentarian-political approach had problems in one way or another and to different degrees regarding openness and accountability of recruitment, agenda setting, openness to the public, conflict of interest, and methods applied. The participatory approach, on the other hand, was open to the public, often gave ordinary citizens an opportunity to set an agenda, often recruited experts and citizens based on open call for tenders and explicit criteria, and used a broad variety of participatory methods.

In summary, comparison shows that in most cases the decision was taken to continue xenotransplantation research in principle. Most often processes of decision-making were bureaucratic-technocratic and – despite the rhetoric of public participation – rarely involved citizens. The processes were only open and accountable to a small degree and framed xenotransplantation rather narrowly in terms of organ shortage and risk. Thereafter, expert bodies rather quickly turned their focus on the concrete details of risk regulation and management.

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[1]This paper is based on results of the project "Impact of Citizen Participation on Decision-Making in a Knowledge Intensive Policy Field" (CIT-PART), which ran from 2009 to 2012.

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[2]The sample included Austria, Canada, Denmark, Latvia, Italy, Netherlands, Sweden, Switzerland, the UK, the Holy See (Vatican), OECD and the European Commission.

Functional evaluation of hTM, CD46 and HLA-E transgenes in vitro and in pig leg xenoperfusion experiments

Anjan K. Bongoni[1,2,3], David Kiermeir[3], Mihai A. Constantinescu[3], Hansjörg Jenni[4], Andrea Bähr[5], Annegret Wünsch[5], David Ayares[6], Eckhard Wolf[5], Esther Voegelin[3], Nikolai Klymiuk[5], Jörg D. Seebach[7], Robert Rieben[1,3]

[1]Department of Clinical Research, University of Bern, Bern; [2]Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern; [3]Clinic of Plastic and Hand Surgery, University Hospital, Bern,; [4]Clinic of Cardiovascular Surgery, University Hospital, Bern, Switzerland; [5]Institute of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilian University, Munich, Germany; [6]Revivicor, Inc., Blacksburg, VA, USA; [7]Division of Immunology and Allergology, University Hospital and Medical Faculty, Geneva, Switzerland

Background: Besides α1,3 galactosyltransferase (Gal) gene knockout several transgene combinations to prevent pig-to-human xenograft rejection are being investigated. hCD46/HLA-E double transgenic pigs were tested for prevention of xenograft rejection in an ex vivo pig-to-human xenoperfusion model. In addition, expression of human thrombomodulin (hTM-) on wild-type and/or multi-transgenic (GalTKO/hCD46) background was evaluated to overcome pig-to-human coagulation incompatibility.
Methods: hCD46/HLA-E double transgenic as well as wild-type pig forelimbs were ex vivo perfused with whole, heparinized human blood and autologous blood, respectively. Blood samples were analyzed for production of porcine and/or human inflammatory cytokines. Biopsy samples were examined for deposition of complement proteins as well as E-selectin and VCAM-1 expression. Serial blood cell counts were performed to analyze changes in human blood cell populations. In vitro, PAEC were analyzed for ASGR1 mediated human platelet phagocytosis. In addition, a biochemical assay was performed using hTM-only and multi-transgenic (GalTKO/hCD46/hTM) pig aortic endothelial cells (PAEC) to evaluate the ability of hTM to generate activated protein C (APC). Subsequently, the anti-coagulant properties of hTM were tested in a microcarrier based coagulation assay with PAEC and human whole blood.
Results: No hyperacute rejection was seen in the ex vivo perfusion model. Extremity perfusions lasted for up to 12 h without increase of vascular resistance and had to be terminated due to continuous small blood losses. Plasma levels of porcine IL1β ($P < 0.0001$), and IL-8 ($P = 0.019$) as well as human C3a, C5a and soluble C5b-9 were significantly ($P < 0.05$–<0.0001) lower in blood perfused through hCD46/HLA-E transgenic as compared to wild-type limbs. C3b/c, C4b/c, and C6 deposition as well as E-selectin and VCAM-1 expression were significantly ($P < 0.0001$) higher in tissue of wild-type as compared to transgenic limbs. Preliminary immunofluorescence staining results showed that the expression of hCD46/HLA-E is associated with a reduction of NK cell tissue infiltration ($P < 0.05$). A rapid decrease of platelets was observed in all xenoperfusions. In vitro findings showed that PAEC express ASGR1 and suggest that this molecule is involved in human platelet phagocytosis. In vitro, we found that the amount of APC in the supernatant of hTM transgenic cells increased significantly ($P < 0.0001$) with protein C concentration in a dose-dependent manner as compared to control PAEC lacking hTM, where the turnover of the protein C remained at the basal level for all of the examined concentration. In further experiments, hTM also showed the ability to prevent blood coagulation by three- to four-fold increased ($P < 0.001$) clotting time as compared to wild-type PAEC. The formation of TAT complexes was significantly lower when hTM-transgenic cells ($P < 0.0001$) were used as compared to wild-type cells.
Conclusions: Transgenic hCD46/HLA-E expression clearly reduced humoral xenoresponses since the terminal pathway of complement, endothelial cell activation, inflammatory cytokine production and NK-cell tissue infiltration were all down-regulated. We also found ASGR1 expression on the vascular endothelium of pigs, and this molecule may thus be involved in binding and phagocytosis of human platelets during pig-to-human xenotransplantation. In addition, use of the hTM transgene has the potential to overcome coagulation incompatibilities in pig-to-human xenotransplantation.

