

CARDIAC XENOTRANSPLANTATION – FROM BENCH TO BEDSIDE

Eckhard Wolf¹, Michael Schmoeckel², Bruno Reichart³

¹ Gene Centre and Centre for Innovative Medical Models (CiMM), Ludwig-Maximilians-Universität München, Munich, Germany; ² Department of Cardiac Surgery, Klinikum Großhadern, Ludwig-Maximilians-Universität München, Munich, Germany; ³ Walter Brendel Centre for Experimental Medicine, Ludwig-Maximilians-Universität München, Munich, Germany

Summary

Heart transplantation is widely regarded as the most effective and durable treatment option for patients with advanced/terminal cardiac failure. However, the need for organs by far surmounts the donations and the corresponding long waiting lists carry a considerable mortality. Currently, mechanical assist devices are the major alternative, but they come with a high complication rate and only moderate improvements in patients' quality of life.

An emerging and promising solution is xenotransplantation of genetically multi-modified (GM) pig hearts. Recent studies have shown long-term success in baboons with both heterotopic (non-life-supporting) abdominal and life-supporting orthotopic porcine heart transplants. Notably, in a recent case of compassionate use, a pig heart with ten genetic modifications was implanted into a terminally ill patient, who survived for two months.

This review examines the concept of pig heart xenotransplantation, focusing on challenges such as immune rejection, coagulation dysregulation, and detrimental overgrowth of the transplanted heart; GM strategies and non-nephrotoxic immunosuppression are discussed to minimize or prevent these issues. This review covers also microbiological (virologic) safety considerations and further requirements for initiating clinical studies.

Key words: xenotransplantation, heart, pig, nonhuman primate, terminal heart failure

Abbreviations

α Gal: galactose- α (1,3)-galactose

ADCC: antibody-dependent cellular cytotoxicity

APC: antigen-presenting cell

ATMP: advanced therapy medicinal product

B2M: β 2-microglobulin

B4GALNT2: β -1,4-N-acetyl-galactosaminyl transferase

BCL2: BCL2 apoptosis regulator

CD39: ectonucleoside triphosphate diphosphohydrolase 1

CD46: membrane cofactor protein (MCP)

CD47: leukocyte surface antigen CD47

CD55: complement decay-accelerating factor (DAF)

CD59: membrane inhibitor of reactive lysis (MIRL)

CMAH: cytidine monophosphate-N-acetylneuraminic acid hydroxylase

CPRP: complement pathway regulatory protein

CRISPR/Cas9: clustered regularly interspaced short palindromic repeats/

CRISPR-associated protein 9

Received: June 22, 2023

Accepted: July 20, 2023

Correspondence

Eckhard Wolf

Gene Centre and Centre for Innovative Medical Models (CiMM), Ludwig-Maximilians-Universität München, Feodor-Lynen-Str. 25, D-81377 Munich, Germany

E-mail: ewolf@genzentrum.lmu.de

How to cite this article: Wolf E, Schmoeckel M, Reichart B. Cardiac xenotransplantation – from bench to bedside. EJT 2023;1:192-206. <https://doi.org/10.57603/EJT-305>

© Copyright by Pacini Editore Srl



OPEN ACCESS

This is an open access article distributed in accordance with the CC-BY-NC-ND (Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International) license. The article can be used by giving appropriate credit and mentioning the license, but only for non-commercial purposes and only in the original version. For further information: <https://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>

CTLA4-Ig: cytotoxic T lymphocyte-associated antigen-4-immunoglobulin (abatacept)
DCD: donation after circulatory death
DPF: designated pathogen-free
EMA: European Medicines Agency
EPCR: endothelial protein C receptor (CD201)
ETV2: ETS variant transcription factor 2
FDA: US Food and Drug Administration
GGTA1: α -1,3-galactosyltransferase
GHR: growth hormone receptor
GM: genetically (multi)modified
hiPSC: human induced pluripotent stem cell
HLA: human leukocyte antigen
HMOX1: haeme oxygenase 1
HTx: heart transplantation
ICU: intensive care unit
IGF1R: insulin-like growth factor 1 receptor
IL6R: interleukin 6 receptor
KDR: kinase insert domain receptor
KO: knockout
LEA29Y: affinity optimized CTLA4-Ig (belatacept)
mAb: monoclonal antibody
MMF: mycophenolate mofetil
mTOR: mechanistic target of rapamycin
MYF5/6: myogenic factor 5/6
MYOD: myogenic differentiation
Neu5Gc: N-glycolylneuraminic acid
NHP: nonhuman primate
NK cell: natural killer cell
PCMV: porcine cytomegalovirus
PCR: polymerase chain reaction
PCXD: perioperative xenograft dysfunction
PD-L1: programmed death-ligand 1
PEI: Paul-Ehrlich-Institute
PERV: porcine endogenous retrovirus
PRA: panel reactive antibodies
Sd(a): blood group antigen
SIRP α : signal regulatory protein alpha
SLA: swine leukocyte antigen
TBM: thrombomodulin (CD141)
TF: tissue factor
TFPI: tissue factor pathway inhibitor
tg: transgenic
TKO: triple-knockout
TM: thrombotic microangiopathy
TNFAIP3: TNF alpha induced protein 3 (A20)
TP53: tumour protein P53

INTRODUCTION

Current medical treatments for advanced heart failure are highly effective (reviewed in Bauersachs, 2021¹). However, when all options fail, heart transplantation (HTx) remains the gold standard for patients with end-stage heart disease, offering a high probability level of an extended life time in good general condition. The world-wide shortage of donated human organs results in waiting lists (Tab. I) with annual numbers being twice as high when compared to those of actually done transplants. Long waiting lists carry a considerable mortality; furthermore, additional patients need to be withdrawn due to severe side effects, which make future transplantations impossible. Taking extended risks in donor selection is one alternative solution², donations after circulatory death (DCD) are another³⁻⁵; DCD is, however, not permitted in Germany. Currently, mechanic assist devices are the major alternative, but they come with a high complication rate and only moderate improvements in patients' quality of life. The one- and five-year survival rates are 83 and 52 percent, respectively, and, when compared with autologous transplantations, are significantly worse after the longer observation time. The hospital readmission rates, mainly due to infections and bleeding events, are high after three and twelve postoperative months (36 and 68 percent, respectively); withdrawal of care is the main cause of death⁶. Significant progress has been made in pig-to-primate cardiac xenotransplantation, using GM donor pigs, improved preservation techniques, optimized transplantation models, and effective immunosuppressive regimens⁷⁻¹⁰, demonstrating the potential for clinical application. In January 2022, the first compassionate use xenotransplantation of a 10xGM pig heart into a patient with

terminal heart failure was done at Maryland University, Baltimore¹¹. The patient died after 2 months due to multiple pre-operative risk factors and portentous complications such as intra-operative acute aortic dissection involving the renal and mesenteric artery branches and their end-organs; a porcine cytomegalovirus (PCMV) infection caused endothelial damage of the transplant and may also have contributed to the patient's demise after two post-operative months¹¹. However, the ability to sustain normal heart function for more than 45 days is widely regarded as a crucial demonstration that clinical cardiac xenotransplantation is feasible.

In this overview, we provide a summary of the background and outline additional steps that we believe are necessary to achieve consistent long-term success and finally translate xenogeneic heart transplantation into clinical reality.

GENETIC MODIFICATION OF SOURCE PIGS TO ALLEVIATE THE PATHOBIOLOGY OF PIG HEART XENOTRANSPLANTATION

Xenogeneic rejection reactions

The intricate nature of organ xenotransplantation's pathobiology surpasses that of allotransplantation, as the innate immune responses assume a more significant role. The factors contributing to xenograft destruction have been comprehensively reviewed by Cooper et al.¹² in this issue. In brief: during infancy, both humans and nonhuman primates (NHPs) develop antibodies that cross-react with carbohydrate antigens found on the surfaces of genetically unmodified pig cells. Hence, when a wild-type pig organ is transplanted into a human or baboon, these

Table I. Active waiting lists for a heart transplant in Europe.

Region	Year	Size of waiting list	Deaths on waiting list
Eurotransplant	2022	1044	116
France	2017	874	43
Spain	2021	495	12
Italy	2022	706	71*
Poland	2017	691	79
United Kingdom	2022	320	22
Austria	2021	112	3
Portugal	2021	93	7
Sweden	2021	90	1
Finland	2021	82	1
Czech Republic	2021	56	20
Denmark	2021	46	1
Ireland	2021	9	1

Source: <https://www.statista.com/statistics/>; *number in 2021.

antibodies swiftly bind to the graft's vascular endothelial cells. In a next quick step, the complement cascade is activated, leukocytes are attracted and infiltrate the porcine heart through (antibody) Fc-receptor-mediated and Fc-independent mechanisms. Consequently, the graft is typically rejected within minutes to hours. This antibody dependent "hyperacute rejection" is characterized by histopathological features such as venous thrombosis, loss of vascular integrity, interstitial haemorrhage, oedema, and infiltration of innate immune cells.

Hyperacute (and later acute vascular) rejections of pig organs in humans or NHPs primarily occur due to (pre-formed) antibodies targeting galactose α -(1,3)-galactose (α Gal). Humans possess additional natural antibodies against N-glycolylneuraminic acid (Neu5Gc) and a glycan resembling the human Sd(a) blood group antigen (often referred to as β 4Gal). In contrast, NHPs only exhibit anti- α Gal and anti-Sd(a) antibodies (reviewed in Byrne et al., 2018; Sykes and Sachs, 2019) ^{13,14}.

To eliminate the α Gal, Neu5Gc, and Sd(a) epitopes as target antigens for xenograft rejection in humans, pigs with inactivated α -1,3-galactosyltransferase (*GGTA1*) ¹⁵, cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH*) ^{16,17}, and β -1,4-N-acetyl-galactosaminyl transferase 2 (*B4GALNT2*)/*B4GALNT2*-like (*B4GALNT2L*) ¹⁸ genes – so-called triple-knockout (TKO) pigs – were generated. However, complement activation can also occur through pathways unrelated to antibody binding, such as ischaemia-reperfusion injury. Therefore, transgenic pigs expressing human complement pathway regulatory (inhibitory) proteins (CPRPs), namely CD46 ¹⁹, CD55 ²⁰, and CD59 ²¹ have been generated; organs derived from animals with transgenic expression of one or more human CPRPs exhibit a significant level of safeguarding against human/NHP complement-mediated injury. In combination with the TKO animals, cell injury is remarkably diminished in these "humanized" porcine organs (reviewed in Galli, 2023 ²²).

Reducing additional detrimental targets diminishes antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells: since swine leukocyte antigen (SLA)-I has limited ability to bind inhibitory NK cell receptors, human/NHP NK cells directly exhibit cytotoxicity against porcine cells. To counter this, transgenic pigs expressing human leukocyte antigen (HLA)-E/ β 2-microglobulin (B2M) have been generated as a strategy ²³.

Furthermore, porcine cells activate macrophages since porcine CD47 fails to bind the "don't eat me" signal regulatory protein alpha (SIRP α) on human macrophages. Consequently, transgenic pigs expressing human CD47 have been developed ²⁴.

The activation of human/NHP T cells against porcine xenotransplants occurs either directly through porcine antigen-presenting cells (APCs) presenting porcine

peptides or indirectly via human/NHP counterparts. These processes involve several co-stimulatory and co-inhibitory signals; direct T cell activation can be reduced by eliminating or downregulating SLA molecules (as reviewed in Ladowski et al., 2021 ²⁵) or by blocking the CD40-CD40L (CD154) costimulatory signal with antibodies (see below). Additionally, transgenic pigs expressing CTLA4-Ig, or its high-affinity derivative LEA29Y ^{26,27}, have been developed to block the CD28-CD80/CD86 co-stimulatory pathway. In another approach, membrane-bound human PD-L1 is expressed on pig cells to activate the inhibitory PD1 receptor on infiltrating human/NHP leukocytes ²⁸. To deplete and inhibit T and NK cells, transgenic pigs have been generated which express a monoclonal anti-human CD2 antibody construct ²⁹.

For LEA29Y, PD-L1 and CD2 transgenic pigs, effective local expression is being investigated as a means to attenuate immune reactions and thus to reduce or even replace the need for systemic immunosuppression.

Xenogeneic coagulation disorders

Coagulation pathway dysregulation is another aspect of the pathobiology associated with pig organ xenotransplantation (as reviewed in Cowan, Robson, 2015; Pierson et al., 2020 ^{30,31}). Contributing factors include the immune responses mentioned earlier, which propagate inflammation, vascular damage, and therefore a procoagulant state of the porcine endothelium; molecular incompatibilities between porcine and human/NHP coagulation regulators are a major cause. Although the measures employed to prevent hyperacute xenograft rejection helped to avert systemic life-threatening consumptive coagulopathy, pig hearts, transplanted long-term into baboons abdominally, displayed signs of thrombotic microangiopathy (TM) – even under clinically approved anticoagulation therapy. In contradistinction, transgenic expression of human thrombomodulin (TBM) in donor pigs (e.g., ³²⁻³⁴) successfully prevented TM by overcoming the inability of porcine TBM – in complex with human/NHP thrombin – to activate human/NHP protein C. Activation of this anticoagulation pathway is necessary to prevent the formation of detrimental fibrin clots in the capillary system of the donor organ and the above mentioned TM.

Although the porcine endothelial protein C receptor (EPCR; supporting the activation of protein C) seems to be compatible with the human/NHP protein C pathway ³⁵, transgenic pigs expressing human EPCR have been created to achieve higher EPCR levels and thereby enhance protective thromboregulation.

Other genetic modifications also aim at addressing coagulation dysregulation: Expression of human tissue factor pathway inhibitor (TFPI) to prevent activation of TF-factor VIIa complexes; expression of human ectonucleoside triphosphate diphosphohydrolase 1 (CD39) to

inhibit platelet aggregation and consecutive thrombus formation (reviewed in Cowan, Robson, 2015; Pierson et al., 2020^{30,31}); and siRNA-mediated suppression of porcine TF expression³⁶.

Additionally, transgenic pigs expressing anti-inflammatory proteins such as human TNF-alpha-induced protein 3 (TNFAIP3 alias A20³⁷) or human haeme oxygenase 1 (HMOX1³⁸) have been generated, aiming to prevent or reduce inflammation that may not be adequately controlled by other genetic modifications.

Lethal overgrowth of the donor heart

Detrimental intrinsic (genetically determined) overgrowth of the porcine organ (the heart of an outgrown German Landrace pig weighs one kg) has consistently been observed in preclinical studies of cardiac xenotransplantation⁷. In the preclinical setting, overgrowth is retarded with rapamycin, a blocker of the activation of mTOR (mechanistic target of rapamycin), which is part of the signalling cascade of many growth stimulating hormones. To address this issue clinically, one approach involves the generation of donor pigs with loss-of-function mutations in the growth hormone receptor (*GHR*) gene^{39,40}. *GHR* inactivation resulted in approximately 50% reduced body and organ weights. A comprehensive proteome analysis of *GHR*-deficient pig hearts revealed no major other molecular abnormalities⁴⁰; *GHR* deficiency, along with other genetic modifications, has contributed to the prolonged survival of orthotopic porcine cardiac xenografts beyond 6 months^{9,41}; in the Maryland case, *GHR* deficiency was one of the 10 GM of the donor organ. However, *GHR* deficiency in donor pigs may have side effects, such as marked obesity³⁹, transient juvenile hypoglycaemia⁴², altered liver metabolism⁴³, and structural/proteomic alterations of the anterior pituitary gland⁴⁴. Thus, it is preferably to use a genetic background of source pigs that fits the size of humans.

TOWARDS PARTIALLY HUMANIZED PIG HEARTS: EXOGENESIS

To enhance compatibility between pig hearts and humans, an additional technique called exogenesis has been proposed⁴⁵. This approach aims to achieve complete humanization of heart structures, specifically the endothelium (Fig. 1). Exogenesis involves the creation of interspecies chimeras using genetically engineered pig embryos (reviewed in Garry DJ, Garry MG, 2019⁴⁶). These embryos lack the ability to generate or sustain certain cell lineages, creating a niche for the introduction of donor human cells or even parts of organs. However, the formation of whole organ chimeras between pigs and humans faces fundamental barriers due to their evolutionary distance. Several mechanisms contribute to this xenogeneic barrier (reviewed in Zheng et al., 2021⁴⁷). Apoptosis and cell competition can eliminate donor cells, ligand-receptor incompatibilities between species pose challenges, differences in developmental timing present obstacles, and mismatches in cell adhesion molecules hinder the formation of adequate junctions between human and pig cells. To overcome these barriers, researchers have explored various genetic manipulations in pig embryos. For instance, overexpressing the antiapoptotic factor BCL2 in human induced pluripotent stem cells (hiPSCs) and engrafting them into pig blastocysts lacking ETV2, a master regulator of haematoendothelial lineages, has facilitated the generation of pig embryos with human endothelium⁴⁵. Knocking out the kinase insert domain receptor (*KDR*) gene is another approach to generate porcine embryos with impaired vasculogenesis⁴⁸. The development of GM pig hearts with humanized endothelium is of particular significance, as the endothelium is the primary target for rejection mechanisms following xenogeneic heart transplantation.