Transplanting allo-islets without immunosuppression

Barbara Ludwig[1,2], Andreas Reichel[1,2], Anja Steffen[1,2], Baruch Zimmermann[3], Clark K. Colton[4], Stefan Ludwig[5], Stephan Kersting[5], Ezio Bonifacio[6], Michele Solimena[2,7], Zohar Gendler[3], Avi Rotem[3], Uriel Barkai[3], Stefan R. Bornstein[1,2]

[1]Department of Medicine III, University Hospital Carl Gustav Carus, Dresden; [2]Paul Langerhans Institute, Dresden, Germany; [3]Beta-O_2 Technologies, Petach-Tikva, Israel; [4]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA; [5]Department of Surgery, University Hospital Carl Gustav, Carus, Dresden; [6]Center for Regenerative Therapies, Dresden; [7]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

Introduction: Pancreatic islet transplantation is currently restricted to patients with critical metabolic lability due to long-term need for immunosuppression and a persistent shortage of donor organs [1–3]. To overcome these obstacles we have developed a strategy for islet macroencapsulation that provides sufficient immune-isolation whereas regulated islet graft function is maintained [4–8].
Case Report and Methods: A 63 year old patient with type 1 diabetes and severe metabolic lability was transplanted with isolated islets (2,000 islets/kgBW) encapsulated in an oxygenated chamber system composed of immune-isolating alginate and polymembrane covers. Via a small abdominal incision, a pre-peritoneal pocket for the chamber was dissected, connected oxygen ports were implanted subcutaneously. No immunosuppressive therapy was applied.
Results: The procedure was surgically straightforward and without complications. We could demonstrate persistent graft function by detection of endogenous insulin and c-peptide secretion proving islet viability and function. This observation was accompanied by persistent lowering in HbA1c despite reduction in insulin requirement.
For oxygenation of the non-vascularized and therefore immune-shielded islet graft, the chamber-integrated gas reservoir was replenished daily via the implanted ports without complications.
Conclusion: This encapsulation strategy was for the first time applied to allogeneic human islet transplantation in man. We demonstrated a persistent graft function with regulated insulin secretion without any immunosuppressive therapy. This novel concept may allow for future widespread application for cell-based therapies.
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Evaluating the functionality of xeno-relevant transgenes

Andrea Baehr[1], Annegret Wuensch[1], Elisabeth Kemter[1], Barbara Kessler[1], Mayuko Kurome[1], Jochen Seissler[2], David Ayares[3], Eckhard Wolf[1,4], Nikolai Klymiuk[1]

[1]Department of Molecular Animal Breeding and Biotechnology, LMU Munich, Munich; [2]Diabetes Center, LMU Munich, Germany; [3]Revivicor Inc.; [4]Laboratory for Functional Genome Analysis, Gene Center Munich, Germany

With the scientific progress made in recent years, clinical application of xenotransplantation has become a realistic goal. Besides optimizing immunosuppressive regimens, the development of geneticallytailored donor pigs contributed to this success. Within the CRC 127 “Biology of xenogeneic cell, tissue and organ transplantation – from bench to bedside” we aim at developing novel transgenic donor

pigs for islet transplantation, and providing existing transgenic donor lines to German as well as international collaboration partners.
Regarding novel donor models, we have established transgenic pigs carrying the x-linked inhibitor of apoptosis (XIAP) under the control of the porcine insulin promoter to achieve beta-cell specific expression, as apoptosis has been postulated to play a major role in islet loss during isolation, in vitro cultivation and engraftment. Expression analyses of the first transgenic founders are presently underway. In addition, an advanced donor pig aiming at the temporary expression of vascular endothelial growth factor (VEGF) for improved engraftment of islets is being established. The model is based on the TetOn principle which requires two independent transgenes. First litters of founder pigs carrying a construct for beta-cell specific expression of the transactivator (TA) have been delivered and qPCR analysis of the pancreas revealed different levels of TA expression in individual animals. Primary cells from a highly expressing founder will be transfected with a construct carrying the regulated VEGF gene under the control of the TA response element.
For detailed pre-clinical evaluation of already characterized transgenic pigs, we have established multitransgenic lines in collaboration with Revivicor Inc. carrying either aGalTKO/CD46/hTM, aGalTKO/CD46/INS-LEA or aGalTKO/CD46/HLA-E. We provide these animals, in addition to single-transgenic INS-LEA pigs, to the groups of P.Brenner and J.Seissler within the consortium. Furthermore, multi-transgenic animals are provided to the groups of M.Mohiuddin, D.K.C. Cooper and R. Pierson by D. Ayares and islets of INS-LEA expressing pigs are supplied to B. Hering in collaboration with J. Seissler.
The ongoing evaluation of existing transgenes or their combination will reveal more detailed insight into the complexity of rejection mechanisms and, thus, support the design of optimized future transgenic approaches.

Bio artificial pancreas – the immune barrier and oxygen deficiency

Uriel Barkai[1], Barbara Ludwig[2], Stefan R. Bornstein[2], Baruch Zimermann[1], Zohar Gendler[1], Avi Rotem[1]
[1]Beta-O_2 Technologies, Petach-Tikva, Israel; [2]Department of Medicine III, University Hospital Carl Gustav Carus, Dresden, Germany

Background: Xenogeneic transplantation of islets of Langerhans into type I diabetes patients imposes a major challenge. This is even more exaggerated when the target is set as xenotransplantation of islets in relatively oxygen poor environment without the use of immunosuppressive therapy. To overcome these hurdles, adequate encapsulation technique and active oxygen supply should be employed.
Methods: A two-article device was developed (the βAir). The first contains an implantable bioreactor module comprising of islets of Langerhans encapsulated in alginate hydrogel and protected from the host immune system by a complex membrane. The second is an external oxygen injection device for replenishing of the islet biomass in the implantable device once a day.
Results: Both small and large diabetic animal models (rats and pigs) were experimented. The βAir device proved: (i) ability to maintain near normal glycemic control in rats transplanted with either isogeneic or allogeneic islets for a period of over 6 months and in pigs transplanted with rat islets using marginal dose of islets (~6,500 IEQ/kg) for a period of up to 75 days; (ii) ability to maintain adequate oxygen tension required to nourish the islets graft for a period of 24 h while maintaining optimal islet's function. (iii) long term separation of transplanted graft from host immune system thus maintaining normal islets function in rat allotype, in rat-to-pig and in human-to-rat transplantation systems and (iv) high level of biocompatibility and tolerability of the device.
Conclusion: The βAir device proved to offer functional solution to islets transplantation in allogeneic and xenogeneic setting. It may provide a solution to transplantation of porcine islets to diabetic patients.
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Reduction of xeno-antigens in porcine pulmonary heart valves by decellularization and glycolytic enzymatic treatment

Andres Hilfiker, Robert Ramm, Katja Findeisen, Tobias Goecke, Axel Haverich
Leibniz Research Labs for Biotechnology and Artificial Organs (LEBAO), Department of Cardiothoracic, Transplantation and Vascular Surgery, MHH, Hannover, Germany