Furthermore, porcine embryos with a human myogenic lineage have been generated by complementing defective

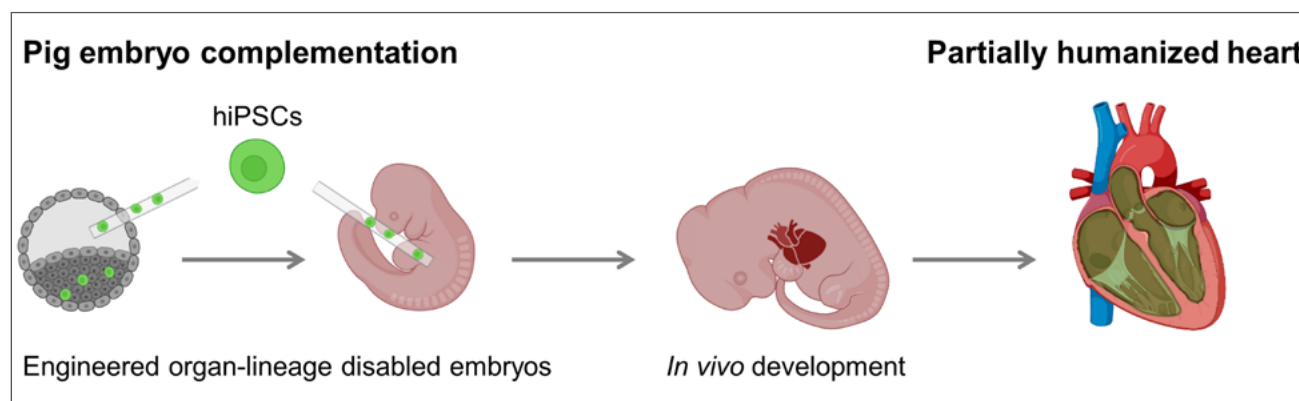


Figure 1. Principle of exogenesis to generate partially humanized pig hearts.

pig blastocysts lacking myogenic factor 5 (MYF5), myogenic differentiation (MYOD), and myogenic factor 6 (MYF6) with *TP53*-null hiPSCs⁴⁹. Inactivation of *TP53* in the donor cells allows adaptation to the low *TP53* expression in porcine host embryos, resulting in increased chimerism. Strategies to enhance the inter-species chimera competency of human stem cells, such as naïve vs. primed vs intermediate hiPSCs, have been extensively reviewed⁴⁷. Additional efforts to improve interspecies chimerism focus on modifying the host embryo, such as inactivating the insulin-like growth factor 1 receptor (*IGF1R*) gene⁵⁰. Systematic analysis of early porcine embryo and heart development is expected to uncover new strategies for enhancing the efficiency of porcine blastocyst complementation with human stem cells. This may involve humanizing essential ligands, receptors, or adhesion molecules in the porcine host embryos⁵¹.

HETEROTOPIC AND ORTHOTOPIC HEART TRANSPLANTATION IN THE PIG-TO-NHP MODEL

Previous comprehensive reviews have examined the early results after pig heart transplantation in NHPs (1968-2013)⁵². The baboon (*Papio anubis* or *hamadryas*) has been the most commonly used recipient species for preclinical porcine cardiac xenotransplantation, and three transplantation models have been established for these animals (as reviewed in Mohiuddin et al., 2015⁵³).

In the abdominal heterotopic cardiac xenotransplantation technique (i), the porcine ascending aorta (with both coronary arteries) is connected to the recipient abdominal aorta, the porcine pulmonary artery (with the coronary venous efflux) to the recipient inferior vena cava (Fig. 2A). Upon release of the aortic clamp, the transplanted heart is perfused and starts pumping. As there is no systemic venous (caval) return, the transplant beats empty; the recipient survives on its own organ, which is left untouched in the chest. This easy to accomplish transplantation model is primarily used to assess the effectiveness of immunosuppressive regimens and new combinations of genetic modifications. With appropriate immunosuppressive therapy, pig hearts lacking α Gal, expressing hCD46 and hTBM have survived for up to 945 days (median 298 days³²).

In the intrathoracic heterotopic cardiac xenotransplantation technique (ii), the xenograft is placed within the right thoracic cavity and to the right of the recipient heart, resulting in compression of parts of the upper and middle lobes of the right lung⁵⁴⁻⁵⁶. Four anastomoses are necessary: The connections between the respective left and right atria to achieve physiologically appropriate bi-atrial “inflow”, as well as end-to-side “outflow” connections

between both ascending aortae and pulmonary artery trunks; the latter requires an extension with an interposition Dacron or Gore-Tex graft (Fig. 2B). In this “piggyback” position, the xeno-heart can fully or partially support the recipient’s organ, and carries under clinical conditions on average 73% of the total cardiac output⁵⁷. The intrathoracic heterotopic cardiac technique has been considered as a potential scenario for an early clinical translation, as the recipient’s native heart can provide the live-saving minimal cardiac output in case a xenograft fails⁵⁸. Re-transplantation may then be considered, utilizing either a human or again a porcine organ.

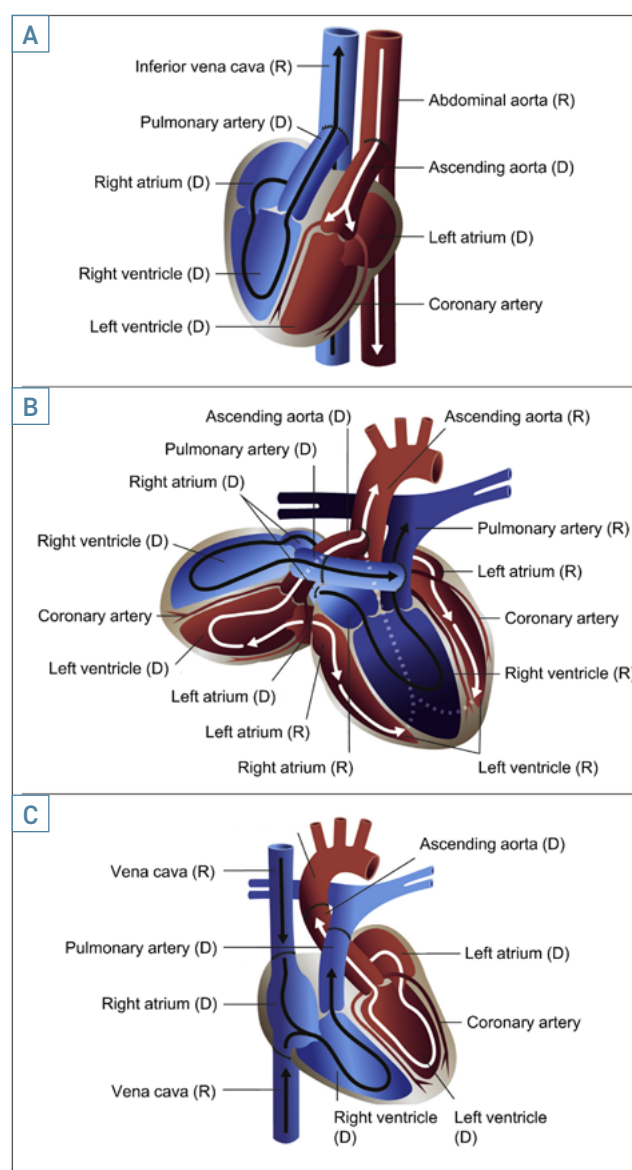


Figure 2. Models of pig-to-baboon cardiac xenotransplantation: A) heterotopic abdominal; B) heterotopic thoracic, and C) orthotopic techniques; D: donor; R: recipient (modified with permission from Mohiuddin et al., 2015)⁵³.

Table II. Orthotopic xenogeneic heart transplantation experiments in baboons (from Reichart et al., mod.) ⁶⁴.

Genetically modified donor pigs											Organ preservation	ISR	Growth control ^a	Survival time [days] ^b	Ref.
Knockouts				Transgenes											
GGTA1	CMAH	B4GALNT2(L)	GHR	hCD46	hCD55	hTBM	hEPCR	hCD47	hHMOX1						
					X						Static	A	-	< 1 (2); 5 (2); 9	65
					X						Static	A	-	< 1 (5); 4; 5 (3); 9	66
					X						Static	B	-	39	67
					X						Static	C	-	9; 25	68
				X							Static	C	-	0; 2 (2); 34; 40; 57	68
X					X						Static	C	-	0; 1; 2; 14	68
X				X		X					Static	D	-	1 (3); 3; 30	7
X				X		X					Perfusion	D	-	4 ^c ; 18; 27; 40	7
X				X		X					Perfusion	D	+	90 ^d (2); 182; 195	7
X				X		X					Perfusion	D	+	15 ^e ; 27 ^e ; 90 ^d (2)	8
X				X		X					Blood cardioplegia	E	-	< 1; 4; 29	9
X				X		X					Perfusion	E		57	9
X				X	X						Perfusion	E	-	6	9
X	X	X		X	X						Perfusion	E	-	< 1	9
X	X	X									Perfusion	E	-	8	9
X		X		X	X	X	X	X	X		Perfusion	E	-	84; 95	9
X		X	X	X		X	X	X			Perfusion	E	-	182 ^f ; 264	9
X					X						Static (UW)	F	+	< 1	10,69
X					X						Static (del Nido)	F	+	90	10,69
X				X		X					Static (del Nido)	F	+	< 1; 241	10,69

A: Treatment with mTOR inhibitor temsirolimus or rapamycin; B: Numbers of animals with the same survival time are given in brackets; C: Technical failure; D: Terminated according to experimental protocol; E: Donor infected with porcine cytomegalovirus (PCMV); F: Elective euthanasia. Immunosuppressive regimens (ISR): A: cyclosporine A (CsA), corticosteroids (Cs), cyclophosphamide (CyP); B: CsA, Cs, CyP, mycophenolate mofetil (MMF); C: anti-thymocyte globulin (ATG), rituximab, tacrolimus, sirolimus, TPC (αGal polymer) or GAS914 some recipients; D: induction: anti-CD20 monoclonal antibody (mAb), anti-CD40 mAb or PASylated anti-CD154 Fab, ATG; maintenance: MMF, anti-CD40 mAb or PASylated anti-CD154 Fab, methylprednisolone (MP; tapered down); anti-inflammatory therapy: interleukin-1 receptor (IL1R) blocker, IL6R blocker, tumour necrosis factor alpha (TNFA) inhibitor; E: induction: anti-CD20 mAb, anti-CD40 mAb, ATG, cobra venom factor (CVF) or C1 esterase inhibitor; maintenance: MMF, anti-CD40 mAb; anti-inflammatory therapy: IL6R blocker, TNFA inhibitor. F: induction: ATG, anti-CD20 mAb, C1-esterase inhibitor; maintenance: anti-CD40 mAb, rapamycin, methylprednisolone; adjunctive medication: etanercept (TNFA antagonist), ganciclovir, famotidine, aspirin, bactrim, erythropoietin, triiodothyronine.

Long-term results after allo-procedures were good, although post-operative anticoagulation is mandatory to avoid thrombus formation within the recipient left ventricle with consecutive systemic emboli ⁵⁹. Our group in Munich carried out pig-to-baboon intrathoracic heterotopic heart experiments between 2009 and 2013. Short-term results (recipient survival, initial xenograft function) were excellent, but long-term results were limited due to the toxic immunosuppressive therapy at that time (e.g. co-stimulation blockade was not available) and no control of the donor organ overgrowth ⁶⁰. The most rigorous preclinical model is the orthotopic cardiac xenotransplantation technique (iii), where the baboon’s own organ is replaced with a GM pig heart using a surgical procedure identical to that of cardiac allotransplantation (Fig. 2C ⁶¹). This model extensively proves the life-supporting function of a xenograft heart, and achieving consistent success in good general

condition is considered a prerequisite before entering clinical studies ⁶². Non-ischaemic preservation of the heart using *ex vivo* perfusion ^(63; XVIVO, Gothenburg, Sweden), non-nephrotoxic immunosuppression ³², and post-implantation growth control of the xenograft heart ⁷ are necessary. Table II summarises the results of orthotopic pig-to-baboon heart transplantation experiments.

LIMITATIONS OF THE PIG-TO-NHP CARDIAC XENOTRANSPLANTATION MODELS, THE ALTERNATIVE DECEDENT EXPERIENCE

While inactivation of *GGTA1* along with expression of hCD46 and hTBM has proven sufficient to achieve long-term survival in the preclinical orthotopic NHP model, the combination of inactivation of *GGTA1*, *CMAH*, and

B4GALNT2/B4GALNT2L (TKO) plus transgenic expression of one or several complement pathway regulatory proteins and human TBM is our preferred minimal set of genetic modifications for clinical cardiac xenotransplantation studies. Testing this combination in baboons is complicated by a significant difference in the innate immune response between humans and NHPs. In contrast to humans, all Old-World monkeys, including baboons, express Neu5Gc, as do pigs. When Neu5Gc is deleted in TKO pigs, it appears that another xenoantigen (sometimes known as the ‘4th xenoantigen’, presumable a glycan) is exposed. The structure and identity of the ‘4th xenoantigen’ remains unknown, but most NHPs express natural antibodies reacting with *CMAH*-KO or TKO cells ¹⁸. Binding of these antibodies to TKO pig grafts is associated with a high level of complement-dependent cytotoxicity ⁷⁰⁻⁷² and reduced graft survival in heart ⁹ and kidney ⁷³ xenotransplantation models. This – in the end clinically irrelevant

– phenomenon has proven to be a major obstacle (for e.g. regulatory authorities) in predicting how a TKO pig organ would work in a human recipient ^{73,74}. Additional inactivation of the *CMAH* gene reduces the antigenicity in a future clinical setting and should therefore be included (but should be avoided in pre-clinical trials ^{18,72}; reviewed in Cooper et al., 2019 ⁷⁵). In that respect, is the recent so-called “decedent model” of interest, meaning the transplantation of clinical-grade GM porcine organs into brain dead persons with still functioning organs. At New York University, Montgomery et al. ⁷⁶ attached *GGTA1*-KO kidneys to groin vessels in two such cases. During the short observation time of 54 h, there were no signs of (hyperacute) rejection and the transplanted organs were passing urine normally. No serum creatinine increases were observed, the recipient’s own kidneys remained however untouched and were working. In the University of Alabama case, J.E. Locke and her

group ⁷⁷ transplanted two 10xGM kidneys (*GGTA1*-KO, *CMAH*-KO, *B4GALNT2*-KO, *GHR*-KO; *hCD46*-tg, *hCD55*-tg; *hTBM*-tg, *hEPCR*-tg, *hCD47*-tg, *hHOMX1*-tg; United Therapeutics (UT)/Revivacor, Blacksburg, Virginia, USA) into a nephrectomised decedent. During the 74-h follow-up time, again, there were no signs of rejection; however, the creatinine clearance did not recover. At autopsy, there were histologic features of thrombotic microangiopathy and tubular necrosis.

In June, July 2022 and again at New York University, two orthotopic heart transplantations were done and allowed to beat for 72 hours; again, 10xGM pigs (UT/Revivacor) served as donors and no signs of rejection were recorded ^{78,79}. Although both xenografts beat normally after starting reperfusion with the recipients' blood, cardiac function decreased in the further follow-up in one case, attributed to a size mismatch between the donor pig (organ too small) and the recipient.

Taken together, such short-term experiments are probably of limited scientific value, but the unstable condition of a brain-dead recipient does not permit easily longer observation times ⁸⁰. For more reliable information, xenotransplantations must be done in living patients.

PREREQUISITES FOR CONSISTENT SUCCESS IN (PRE-) CLINICAL CARDIAC XENOTRANSPLANTATIONS

Stable genetic modifications of the source pigs

These aspects have already been discussed in chapter "Genetic modification of source pigs to alleviate the pathobiology of pig heart xenotransplantation".

Non-ischaemic perfusion preservation of the donor heart

For over 20 years, the preclinical results after orthotopic xenogeneic heart transplantations were unpredictable with a 40-60% peri-operative mortality, inconsistent, despite the use of clinically approved cold static preservation techniques (reviewed in Shu et al., 2022 ⁸¹). This phenomenon was termed "Peri-operative Cardiac Xenograft Dysfunction" (PCXD) and was thought to be due to ischaemia/reperfusion injury ^{53,82}; when compared to humans, porcine hearts are obviously less resistant against ischaemia. Since December 2015, PCXD has been consistently prevented by a continuous (non-ischaemic) perfusion of the grafts with an 8°C hyperoncotic, oxygenated cardioplegic solution containing erythrocytes, nutrition and hormones ^{63,83}; the perfusion was intermittently continued even during implantation. This perfusion preservation technique was also employed in the clinical Maryland case ¹¹.