Background: Heart valve replacement therapy with allogenic decellularized heart valve matrices allows an in vivo autologisation by repopulation of the foreign matrix with autologous cells. This method, which is superior based on experimental evidence to traditional heart valve replacements either of mechanical or glutaraldehyde fixated heart valve grafts, is current object of a multicentric international clinical trial (ESPOIR) granted by the EU. To circumvent the limited availability of allogenic heart valve matrices, we aim to the reduction of immunogenic epitops common to xenogeneic materials by high efficient decellularization paralleled by enzymatic treatment of the glycocalyx.
Methods: Porcine pulmonary heart valves of landrace pigs harvested at the local slaughterhouse were subjected to detergent and proteinase based decellularization protocols widely described in the literature. In a second step decellularized matrices were exposed to α1-3 Galactosidase or PNGase F digestion. Alterations in αGal epitope levels and glycocalyx structures were investigated by inhibitory ELISAs with anti-αGal antibodies (M86) and IgGs of unconditioned human sera on crushed matrix, and histochemical stains on cryosections exploiting lectins as isolectin B4 (staining αGal), wheat germ agglutinin (staining GalNAc), and Datura stramonium lectin (staining GlcNAc). Equally treated GalT-KO pig and human pulmonary artery tissues served as controls.
Results: Inhibitory ELISA results show that all decellularization protocols reduced αGal epitope levels significantly, some down to <30% of native porcine tissue. Further reduction down to control levels was achieved by α1-3 Galactosidase digestion. The absence of preformed non- αGal xenoantibodies in human IgG of unconditioned donors is evident by equal binding of human IgG to decellularized tissues of GalT-KO and human origin.
Histochemistry reveals that decellularization results in reduced Isolectin B4 (αGal) and WGA (GalNAc) staining, but not of DSL (GlcNAc) staining. Additional enzymatic treatment of decellularized matrix with PNGase F reduces binding of all lectins, whereas digestion with α1-3 Galactosidase only affects Isolectin B4 staining (αGal).
Conclusion: The reduction of αGal epitope levels is strongly dependent on the decellularization method. A further decrease can be achieved by enzymatic digestion of carbohydrates present on the decellularized matrix. Sugar structures of the glycocalyx are believed to be strongly immunogenic, therefore, their general removal by PNGase F digestion may produce non-immunogenic heart valve matrices. However, the lack of preformed human non- αGal xenoantibodies in non conditioned sera requires the testing for immunogenicity in an in vivo system. Thus, in future decellularized and enzymatically treated GalT-KO matrices will be tested in a humanized mouse model.

Ex-vivo testing and preclinical heterotopic thoracic cardiac xenotransplantation of multi-transgenic organs

Jan-Michael Abicht[1], Tanja Mayr[2], Sonja Guethoff[2], Stefan Buchholz[2], Fabian Werner[2], Andrea Baehr[3], Nikolai Klymiuk[3], Annegret Wuensch[3], Eckhart Wolf[3], Claus Belka[4], David Ayares[5], Björn Petersen[6], Heiner Niemann[6], Anjan K. Bongoni[7], Robert Rieben[7], Christopher McGregor[8], Bruno Reichart[9,10], Paolo Brenner[2]
[1]Department of Anaesthesiology, Ludwig-Maximilians University, Munich; [2]Department of Cardiac Surgery, Ludwig-Maximilians University, Munich; [3]Institute of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians, Munich; [4]Department of Radiation Oncology, Ludwig-Maximilians University, Munich; [5]Revivicor Inc., Blacksburg, VA, USA; [6]Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute (FLI), Mariensee, Germany; [7]Department of Clinical Research, and Graduate School of Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland; [8]Division of Cardiovascular Surgery, Mayo Clinic, Rochester, MN, USA; [9]Walter-Brendel-Centre, Ludwig-Maximilians University Munich, Munich; [10]University Munich, Munich, Germany

The heterotopic thoracic pig-to-baboon heart transplantation has been established by our group as a safe preclinical model. Since the recipient's own heart remains in place, it is possible to evaluate immunological reactions of various types and the symptoms of the thrombotic microangiopathy under working heart conditions. However, these experiments are complex and costly. In order to limit the number of transplants, we tested our multi-transgenic porcine donor hearts in an ex-vivo perfusion model first.

Ex-vivo model: Both beating ventricles were perfused with heparinized freshly drawn human whole blood using a centrifugal pump and a membrane oxygenator. During 3 h of observation, cardiac function parameters were obtained continuously; specimens from the perfusate, coronary sinus blood, and myocardial biopsies were assessed. Various genetically modified porcine donor hearts were tested; we are currently evaluating the impact of human thrombomodulin on the porcine microcirculation; the efficacy of complement regulation is another work package.
Pig-to-baboon heterotopic cardiac xenotransplantation: In our latest group, seven baboons received five double (Gal-KO/hCD46), and two triple (Gal-KO/hCD46/hTM resp./HLA-E) transgenic pig hearts. Our immunosuppressive regimen included preoperative anti-CD20-antibody, bortezomib, dexamethasone and cyclophosphamide; postoperatively, ATG, tacrolimus, MMF, anti-CD20-antibody, bortezomib and dexamethasone were administered. Total lymph node irradiation (6 Gy) was applied on postoperative day five.
The triple transgenic hearts survived 35 and 37 days respectively. Both hearts maintained function throughout the experiment. Both recipients succumbed to fungal infections. Humoral rejection was seen only once. The next barrier appears to be the complex event of thrombotic microangiopathy that needs to be addressed with the additional expression of human thrombomodulin strategies.

Production of multi-transgenic pigs tailored for xenotransplantation by using zinc-finger nucleases and sleeping beauty transposons

Björn Petersen[1], Stoyan Petkov[1], Janet Hauschild[1], Hellen Ahrens[1], Andrea Lucas-Hahn[1], Petra Hassel[1], Maren Ziegler[1], Klaus-Gerd Hadeler[1], Reinhard Schwinzer[2], Heiner Niemann[1]
[1]Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Mariensee;
[2]Transplantation Laboratory, Hanover Medical School, Hanover, Germany

Multi-transgenic pigs are required for prolonging survival of porcine xenografts after transplantation into primate recipients. A homozygous knockout of the alpha1,3-galactosyltransferase gene (GGTA-1, encoding for Gal-epitopes) is critical for controlling the hyperacute rejection response. Nevertheless, the additional expression of complement regulatory proteins such as CD55 (decay accelerating factor, DAF) on a GGTA-1 KO background is thought to further improve survival after xenotransplantation. The next hurdle is the acute vascular rejection (AVR), which is primarily caused by endothelial cell activation. Previous experiments had shown that expression of anti-apoptotic/anti-inflammatory genes, such as human heme oxygenase-1 (hHO-1) or human A20 in pig organs may overcome AVR (1, 2). Adult fibroblasts from previously generated hHO-1 transgenic pigs were transfected with Zinc-finger nuclease plasmids directed towards knockout of the GGTA-1 gene. Transfected cells were selected via a magnetic bead selection approach as previously described (3). GGTA-1-KO/hHO-1 cells were used as donor cells in somatic cell nuclear transfer (SCNT). The first pregnancy was terminated at day 25 of gestation and 11 healthy fetuses were obtained. All fetuses were GGTA-1-KO and expressed hHO-1 at similar levels as the hHO-1 transgenic cell donors. Next, fetal GGTA-1-KO/hHO-1 cells from one fetus (C6F5) were transfected with a Sleeping Beauty (SB) transposon (kindly provided by Dr. Zoltan Ivics) coding for hCD55. Two positive cell clones were pooled and used as donor cells for SCNT. One pregnancy was terminated on day 25 and four healthy fetuses were obtained which showed the desired GGTA-1 KO/HO-1/CD55 genotype. Real-time PCR detected a 0.9 fold expression of CD55 over the housekeeping gene GAPDH. Compared to human umbilical vein cells (HUVEC), GGTA-1 KO/HO-1/CD55 fetal fibroblasts expressed CD55 10–15 fold higher than HUVEC. Two fetuses showed a slightly higher expression of CD55 and are currently used as cell donors for recloning to produce live triple transgenic offspring.
To solve the problem of transgene segregation occurring after mating of multitransgenic pigs, we generated two tri-cistronic SB vectors coding for A20 and CD55 separated by the self-cleaving peptide E2 site. To select for transfected cells, one vector carried a Tomato-fluorescence cassette whereas the second conferred puromycin resistance. Red fluorescent/puromycin resistant cells served as donor cells for SCNT. Six healthy fetuses were collected on day 25 of pregnancy; all showed high expression of A20 and CD55 as detected by Real-time PCR and FACS. One of the fetuses was used as cell donor for recloning. Currently, three pregnancies with A20/CD55 fetuses are going on and will be allowed to go to term. These results show that molecular scissors, including ZFNs and TALENS, and expression vectors such as the Sleeping beauty system are valuable tools to enhance and accelerate the generation of multi-transgenic pigs for xenotransplantation.
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Effective combination of xenoprotective transgenes

Konrad Fischer, Simone Kraner-Scheiber, Tatiana Flisikowska, Angelika Schnieke
Department of Livestock Biotechnology, Technische Universität München, Freising, Germany

Abundant expression of a series of xenoprotective transgenes is vital for clinically useful xeno-donor pigs. Breeding of such multi-transgenic animals requires that xeno-transgenes be placed at a single, or a small number of loci in the pig genome to avoid or minimise separation by genetic segregation. We are investigating methods that support transgene expression and also enable transgenes to be “stacked” at a single locus. These include the use of BAC based multi-transgene vectors, and very recently serial transgene placement at a permissive locus by homologous recombination supported by TALENs [1].
BAC vector constructs containing genomic sequences for the complement activation inhibitors CD55, CD46 and CD59 were assessed to determine the minimum size without reduction of expression level. These constructs enable us to express all biologically active CD55 splice variants (membrane bound and soluble forms). Effective xenoprotection also requires ubiquitous expression. We are thus examining endogenous promoter sequences of various lengths as well as the widely used CAGGs (CMV enhancer/chicken β actin) promoter to direct complement inhibitory gene expression levels in all porcine tissues.
To investigate means of coexpressing groups of transgenes that provide xeno-protection at different levels, we developed a series of BAC vector constructs containing the CD55 genomic sequence plus two additional xeno-transgene cDNAs, including A20 [2], HO1 [3], thrombomodulin [4] and CTLA4-Ig (LEA29Y) [5]. Analysis of porcine adipose MSC stably transfected cell clones revealed expression of all inserted xeno-transgenes most importantly high expression of complement inhibitory genes (Fig. 1). This demonstrates that xeno-transgenes can be effectively combined in a single construct, thus minimising the number of transgene loci in the final donor pigs. The huge carrying capacity of BAC vectors enables the addition of further xeno-transgenes in a single vector construct.
We have further examined the system in vivo. Primary cells transfected with up to three BAC constructs were used for nuclear transfer, fetuses explanted and examined for expression with very promising results.
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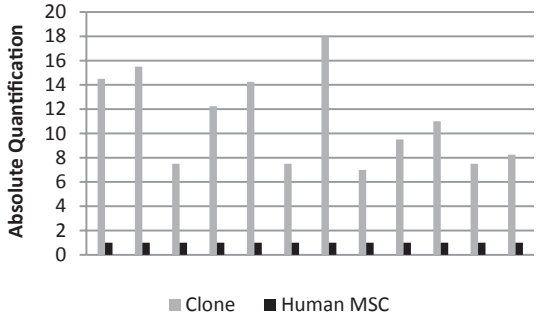
Fig. 1. Q-RT-PCR analysis of CD55-CAGGs (left) and CD46-CAGGs (right) vector constructs compared to the expression levels of human MSCs.



Symposium on Xenotransplantation

Theological-ethical legitimization of xenotransplantation and challenges regarding the hospital chaplaincy including the pastoral psychological companionship

Jochen Sautermeister
Department of Moral Theology, Catholic Theological Faculty, LMU Munich, Munich, Germany