Development of a non-nephrotoxic immunosuppressive regimen with CD40-CD154 co-stimulation blockade

Initial pig-to-baboon cardiac xenotransplantation studies used conventional immunosuppressive regimens including cyclophosphamide, cyclosporine A or tacrolimus, mycophenolate mofetil, and corticosteroids without success. Since 2000, co-stimulation blockade – first with anti-CD154 mAb – was applied in abdominal heterotopic heart transplantation experiments (⁸⁴; reviewed in Bühler et al., 2000 ⁸⁵). After anti-CD154 mAb was found to be thrombogenic in humans, the chimeric anti-CD40 2C10 mAb-based regimen was introduced and has since then contributed to the longest reported cardiac xenograft survivals after heterotopic ³² and orthotopic transplantations in baboons ^{7,9}.

The humanized anti-CD40 antibody KPL-404 (Kiniksa Pharmaceuticals, Lexington, Massachusetts, USA) was given in the recent Maryland case ¹¹.

The following immunosuppressive drugs complemented the treatment: for induction, cortisone, ATG and anti-CD20 antibody; for maintenance, cortisone tapered down, MMF and/or rapamycin (also necessary for graft overgrowth control).

Post-implantation growth control of the xeno-heart

Domestic pig breeds used for xenotransplantation experiments, such as German Landrace or Large White, weigh outgrown 200-300 kg; heart sizes increase proportionally and reach then approximately one kg. This size mismatch is of great importance for both preclinical experiments as well as clinical applications.

For many years, it was believed that after xenogeneic transplantations, the grafts would adapt to the growth regulation of the recipient under the influence of extrinsic (recipient dependent) factors such as hormones and growth factors (reviewed in Lui and Baron, 2011; Penzo-Méndez and Stanger, 2015 ^{86,87}). However, Längin et al. ⁷ could demonstrate more recently that the donor organ growth is regulated intrinsically, which means genetically, or in other words: the xeno-heart in the primate body increases its size as if it still would be within the porcine chest (this "overgrowth" was also observed in xenogeneic kidney transplantation experiments ^{88,89}). After heterotopic thoracic xeno-heart transplantation ⁶⁰, this phenomenon caused a reduction in pulmonary function, and in the orthotopic model diastolic pump failure and subsequent congestive liver damage ⁷.

In the preclinical setting, cardiac overgrowth was successfully prevented by decreasing the blood pressure (baboons have a higher blood pressure than pigs), early weaning from cortisone, and treatment with sirolimus (or the i.v. donation of the prodrug temsirolimus), which inhibits activation of the mechanistic target of rapamycin (mTOR) and thereby cardiomyocyte hypertrophy.

An alternative would be use of *GHR*-KO pigs as described before; *GHR*-KO was included in the 10xGM pig (United Therapeutics/Revivicor, Blacksburg, VA) used as donor for the already mentioned Maryland case.

In future clinical applications, however, smaller donor animal breeds will be preferred, such as the Auckland Island pigs from New Zealand (NZeno, Auckland), which weigh outgrown between 70 and 90 kg, i.e. perfect for adult humans. In the meantime, a small porcine endogenous retrovirus-C (PERV-C) free herd has been established in a farm near Munich. GM Yucatan minipigs were used by Ma et al., 2022 ⁹⁰.

SAFETY OF XENOTRANSPLANTATION, AVOIDING POTENTIAL INFECTIOUS COMPLICATIONS

The microbiologic and virologic safety profile of porcine xenotransplants is high, since GM donor pigs must be maintained in designated pathogen-free (DPF) barrier facilities ensuring the absence of zoonotic pathogens (reviewed in Fishman, 2018;2020 ^{91,92}). In addition, highly sensitive and specific assays have been established for specific pathogens which must be absent from the donor pigs ⁹³. Some of them like the porcine cytomegalovirus (PCMV) had a significant negative effect on cardiac xenograft survival times in preclinical transplantation experiments ⁹⁴ and may have contributed to the Maryland heart xenograft recipient's demise ¹¹. It is thus mandatory to use only PCMV negative donor pigs confirmed by negative PCR assays and, more importantly, by absent serological antibodies ^{95,96}. Since there exist no drugs or vaccines, "early weaning" is the only solution at the present time, as it prevents the nasal transmission from the mother to the piglets while sucking milk.

Of special importance are the porcine endogenous retroviruses (PERVs) since they are integrated in the porcine genome and will remain there: PERV-A and PERV-B are present in all pigs, whereas PERV-C is in the genome of most, but not all animals. PERV-A and PERV-B are polytropic and can infect human cells only in vitro, whereas PERV-C is ecotropic and infects only pig cells ⁹⁷. When compared with PERV-A, recombinants between PERV-A and -C carry higher replication rates ^{98,99} and must be avoided.

PERV-A, -B and -A/C have been shown to infect human tumour and immortalized cell cultures, but rarely primary cells. To date, PERV transmission has not been detected in numerous preclinical xenotransplantation nor in infection experiments in different species ¹⁰⁰ – nor in clinical trials with encapsulated porcine islets in diabetic patients ^{101,102}. Virologists are now of the opinion, that PERVs don't pose

the tremendous risk, as more than 25 years ago researchers thought it would be ¹⁰³. Since, however, a PERV infection cannot be ruled out for sure, several strategies have been proposed to prevent an (unlikely) PERV transmission:

- selection of pigs with low expression of PERVs and therefore a low probability to release infectious particles;
- selection of PERV-C-negative animals to prevent PERV-A/C recombination (our strategy, which is accepted by both FDA and EMA);
- inhibition of PERV expression by RNA interference ⁹⁷;
- inactivation of PERVs using CRISPR/Cas9 technology ¹⁰⁴; this strategy may however be associated with off-target effects ^{105,106}; and
- use of antiviral drugs.

WHAT EXPERIMENTAL RESULTS WOULD JUSTIFY A FORMAL CLINICAL TRIAL?

In 2000, the ad hoc Xenotransplantation Advisory Committee of the International Society for Heart and Lung Transplantation recommended that consistent survival of NHPs supported by pig orthotopic heart transplants for 3 months would be sufficient to warrant moving to a clinical trial ⁶². That recommendation was made at a time when even 3-month survivals had been unobtainable.

The state of the science has changed dramatically since those pioneering days, and consequently the experimental evidence needs to be stronger, and has indeed already been achieved ⁷⁻⁹. We therefore suggest that consistent survival of up to 6 months, in the absence of features of irreversible rejection or infection, would be sufficient to warrant moving towards a clinical trial in carefully selected patients. Achieving survival for longer durations, with one or two recipients being followed for nine or even 12 months would be reassuring.

However, we would emphasize that any clinical trials should be carried out by teams with experience of both clinical orthotopic heart transplantation and the preclinical setting of a pig-to-NHP model.

REGULATORY ASPECTS

National regulatory bodies (FDA, EMA; in Germany the Paul-Ehrlich-Institute, PEI) have the authority to determine what experimental benchmarks in preclinical studies are appropriate, what kind of (microbiologic/virologic) safeguards are necessary, before approving clinical trials of xenotransplantation. The necessary regulatory framework in the United States has been summarized in a recent letter ¹⁰⁷. Within Europe, guidelines on Advanced

Table III. Potential indications for the initial clinical trials of pig heart transplantation*.

1.	Relative or absolute contraindications to mechanical circulatory support, <i>e.g.</i> (a) restrictive or hypertrophic cardiomyopathy (b) presence of a dysfunctional mechanical valve prosthesis or degenerated bio-prosthesis (c) atrial or ventricular septal defect
2.	High titres of broadly panel-reactive anti-HLA antibodies (high PRA) that do not cross-react with swine leukocyte antigens (SLA) of the donor animal
3.	Chronic rejection after cardiac allotransplantation
4.	Infants and children with complex congenital heart disease, <i>e.g.</i> , after atrial correction of a transposition of the great arteries, single ventricle circulation after right ventricular Fontan (Norwood III) procedures

*Based on Chaban et al., 2023¹⁰⁸.

Therapy Medicinal Products (ATMP; EC/1394/2007) must be adhered. In addition, national laws, such as the AMG (Arzneimittelgesetz, law for medicinal products, which includes bioengineering) in Germany, will be additionally implemented.

It should be remembered that, to date, no NHP has survived longer than 9 months after orthotopic pig heart transplantation^{7,9}. Consequently, regulatory authorities like the FDA or EMA may decide that (compassionate use) pig heart transplantations should initially be offered as a bridge-to-allotransplantation; in other words: after several months, a human heart-transplantation would be done if clinically indicated.