Background: In contrast to allotransplantation, xenotransplantation has thus far attracted lit-tle interest within the field of theological ethics [6]. This can essentially be attributed to the fear of considerable problems regarding the medical feasibility due to possible immune reac-tions and the risk of infection [10,11]. Consequently, at the beginning of the 21st century a recommendation was issued to stop all relevant research; this also led to a retardation of theo-logical-ethical reflections on xenotransplantation.
Ethical questions: Irrespective of technical feasibility, the question whether xenotransplanta-tion is ethically justifiable or not can systematically be divided into three areas [10]: (i) Ethics regarding animals and nature are concerned with the significance of non-human rights; (ii) ethics in the areas of medicine, biomedicine and research deal with the importance of human rights; (iii) within the frame of a social-ethical perspective, these rights need to be debated within the context of distributive justice. The prevalent ethical discussions regarding xeno-transplantation focus on the following issues [2, 10]: protection of animals, risks of infection and informed consent. These have been the main reasons for the reluctance regarding human experiments [3, 5, 8]. Another range of concerns centers on the creation of human/animal chimeras by Xenotransplantation and the psychosocial and sociocultural acceptance of such procedures. These reservations are especially strong where the heart is concerned, since it has a special value in terms of personal and cultural identity, and a strong symbolic meaning. The morality of breeding transgenic pigs as donor animals has also recently been questioned, rais-ing the question of whether it is legitimate to transgress the human-animal boundary [1].
Since chimerism raises questions regarding its psychosocial and sociocultural acceptance, the basic levels of the bioethical discourse that underlie the specific normative regulations are addressed. This concerns the anthropological question of human self-conception and ques-tions regarding fundamental interpretations of life and essential beliefs regarding the meaning of life. To the extent that the last mentioned levels are inevitably incorporated into the norma-tive judgments of applied ethics and relate to the psychosocial and sociocultural acceptance of the xenotransplantation of the heart, it is necessary to bear in mind the fundamental anthropo-logical and ideological implications [4, 9]. These implications are reflected by theological ethics. From a perspective of theological ethics the question of identity also has to be debated within a theological anthropological framework.
Most ethical perspectives on xenotransplantation refer explicitly or implicitly to the question of the psychosocial and sociocultural acceptance of Xenotransplantation. As a consequence, the question of personal [7], social and cultural identity as well as the pivotal question con-cerning the significance of the acceptance category for the ethical argumentation and ethical legitimization of standards are addressed. According to the ethical standards definition, validi-ty of a standard is also characterized by an opportunity to achieve social recognition and ac-ceptance.
Research areas: The relationship between psychosocial and sociocultural acceptance and ethical legitimization shall be discussed within the context of xenotransplantation, and the relevance of public opinion shall be mapped out with the goal of achieving social acceptance and thereby facilitating the success of xenotransplantation. With reference to the psychosocial conditions for acceptance and success, the relevance as well as the tasks of pastoral compan-ionship and pastoral-psychological support for potential organ recipients shall be debated and guidelines shall be drafted. In light of the identity-related challenges, an ethical and theologi-cal request for psychosocial counseling and pastoral companionship. Furthermore, special focus shall be given to the particular importance of the spiritual dimension of counseling.
This project is funded by DFG (TR CRC 127 “Biology of xenogeneic cell, tissue and organ transplantation – from bench to bedside”).
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B cell activation and induced antibody responses to porcine antigen can be diminished by PD-L1-mediated triggering of PD-1

Anna Buermann, Katja Borns, Dorothee Römermann, Annegret Plege-Fleck, Reinhard Schwinzer
Transplant Laboratory, Department of General-, Visceral-, and Transplantation Surgery, Hannover Medical School, Hannover, Germany

Background: T cell activation is regulated by the integration of positive and negative signals. A strategy to down-regulate human immune responses to a porcine xenograft is the enhancement of negative costimulatory signals. The programmed death-1 receptor (PD-1) is one of the known negative regulators for T cell activation and we have previously shown that human in vitro T cell responses to porcine transfectants over-expressing human PD-Ligand 1 (PD-L1) are reduced as compared to responses triggered by control cells. However, PD-1 is also expressed on B cells and its role in B cell activation is not well understood so far. Thus, the present study focused on the effects of PD-1/PD-L1 targeting on the humoral immune response to xenoantigens.
Methods: In vitro activation of human B cells was induced by co-culturing with porcine L23 cells (B cell line) genetically engineered to over-express human PD-L1 (L23-PD-L1 cells) or “mock” -transfected controls. In vivo immune responses to the two cell types were characterized in a rat model by immunization experiments. The anti-pig antibody titer in the culture supernatant or the sera of rats was examined by binding to L23 cells and visualized by flow cytometry using FITC-conjugated IgM and IgG.
Results: Stimulation of human peripheral blood mononuclear cells (hPBMC) with L23 cells resulted in up-regulation of the early activation marker CD69 on $CD19^+$ B cells. Furthermore, we observed T cell dependent proliferation of B cells and differentiation to antibody secreting cells in vitro. This was shown by the detection of anti-pig antibodies in the culture supernatant of hPBMCs stimulated with xenoantigen after 7 days and the titer increased till day 12, with higher amounts of IgM than IgG antibodies. When the activation of B cells was induced by L23-PD-L1 cells, lower antibody titers were determined in comparison to B cell activation induced by L23 control cells. Moreover, the humoral immune response was reduced when a soluble PD-L1 molecule was applied to hPBMCs stimulated with porcine cells. To further characterize the antibody responses to L23-PD-L1 and control cells in vivo, we analyzed the level of anti-pig antibodies in rats after cell transplantation under the kidney capsule. In line with the in vitro data we found lower levels of antibodies in the serum of rats immunized with L23-PD-L1 cells than in rats receiving L23 control cells.
Conclusion: These data suggest that the antibody response to porcine xenoantigen is diminished in the presence of the inhibitory ligand PD-L1. Application of PD-L1 might be a strategy to prevent induction of anti-graft antibody responses, which are particularly strong in the pig-to-primate combination and represent a significant immunologic barrier for clinical xenotransplantation.

Decellularization followed by PNGase F treatment efficiently removes immunogenic αGal epitopes and other *N*-acetylglucosamine structures on the glycocalyx of porcine pulmonary heart valve matrices

Katja Findeisen, Robert Ramm, Andres Hilfiker
Leibniz Research Labs for Biotechnology and Artificial Organs (LEBAO), MHH, Hannover, Germany

Background: Xenotransplantation leads to hyperacute or acute graft reaction due to interaction of preformed human antibodies, mainly αGal-antibodies, with carbohydrate structures present on non-human tissue. By removing these carbohydrate structures from porcine decellularized pulmonary heart valves (PHV), grafts might be generated that allow heart valve replacement therapy as with allogeneic PHV matrices.
Methods: Thus, after detergents based decellularization (0.5%SDS/0.5%Triton-X100) cell free PHV matrices were enzymatically treated with α1-3,6-galactosidase or PNGase F. The potential impact on the glycocalyx was investigated by histochemical stains utilizing isolectin B4 (IL-B4), wheat germ agglutinin (WGA), Datura stramonium lectin (DSL), and Ricinus communis agglutinin (RCA I) on decellularized only, and decellularized and enzymatically treated specimens. Native PHV tissue served as controls.
Results: All used lectins stained native heart valve tissue. Decellularization resulted in reduced IL-B4 and WGA staining, whereas cell removal had no effect on DSL and RCA I staining. Enzymatic PNGase F treatment resulted in a further decrease of IL-B4 and WGA staining whereas initially not affected DSL stain was reduced as well. Enzymatic treatment with α1-3,6-galactosidase led to a reduction of IL-B4 stain only.
Conclusion: Decellularization per se is able to reduce αGal epitopes as demonstrated by IL-B4 staining. This reduction of αGal epitopes can be enhanced by α1-3,6-galactosidase digestion as expected. Enzymatic treatment with PNGase F that recognizes GlcNacβ(1-N)Asn sites results in removal of carbohydrate structures as αGal and *N*-acetyl-glucosamines to a high degree as demonstrated by IL-B4, WGA, and DSL stains.
In summary, detergent based decellularization followed by PNGase F treatment resembles an efficient way to remove immunogenic epitopes from porcine pulmonary heart valve matrices, thus potentially enabling the generation of xenogeneic PHV matrices for clinical application.

After decellularization of porcine heart valves: no non-Gal antigeneic epitopes detectable by non conditioned human sera

Robert Ramm, Andres Hilfiker
Leibniz Research Labs for Biotechnology and Artificial Organs (LEBAO), Department of Cardiothoracic, Transplantation and Vascular Surgery, MHH, Hannover, Germany

Background: Patients that undergo heart valve replacement surgery with glutaraldehyde fixated porcine grafts do not face acute rejection as recipients of native porcine organs and tissues do, however, grafts calcify over time and fail after 10–20 years in adults and 3–4 years in children. Responsible for hyperacute and acute rejection of unfixated tissue are preformed antibodies that recognize xenoantigens. Major targets are carbohydrates like the αGal epitope or Neu5Gc linked to proteins as well as to lipids on cells and extracellular matrix. To overcome the limited availability of decellularized allogeneic heart valve grafts, which show the ability to remodel and to grow by ingrowth of autologous cells, a characteristics lacking in glutaraldehyde fixated heart valve grafts, we want to develop a porcine derived decellularized matrix that is well tolerated in humans.
Methods: The generation of such optimal porcine heart valves matrices is dependent on a successful elimination of bound xenoantigens. To quantify the amount of xenoantigens present on decellularized matrix, we established an inhibition ELISA by exposing human IgG to crushed matrix and measuring unbound IgG in an ELISA set up. High levels of measured IgG means low levels of xenoantigens present on the decelllularized matrix. To enhance the specificity of IgG tested in the ELISA, human sera were perfused through porcine kidneys, followed by intensive washing and elution of bound xenoantibodies. Total amount of IgG and αGal specific antibodies was monitored to control the purification steps. Inhibition ELISA was performed with matrices derived from Landrace pigs, GalT-KO pigs and humans. Binding of total IgG as well as αGal specific antibodies was assayed.
Results: The amount of total IgG binding correlates with the amount of αGal specific antibodies binding to decellularized porcine matrix indicating the presence of remaining αGal epitopes on the porcine matrix after decellularization. However, decellularized GalT-KO pig and decellularized human matrix showed no binding of αGal specific antibodies and comparable amounts of total IgG binding.
Conclusion: Our results indicate that only αGal epitopes present on decellularized porcine heart valve matrixes are recognized by preformed xenoantibodies while no other xenoantigens are detected. Therefore, further evaluation focusing on induction of immunogeneic reactions has to be conducted in an in vivo model like the humanized mouse utilizing decellularized heart valve material derived from GalT-KO pigs.

Production of high expressing A20/DAF- transgenic pigs on a GGTA1-KO background

Hellen E. Ahrens[1], Bjoern Petersen[1], Stoyan Petkov[1], Doris Herrmann[1], Antje Frenzel[1], Janet Hauschild-Quintern[1], Andrea Lucas-Hahn[1], Petra Hassel[1], Maren Ziegler[1], Wiebke Baars[2], Reinhard Schwinzer[2], Heiner Niemann[1]
[1]Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Mariensee, Neustadt, Germany; [2]Transplant Laboratory, Hannover Medical School, Hannover, Germany

Background: Pig-to-human xenotransplantation is promising to ameliorate the worldwide shortage of suitable human donor organs. The transplantation of xenografts evokes severe immunological rejection responses. Different approaches led to considerable progress in this respect: transgenic expression of human complement regulatory proteins such as human decay-accelerating factor (hCD55), membrane co-factor protein (hCD46, MCP) or hCD59 and/or knockout of the porcine α1,3-galactosyltransferase gene (GGTA1-KO) prevent the hyperacute rejection response (HAR) [1, 2]. Moreover, human anticoagulant and/or anti-apoptotic and anti-inflammatory transgenes, such as human A20 (hA20) and human heme oxygenase-1 (hHO-1), could protect porcine xenografts from being rejected by the acute vascular rejection response [3, 4].
Recently, we produced pigs transgenic for hA20 which provides cytoprotective properties in porcine aortic endothelial cells in vitro [5]. However, hA20 was expressed only at low levels in heart, skeletal muscle and porcine aortic endothelial cells. Here, we generated a new tri-cistronic hA20/hCD55/Puro vector based on the Sleeping Beauty system (kindly provided by Dr. Zoltán Ivics) and transfected it into porcine GGTA1-KO cells that served as donor cells for somatic cell nuclear transfer (SCNT).
Methods: We co-transfected GGTA1-KO [6] porcine fibroblasts with a tri-cistronic cDNA expression vector coding for hA20/hCD55/Puro and the SB transposase 100X plasmid. The advantage of this vector is a stable genomic integration without any plasmid-based vector backbone and the combined integration of all transgenes at a single genomic locus which avoids transgene segregation after mating. Following puromycin selection (5 µg/ml), cells were screened by PCR for genomic integration of the vector and used for SCNT.
Results: In total, 1,028 embryos were transferred to 13 recipients of which five remained pregnant. Two pregnancies were in too early stages for ultrasound diagnosis at the time of writing. One pregnancy was terminated on day 25 of gestation and eight fetuses were obtained. The hA20 mRNA levels of these fetuses were increased 21- to 44-fold compared to another line (hA20/hHO-1/GGTA1-KO) and even higher than in the first generation hA20 transgenic pigs. Preliminary immunofluorescence analysis data showed delta mean fluorescence intensities (MFI) for hCD55 (hCD55 MFI minus control antibody MFI) of 1,016 in average in transgenic fibroblasts in comparison to delta MFI 299 in HUVECs and 114 in PBMCs. The susceptibility of the cells to NK cell- and T cell-mediated cell death compared to wild-type fibroblasts will be determined in a ^{51}chromium release assay. Three gilts are expected to deliver piglets in November of this year. The expression and organ distribution of hA20 and hCD55 will be characterised subsequently.
Conclusions: Our goal is the production of multi-transgenic pigs with an increased expression of xeno-relevant transgenes. The combination of ubiquitous, high expression of anti-apoptotic and-inflammatory transgenes such as hA20 together with a complement regulatory protein like hCD55 on a GGTA1-KO background has the potential to prolong the survival of porcine grafts after pig-to-primate xenotransplantation.
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Generation of transgenic pigs carrying siRNA vector directed against human tissue factor expression