"Compassionate use" means in this context that a specific patient has no realistic chance to receive an allograft during lifetime. After an in-depth informed consent, the decision for a cardiac xenotransplant is made. Since under these circumstances the whole responsibility remains within the treating team, no permission from regulatory authorities is necessary.

This is in contradistinction to small pivotal or pilot studies, which need such a permission and which is only granted after a thorough (and lengthy) investigation (in that case primarily, the high level exclusion of the transmission of pathogenic microorganisms).

The FDA recognizes the need for xenotransplantation and accepts that:

- i) pigs with multiple genetic modifications would be required;
- ii) cloned pigs could be used for the initial studies; and
- iii) complete inactivation of porcine endogenous retroviruses is not required.

There is the necessity for biobanking in order to be prepared for the emergence of unknown micro-organisms.

FIRST PATIENT SELECTION

The patients for the first clinical cardiac xenotransplantation trials must be carefully selected to justify these

interventions and ensure very likely favourable outcomes. Initial candidates could be intensive care (ICU) patients who are poor candidates for mechanical circulatory support, such as those with hypertrophic cardiomyopathy, prior mechanical valve replacements, deteriorated aortic bio-protheses, post-infarction ventricular septal defects. These high-risk patients become increasingly unstable due to inotrope requirements and arrhythmias; secondary liver and kidney damages must be considered reversible and pulmonary hypertension medically treatable (reviewed in Reichart et al., 2021⁵⁸; Table III).

Paediatric patients with complex congenital heart diseases are of special interest, particularly those with single right ventricular pathophysiology. Although palliative surgical techniques (Norwood, Fontan) provide adequate results in some patients, survival and quality of life are limited, particularly in patients with high-risk anatomic lesions or complex arrhythmias. In contradistinction, these high-risk patients do well after allotransplantation¹⁰⁹, but have a high mortality while waiting for a (usually small) heart. In single-ventricle patients, mechanical circulatory devices are associated with little success¹¹⁰. In addition, total implantable systems are impossible in those below a bodyweight of 40 kg. In these patients, paracorporeal pump chambers are implanted¹¹¹.

HOW DO WE PREDICT THE FUTURE OF CARDIAC XENOTRANSPLANTATION DURING THE NEXT 5-10 YEARS?

For humans with advanced/terminal myocardial disease, allografts will always be preferable. Due to the long waiting lists, and as an alternative, we suggest that bridging with a pig heart xenograft will be introduced into the clinic within the next two years, possibly initially again on an individual compassionate basis, but preferably as part of a formal clinical trial. We expect that trials in both infant and adult patients will be approved. With successful longer-term experience, we predict that cardiac xenotransplantation as destination therapy will soon be an accepted treatment form.

We firmly anticipate that advances will be made in the field of xenotransplantation during the next decade that will surpass those of mechanical assist devices, stem cell technology, and regenerative medicine.

Conflict of interest statement

BR and EW are cofounders of XTransplant GmbH, Starnberg, Germany.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG; CRC/TR 127 to BR and EW) and by the Swiss National Science Foundation (SNSF; Sinergia grant CRSII5_198577/1 to EW).

Author contributions

EW, BR: drafted the initial manuscript; EW, MS, BR: reviewed and edited the draft. All authors approved the final manuscript.

Ethical consideration

Not applicable.

References

- Bauersachs J. Heart failure drug treatment: the fantastic four. *Eur Heart J* 2021;42:681-683. <https://doi.org/10.1093/eurheartj/ehaa1012>
- Ruck JM, Zhou AL, Zeiser LB, et al. Trends and three-year outcomes of hepatitis C virus-viremic donor heart transplant for hepatitis C virus-seronegative recipients. *JTCVS Open* 2022;12:269-279. <https://doi.org/10.1016/j.xjon.2022.10.007>
- Ayer A, Schroder JN, Casalino S, et al. The future of heart procurement with donation after circulatory death: current practice and opportunities for advancement. *J Heart Lung Transplant* 2022;41:1385-1390. <https://doi.org/10.1016/j.healun.2022.06.016>
- Messer S, Rushton S, Simmonds L, et al. A national pilot of donation after circulatory death (DCD) heart transplantation within the United Kingdom. *J Heart Lung Transplant* 2023. <https://doi.org/10.1016/j.healun.2023.03.006> [Epub Ahead of Print]
- Joshi Y, Scheuer S, Chew H, et al. Heart transplantation from DCD donors in Australia: lessons learned from the first 74 cases. *Transplantation* 2023;107:361-371. <https://doi.org/10.1097/tp.0000000000004294>
- Yuzefpolskaya M, Schroeder SE, Houston BA, et al. The Society of Thoracic Surgeons Intermacs 2022 Annual Report: Focus on the 2018 Heart Transplant Allocation System. *Ann Thorac Surg* 2023;115:311-327. <https://doi.org/10.1016/j.athoracsur.2022.11.023>
- Längin M, Mayr T, Reichart B, et al. Consistent success in life-supporting porcine cardiac xenotransplantation. *Nature* 2018;564:430-433. <https://doi.org/10.1038/s41586-018-0765-z>
- Reichart B, Längin M, Radan J, et al. Pig-to-non-human primate heart transplantation: the final step toward clinical xenotransplantation? *J Heart Lung Transplant* 2020;39:751-757. <https://doi.org/10.1016/j.healun.2020.05.004>
- Mohiuddin MM, Goerlich CE, Singh AK, et al. Progressive genetic modifications of porcine cardiac xenografts extend survival to 9 months. *Xenotransplantation* 2022;E12744. <https://doi.org/10.1111/xen.12744>
- Litovsky SH, Foote JB, Jagdale A, et al. Cardiac and pulmonary histopathology in baboons following genetically-engineered pig orthotopic heart transplantation. *Ann Transplant* 2022;27:E935338. <https://doi.org/10.12659/aot.935338>
- Griffith BP, Goerlich CE, Singh AK, et al. Genetically modified porcine-to-human cardiac xenotransplantation. *N Engl J Med* 2022;387:35-44. <https://doi.org/10.1056/NEJMoa2201422>
- Cooper DKC, Wang L, Kinoshita K, et al. Immunobiological barriers to pig organ xenotransplantation. *EJT* 2023;1:168-185. <https://doi.org/10.57603/EJT-266>
- Byrne G, Ahmad-Villiers S, Du Z, et al. B4GALNT2 and xenotransplantation: a newly appreciated xenogeneic antigen. *Xenotransplantation* 2018;25:E12394. <https://doi.org/10.1111/xen.12394>
- Sykes M, Sachs DH. Transplanting organs from pigs to humans. *Sci Immunol* 2019;4:eaau6298. <https://doi.org/10.1126/sciimmunol.aau6298>
- Phelps CJ, Koike C, Vaught TD, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science* 2003;299:411-414. <https://doi.org/10.1126/science.1078942>
- Kwon DN, Lee K, Kang MJ, et al. Production of biallelic CMP-Neu5Ac hydroxylase knock-out pigs. *Sci Rep* 2013;3:1981. <https://doi.org/10.1038/srep01981>
- Lutz AJ, Li P, Estrada JL, et al. Double knockout pigs deficient in N-glycolylneuraminic acid and galactose alpha-1,3-galactose reduce the humoral barrier to xenotransplantation. *Xenotransplantation* 2013;20:27-35. <https://doi.org/10.1111/xen.12019>
- Estrada JL, Martens G, Li P, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/beta4GalNT2 genes. *Xenotransplantation* 2015;22:194-202. <https://doi.org/10.1111/xen.12161>
- Diamond LE, Quinn CM, Martin MJ, et al. A human CD46 transgenic pig model system for the study of discordant xenotransplantation. *Transplantation* 2001;71:132-142.
- Cozzi E, White DJ. The generation of transgenic pigs as potential organ donors for humans. *Nat Med* 1995;1:964-966. <https://doi.org/10.1038/nm0995-964>
- Fodor WL, Williams BL, Matis LA, et al. Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection. *Proc Natl Acad Sci U S A* 1994;91:11153-11157.
- Galli C. Animal engineering for xenotransplantation: genome editing and cloning in pigs for xenotransplantation. *EJT* 2023;1:186-195. <https://doi.org/10.57603/EJT-307>
- Weiss EH, Lilienfeld BG, Müller S, et al. HLA-E/human beta2-microglobulin transgenic pigs: protection against xenogeneic human anti-pig natural killer cell cytotoxicity. *Transplantation* 2009;87:35-43. <https://doi.org/10.1097/TP.0b013e318191c784>
- Tena A, Kurtz J, Leonard DA, et al. Transgenic expression of human CD47 markedly increases engraftment in a murine model of pig-to-human hematopoietic cell transplantation. *Am J Transplant* 2014;14:2713-22. <https://doi.org/10.1111/ajt.12918>
- Ladowski JM, Hara H, Cooper DKC. The role of SLAs in xenotransplantation. *Transplantation* 2021;105:300-307. <https://doi.org/10.1097/tp.0000000000003303>
- Klymiuk N, van Buerck L, Bahr A, et al. Xenografted islet cell clusters from INSLEA29Y transgenic pigs rescue diabetes and prevent immune rejection in humanized mice. *Diabetes* 2012;61:1527-1532. <https://doi.org/10.2337/db11-1325>
- Bähr A, Kaser T, Kemter E, et al. Ubiquitous LEA29Y expression

- blocks T Cell co-stimulation but permits sexual reproduction in genetically modified pigs. *PLoS One* 2016;11:E0155676. <https://doi.org/10.1371/journal.pone.0155676>
- 28 Buermann A, Petkov S, Petersen B, et al. Pigs expressing the human inhibitory ligand PD-L1 (CD 274) provide a new source of xenogeneic cells and tissues with low immunogenic properties. *Xenotransplantation* 2018;25:E12387. <https://doi.org/10.1111/xen.12387>
 - 29 Nottle MB, Salvaris EJ, Fiscaro N, et al. Targeted insertion of an anti-CD2 monoclonal antibody transgene into the GGTA1 locus in pigs using FokI-dCas9. *Sci Rep* 2017;7:8383. <https://doi.org/10.1038/s41598-017-09030-6>
 - 30 Cowan PJ, Robson SC. Progress towards overcoming coagulopathy and hemostatic dysfunction associated with xenotransplantation. *Int J Surg* 2015;23:296-300. <https://doi.org/10.1016/j.ijsu.2015.07.682>
 - 31 Pierson RN, 3rd, Fishman JA, Lewis GD, et al. Progress toward cardiac xenotransplantation. *Circulation* 2020;142:1389-1398. <https://doi.org/10.1161/circulationaha.120.048186>
 - 32 Mohiuddin MM, Singh AK, Corcoran PC, et al. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. *Nat Commun* 2016;7:11138. <https://doi.org/10.1038/ncomms11138>
 - 33 Iwase H, Ekser B, Satyananda V, et al. Pig-to-baboon heterotopic heart transplantation – exploratory preliminary experience with pigs transgenic for human thrombomodulin and comparison of three costimulation blockade-based regimens. *Xenotransplantation* 2015;22:211-220. <https://doi.org/10.1111/xen.12167>
 - 34 Wuensch A, Baehr A, Bongoni AK, et al. Regulatory sequences of the porcine THBD gene facilitate endothelial-specific expression of bioactive human thrombomodulin in single- and multi-transgenic pigs. *Transplantation* 2014;97:138-147. <https://doi.org/10.1097/TP.0b013e3182a95cbc>
 - 35 Salvaris EJ, Moran CJ, Roussel JC, et al. Pig endothelial protein C receptor is functionally compatible with the human protein C pathway. *Xenotransplantation* 2020;27:E12557. <https://doi.org/10.1111/xen.12557>
 - 36 Ahrens HE, Petersen B, Herrmann D, et al. siRNA mediated knockdown of tissue factor expression in pigs for xenotransplantation. *Am J Transplant* 2015;15:1407-1414. <https://doi.org/10.1111/ajt.13120>
 - 37 Oropeza M, Petersen B, Carnwath JW, et al. Transgenic expression of the human A20 gene in cloned pigs provides protection against apoptotic and inflammatory stimuli. *Xenotransplantation* 2009;16:522-534. <https://doi.org/10.1111/j.1399-3089.2009.00556.x>
 - 38 Petersen B, Ramackers W, Lucas-Hahn A, et al. Transgenic expression of human heme oxygenase-1 in pigs confers resistance against xenograft rejection during ex vivo perfusion of porcine kidneys. *Xenotransplantation* 2011;18:355-368. <https://doi.org/10.1111/j.1399-3089.2011.00674.x>
 - 39 Hinrichs A, Kessler B, Kurome M, et al. Growth hormone receptor-deficient pigs resemble the pathophysiology of human Laron syndrome and reveal altered activation of signaling cascades in the liver. *Mol Metab* 2018;11:113-128. <https://doi.org/10.1016/j.molmet.2018.03.006>
 - 40 Hinrichs A, Riedel EO, Klymiuk N, et al. Growth hormone receptor knockout to reduce the size of donor pigs for pre-clinical xenotransplantation studies. *Xenotransplantation* 2021;28:E12664. <https://doi.org/10.1111/xen.12664>
 - 41 Goerlich CE, Griffith B, Hanna P, et al. The growth of xenotransplanted hearts can be reduced with growth hormone receptor knockout pig donors. *J Thorac Cardiovasc Surg* 2023;165:E69-E81. <https://doi.org/10.1016/j.jtcvs.2021.07.051>
 - 42 Hinrichs A, Renner S, Bidlingmaier M, et al. MECHANISMS IN ENDOCRINOLOGY: transient juvenile hypoglycemia in growth hormone receptor deficiency – mechanistic insights from Laron syndrome and tailored animal models. *Eur J Endocrinol* 2021;185:R35-R47. <https://doi.org/10.1530/eje-21-0013>
 - 43 Riedel EO, Hinrichs A, Kemter E, et al. Functional changes of the liver in the absence of growth hormone (GH) action – proteomic and metabolomic insights from a GH receptor deficient pig model. *Mol Metab* 2020;36:100978. <https://doi.org/10.1016/j.molmet.2020.100978>
 - 44 Shashikadze B, Franzmeier S, Hofmann I, et al. Structural and proteomic repercussions of growth hormone receptor deficiency on the pituitary gland: lessons from a translational pig model. *J Neuroendocrinol* 2023;E13277. <https://doi.org/10.1111/jne.13277>
 - 45 Das S, Koyano-Nakagawa N, Gafni O, et al. Generation of human endothelium in pig embryos deficient in ETV2. *Nat Biotechnol* 2020;38:297-302. <https://doi.org/10.1038/s41587-019-0373-y>
 - 46 Garry DJ, Garry MG. Interspecies chimeras and the generation of humanized organs. *Circ Res* 2019;124:23-25. <https://doi.org/10.1161/circresaha.118.314189>
 - 47 Zheng C, Ballard EB, Wu J. The road to generating transplantable organs: from blastocyst complementation to interspecies chimeras. *Development* 2021;148:dev195792. <https://doi.org/10.1242/dev.195792>
 - 48 Matsunari H, Watanabe M, Hasegawa K, et al. Compensation of disabled organogenesis in genetically modified pig fetuses by blastocyst complementation. *Stem Cell Reports* 2020;14:21-33. <https://doi.org/10.1016/j.stemcr.2019.11.008>
 - 49 Maeng G, Das S, Greising SM, et al. Humanized skeletal muscle in MYF5/MYOD/MYF6-null pig embryos. *Nat Biomed Eng* 2021;5:805-814. <https://doi.org/10.1038/s41551-021-00693-1>
 - 50 Nishimura T, Suchy FP, Bhadury J, et al. Generation of functional organs using a cell-competitive niche in intra- and inter-species rodent chimeras. *Cell Stem Cell* 2021;28:141-149.E3. <https://doi.org/10.1016/j.stem.2020.11.019>
 - 51 Wolf E, Reichart B, Moretti A, et al. Designer pigs for xenogeneic heart transplantation and beyond. *Dis Model Mech* 2023;16. <https://doi.org/10.1242/dmm.050177>
 - 52 Cooper DK, Satyananda V, Ekser B, et al. Progress in pig-to-non-human primate transplantation models (1998-2013): a comprehensive review of the literature. *Xenotransplantation* 2014;21:397-419. <https://doi.org/10.1111/xen.12127>
 - 53 Mohiuddin MM, Reichart B, Byrne GW, et al. Current status of pig heart xenotransplantation. *Int J Surg* 2015;23:234-239. <https://doi.org/10.1016/j.ijsu.2015.08.038>
 - 54 Barnard CN, Losman JG, Curcio CA, et al. The advantage of heterotopic cardiac transplantation over orthotopic cardiac transplantation in the management of severe acute rejection. *J Thorac Cardiovasc Surg* 1977;74:918-924.
 - 55 Novitzky D, Cooper DK, Barnard CN. The surgical technique of heterotopic heart transplantation. *Ann Thorac Surg* 1983;36:476-482. [https://doi.org/10.1016/s0003-4975\(10\)60492-4](https://doi.org/10.1016/s0003-4975(10)60492-4)
 - 56 Cooper DK, Novitzky D, Becerra E, et al. Are there indications for heterotopic heart transplantation in 1986? A 2- to 11-year follow-up of 49 consecutive patients undergoing heterotopic heart transplantation. *Thorac Cardiovasc Surg* 1986;34:300-304. <https://doi.org/10.1055/s-2007-1022159>
 - 57 Hildebrandt A, Reichenspurner H, Gordon GD, et al. Heterotopic heart transplantation: mid-term hemodynamic and