Hellen Ahrens, Björn Petersen, Doris Herrmann, Andrea Lucas-Hahn, Petra Hassel, Maren Ziegler, Wilfried Kues, Heiner Niemann
Institute of Farm Animal Genetics, Friedrich-Loeffler Institut, Mariensee, Neustadt, Germany

Background: The acute vascular rejection (AVR) is the major obstacle to successful pig-to-primate xenotransplantation. Changes of the endothelium to a procoagulant state which occur after xenotransplantation entail an increased expression of Tissue Factor (TF). This initiates the extrinsic coagulation cascade and results in microvascular thrombosis and rapid loss of organ function. Organs from pigs with a decreased expression of TF could be promising to overcome AVR. Since a homozygous TF knockout had shown to be lethal in mice [1], we decided to use siRNA-technology to knockdown TF to a basal level.
Methods: Different siRNAs directed against TF expression were tested in initial studies for their efficacy. A vector coding for the siRNA providing the highest decrease of TF mRNA levels was kindly provided by Dr. Joachim Denner, RKI Berlin. Porcine fetal fibroblasts were co-transfected with this plasmid and a vector (DsRed, Clontech) which confers neomycin resistance and red fluorescence. After 14 days of selection with 800 µg/ml Geneticin (G418), cells were screened for siRNA expression prior to somatic cell nuclear transfers (SCNTs). Subsequently, the reconstructed embryos were transferred to synchronised recipient sows. Live-born offspring were analysed by real-time PCR for TF mRNA quantification. The production of the siRNA was detected via RT-PCR followed by real-time PCR.
Results: An average of 95 embryos per sow was transferred to 28 synchronized recipients. The pregnancy rate was 54%. One sow was sacrificed on day 25 of pregnancy and one fetus (#893/1) was obtained. The candidate siRNA could be detected fetal tissues. Real-time PCR analysis revealed decreased TF mRNA expression in fibroblasts of fetus #893/1 compared to wild-type fibroblasts. Therefore, somatic cells from fetus #893/1 was used for recloning. A total of 12 live born piglets could be obtained; five piglets died within few days after delivery. The mRNA levels and production of the siRNA are currently determined. Protein expression analysis by fluorescence cytometry and functionality assessment by coagulation assays are planned for the near future with collaborating laboratories at the Medical School Hannover.
Conclusions: The AVR is currently the bottleneck in porcine-to-primate organ xenotransplantation. Controlling TF-induced coagulation might provide protective effects for porcine xenografts. Thus, TF knockdown pigs could be critical in producing the ultimate multi-transgenic pig as organ donor for human patients.
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Cloning and characterization of replication-competent ecotropic porcine endogenous retroviruses (PERV-C) in the genome of pigs used and intended for clinical pig-to-human xenotransplantation

Michael Rodrigues Costa, Nicole Fischer, Barbara Gulich, Hanna Belschner, Antonia Gronewold, Ralf R. Tönjes
Division of Medical Biotechnology, Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Langen, Germany

Porcine endogenous retroviruses (PERV) pose a xenozoonotic risk when applying pig organs, tissues and cells in clinical xenotransplantation. Three classes of replication-competent PERV were described in host range and interference studies [1]. Polytropic PERV-A and PERV-B are able to infect not only human cells but also various cell lines in vitro using host membrane proteins as receptors [2]. Although cell tropism of ecotropic PERV-C is mainly restricted to porcine cells, PERV-C represents a considerable infectious risk. On the one hand, PERV-C serves as template for recombination with PERV-A resulting in highly infectious human-tropic PERV-A/C [3]. On the other hand, PERV-C may evolve towards an infectious human-tropic variant since the PERV-C receptor-binding domain could be bound to human cells and solely four amino acid exchanges in the surface unit of the *env*C gene are sufficient to permit receptor-mediated membrane fusion and virus entry [4]. To guarantee retroviral safety in pig-to-human xenotransplantation generation of appropriate pigs free from replication-competent PERV-A and PERV-B as well as ecotropic PERV-C is required.
Using PCR-based methods and directional cloning strategies, we cloned and characterized PERV-C. Genomic DNA was prepared from peripheral blood mononuclear cells derived from three different pig subspecies which are either already used or intended for clinical xenotransplantation. One PERV-C clone was reconstructed using genomic DNA of an Auckland Islands pig derived from a DPF herd in New Zealand. Islet cell clusters of these PERV non-transmitters are used in clinical trials to treat diabetes type I patients. Moreover, the potential of brain cells from these animals to treat Parkinson's and Huntington's disease is currently tested clinically. Similarly, a PERV-C clone was reconstructed from genomic DNA of Göttingen minipig which is a PERV non-transmitter as tested in co-cultures with susceptible human HEK 293 cells [5]. Finally, a bacteriophage lambda library was constructed from genomic DNA of a *d/d* haplotype miniature pig. The individuals of this pig line show lower PERV transmission levels in vitro [6]. We isolated a λ-clone containing PERV-C 5′-LTR, *gag*, *pro/pol* as well as large part of the *env*C gene compared to replication-competent PERV-C(1312) [7]. The provirus is truncated due to our cloning strategy. Nonetheless, using PCR and PERV-C specific primers the missing part of the *env*C gene and 3′-LTR was amplified and ligated to the proviral fragment present in the λ-clone. Replication-competence of reconstructed full-length viruses is currently tested in susceptible ST-Iowa cells.
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RNase A in (Xeno)Transplantation

Eike Kleinert[1], Bruno Reichart[2], Tanja Mayr[2,3], Jan-Michael Abicht[2,4], Paolo Brenner[2,3], Christian Hagl[3], Martin Langenmayer[5], Ruediger Wanke[5], Elisabeth Deindl[1], Sonja Guethoff[2,3]
[1]Walter-Brendel-Centre of Experimental Medicine, Ludwig-Maximilians University, Munich; [2]Transregio Collaborative Research Center 127, Ludwig-Maximilians University, Munich; [3]Department of Cardiac Surgery, Ludwig-Maximilians University, Munich; [4]Department of Anaesthesiology, Ludwig-Maximilians University, Munich; [5]Institute of Veterinary Pathology, Ludwig-Maximilians University, Munich, Germany

Background: Cell injury, particularly as consequence of ischemia/reperfusion or acute rejection after (xeno)transplantation, leads to release of intracellular components like RNA. Extracellular RNA, (i) causes edema as a result of increasing permeability of blood vessels [1], (ii) is described as a procoagulation factor by activation of the contact phase system leading to thrombus formation and vessel occlusion [2], and (iii) promotes leukocyte recruitment to the vascular system by mobilising proinflammatory cytokines [3], and thus, triggers immune response. In rodent models of stroke and thrombosis, treatment with counteracting RNase A, a ribonuclease that specifically degrades single-stranded RNA, was shown to result in less edema formation and vessel occlusion [4]. In search of additive drugs to protect (xeno)grafts from dysfunction, we investigated if treatment with RNase A in a rat model of heterotopic heart transplantation results in less edema and less coronary occlusion, and, therefore, in improved graft survival.

Methods: Brown Norway rat cardiac allografts were transplanted into the abdomen of Lewis rats after perfusion with Bretschneider cardioplegic solution. Microvascular technique for aorto-aortic anastomosis and pulmonary artery to inferior vena cava anastomosis (Langendorff) was used. Recipients were intravenously treated directly before transplantation and every other day with RNase A (50 μg/kg) or vehicle (saline, n = 6 in each group). In addition, in six cases the donor graft was perfused with Bretschneider solution containing RNase A (8 μg in 10 ml), and afterwards transplanted into RNase A treated recipients. The primary end point of the study was graft survival, which was determined by daily examination of the graft function by palpation and in case of inconclusiveness by echocardiography. A tolerance study was performed treating animals daily with 50 μg/kg of RNase A, with 1,000 μg/kg of RNase A, or vehicle, respectively, for 28 days (n = 3 in each group). All explanted hearts were collected for histological evaluation.
Results: Mean graft survival was 6.5 ± 0.55 day (range 6–7 day) in vehicle treated animals vs. 10.2 ± 1.0 day (range 9–11 day) in RNase A treated animals (P = 0.002). Directly after explantation donor grafts had a mean weight of 0.94 ± 0.03 g (range 0.89–0.98 g). After been rejected, donor graft weight increased to 2.06 ± 0.52 g (range 1.59–2.77 g) in vehicle treated recipients, in contrast to 1.28 ± 0.27 g (1.05–1.61 g) in RNase A treated recipients (P = 0.017). No supplemental benefit was observed by additive using of RNase A in Bretschneider solution (graft survival 9.50 ± 1.52 day, graft weight 1.38 ± 0.15 g) compared to single treatment of the recipient (graft survival P = 0.394, graft weight P = 0.589). Tolerance studies showed neither macroscopic nor histological differences compared to saline treated animals.
Outlook: Further histological analyses will help to clarify if RNase A will keep the promise to reduce edema formation even in the field of transplantation, as suggested by the significant less graft weight in RNase A treated recipients. Moreover, we will investigate, if RNase A prevents leukocyte recruitment in the context of rejection reaction. Therefore, grafts of RNase A treated and untreated rats will be explanted on day 4 after transplantation.
Conclusions: RNase A significantly improved graft survival. On the basis of these sweeping results, however, we suppose that RNase A could be an important adjuvant drug not only in allotransplantation but even in xenotransplantation.
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Requirements of informed-consent to xenotransplantation: a qualitative interview study

Sandra Thiersch, Georg Marckmann
Institut for Ethics, History and Theory of Medicine, Ludwig-Maximilan-Universität, Munich, Germany

Background: The aim is to establish xenotransplantation as a possible alternative to allotransplantation. The clinical application requires that patients give their informed consent before the transfer of the xenograft. The first informed-consent-documents have already been developed. The International Xenotransplantation Association has formulated recommendations for the informed consent process [1]. Nevertheless, up to now it has not yet been examined empirically under which conditions people are ready to consent to a xenotransplantation and how these can be implemented appropriately in the informed consent process.
Aims of the qualitatve study: The aims of the study include:

1. to find out which information patients need to be able to make a decision for or against a xenotransplantation;
2. to investigate how the partners and other family members of possible xenotransplantation patients handle their role and the responsibilites linked to it;
3. to ask physicians about their attitude to xenotransplantations and their opinion about which information patients need to be able to make a decision for or against a xenotransplantation;
4. to contribute to improving informed- consent-documents for xenotransplantation patients.

Concept and methodology of the study: People should receive the possibility to express their personal considerations and appraisals of the possibility of a xenotransplantation. Hence, we have chose a qualitative study design. Members of the following groups will be interviewed:

1. patients who are waiting for a suitable organ
2. their family members
3. patients who have already received an organ
4. healthy subjects
5. healthy care providers (especially transplant surgeons)

On the one hand, the composition of the sample allows us to gather different perspectives on the requirements of participating in a xenotranplantation trial. On the other hand, we include groups which so far have played a minor role in the socio-empirical research in the field of xenotransplantation (physicians and members). Prior to the interview, the study participants receive an informed-consent document for a xenotransplantation. The interview guide covers the following questions:

1. How do people get along with the informed-consent-document? What do they think about the possibilities and restrictions linked to the xenotransplantation?
    a. Which questions are relevant for potential xenotransplantation patients to make an informed decision?
    b. What do family members think about the possibility of a xenotransplantation? How do they handle the restrictions? (e.g. life-long monitoring)
    c. What attitudes have physicians and nursing staff to xenotransplantations?

Discussion: The study is still in the conceptual phase. To improve the study design and the interview guide, a discussion with experts from the field of xenotransplantation is important.
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