- echocardiographic analysis – the concern of arteriovenous-valve incompetence. *J Heart Transplant* 1990;9:675-681; discussion 682
- 58 Reichart B, Längin M, Denner J, et al. Pathways to clinical cardiac xenotransplantation. *Transplantation* 2021;105:1930-1943. <https://doi.org/10.1097/tp.0000000000003588>
 - 59 Reichenspurner H, Odell JA, Cooper DK, et al. Twenty years of heart transplantation at Groote Schuur Hospital. *J Heart Transplant* 1987;6:317-323.
 - 60 Abicht JM, Mayr T, Reichart B, et al. Pre-clinical heterotopic intrathoracic heart xenotransplantation: a possibly useful clinical technique. *Xenotransplantation* 2015;22:427-442. <https://doi.org/10.1111/xen.12213>
 - 61 Lower RR, Shumway NE. Studies on orthotopic homotransplantation of the canine heart. *Surg Forum* 1960;11:18-19.
 - 62 Cooper DK, Keogh AM, Brink J, et al. Report of the xenotransplantation Advisory Committee of the International Society for Heart and Lung Transplantation: the present status of xenotransplantation and its potential role in the treatment of end-stage cardiac and pulmonary diseases. *J Heart Lung Transplant* 2000;19:1125-1165. [https://doi.org/10.1016/s1053-2498\(00\)00224-2](https://doi.org/10.1016/s1053-2498(00)00224-2)
 - 63 Steen S, Paskevicius A, Liao Q, et al. Safe orthotopic transplantation of hearts harvested 24 hours after brain death and preserved for 24 hours. *Scand Cardiovasc J* 2016;50:193-200. <https://doi.org/10.3109/14017431.2016.1154598>
 - 64 Reichart B, Cooper DKC, Längin M, et al. Cardiac xenotransplantation: from concept to clinic. *Cardiovasc Res* 2023;118:3499-3516. <https://doi.org/10.1093/cvr/cvac180>
 - 65 Waterworth PD, Dunning J, Tolan M, et al. Life-supporting pig-to-baboon heart xenotransplantation. *J Heart Lung Transplant* 1998;17:1201-1207.
 - 66 Schmoekel M, Bhatti FN, Zaidi A, et al. Orthotopic heart transplantation in a transgenic pig-to-primate model. *Transplantation* 1998;65:1570-1577. <https://doi.org/10.1097/00007890-199806270-00006>
 - 67 Vial CM, Ostlie DJ, Bhatti FN, et al. Life supporting function for over one month of a transgenic porcine heart in a baboon. *J Heart Lung Transplant* 2000;19:224-229. [https://doi.org/10.1016/s1053-2498\(99\)00099-6](https://doi.org/10.1016/s1053-2498(99)00099-6)
 - 68 Byrne GW, Du Z, Sun Z, et al. Changes in cardiac gene expression after pig-to-primate orthotopic xenotransplantation. *Xenotransplantation* 2011;18:14-27. <https://doi.org/10.1111/j.1399-3089.2010.00620.x>
 - 69 Cleveland DC, Jagdale A, Carlo WF, et al. The genetically engineered heart as a bridge to allotransplantation in infants just around the corner? *Ann Thorac Surg* 2022;114:536-544. <https://doi.org/10.1016/j.athoracsur.2021.05.025>
 - 70 Yamamoto T, Iwase H, Patel D, et al. Old World Monkeys are less than ideal transplantation models for testing pig organs lacking three carbohydrate antigens (Triple-Knockout). *Sci Rep* 2020;10:9771. <https://doi.org/10.1038/s41598-020-66311-3>
 - 71 Yamamoto T, Hara H, Iwase H, et al. The final obstacle to successful pre-clinical xenotransplantation? *Xenotransplantation* 2020;27:E12596. <https://doi.org/10.1111/xen.12596>
 - 72 Cui Y, Yamamoto T, Raza SS, et al. Evidence for GTKO/4GalNT2KO pigs as the preferred organ-source for old world nonhuman primates as a preclinical model of xenotransplantation. *Transplant Direct* 2020;6:E590. <https://doi.org/10.1097/txd.0000000000001038>
 - 73 Iwase H, Jagdale A, Yamamoto T, et al. Evidence suggesting that deletion of expression of N-glycolylneuraminic acid (Neu5Gc) in the organ-source pig is associated with increased antibody-mediated rejection of kidney transplants in baboons. *Xenotransplantation* 2021;28:E12700. <https://doi.org/10.1111/xen.12700>
 - 74 Foote JB, Jagdale A, Yamamoto T, et al. Histopathology of pig kidney grafts with/without expression of the carbohydrate Neu5Gc in immunosuppressed baboons. *Xenotransplantation* 2021;28:E12715. <https://doi.org/10.1111/xen.12715>
 - 75 Cooper DKC, Hara H, Iwase H, et al. Justification of specific genetic modifications in pigs for clinical organ xenotransplantation. *Xenotransplantation* 2019;26:E12516. <https://doi.org/10.1111/xen.12516>
 - 76 Montgomery RA, Stern JM, Lonze BE, et al. Results of two cases of pig-to-human kidney xenotransplantation. *N Engl J Med* 2022;386:1889-1898. <https://doi.org/10.1056/NEJMoa2120238>
 - 77 Porrett PM, Orandi BJ, Kumar V, et al. First clinical-grade porcine kidney xenotransplant using a human decedent model. *Am J Transplant* 2022;22:1037-1053. <https://doi.org/10.1111/ajt.16930>
 - 78 Patel NG, Langmaid V. Genetically modified pig heart transplanted into deceased recipient, researchers say. Vol. 2023, CNN, 2022.
 - 79 Moazami N, Stern JM, Khalil K, et al. Pig-to-human heart xenotransplantation in two recently deceased human recipients. *Nat Med* 2023;Jul 24. <https://doi.org/10.1038/s41591-023-02471-9> [Epub Ahead of Print]
 - 80 Novitzky D. Electrocardiographic, hemodynamic and endocrine changes occurring during experimental brain death in the Chacma baboon. *Heart Transpl* 1984;4:63-69.
 - 81 Shu S, Ren J, Song J. Cardiac xenotransplantation: a promising way to treat advanced heart failure. *Heart Fail Rev* 2022;27:71-91. <https://doi.org/10.1007/s10741-020-09989-x>
 - 82 Byrne GW, McGregor CG. Cardiac xenotransplantation: progress and challenges. *Curr Opin Organ Transplant* 2012;17:148-154. <https://doi.org/10.1097/MOT.0b013e3283509120>
 - 83 Längin M, Reichart B, Steen S, et al. Cold non-ischemic heart preservation with continuous perfusion prevents early graft failure in orthotopic pig-to-baboon xenotransplantation. *Xenotransplantation* 2021;28:E12636.
 - 84 Bühler L, Basker M, Alwayn IP, et al. Coagulation and thrombotic disorders associated with pig organ and hematopoietic cell transplantation in nonhuman primates. *Transplantation* 2000;70:1323-1331. <https://doi.org/10.1097/00007890-200011150-00010>
 - 85 Samy KP, Butler JR, Li P, et al. The role of costimulation blockade in solid organ and islet xenotransplantation. *J Immunol Res* 2017;2017:8415205. <https://doi.org/10.1155/2017/8415205>
 - 86 Lui JC, Baron J. Mechanisms limiting body growth in mammals. *Endocr Rev* 2011;32:422-440. <https://doi.org/10.1210/er.2011-0001>
 - 87 Penzo-Méndez AI, Stanger BZ. Organ-size regulation in mammals. *Cold Spring Harb Perspect Biol* 2015;7:A019240 <https://doi.org/10.1101/cshperspect.a019240>
 - 88 Soin B, Ostlie D, Cozzi E, et al. Growth of porcine kidneys in their native and xenograft environment. *Xenotransplantation* 2000;7:96-100. <https://doi.org/10.1034/j.1399-3089.2000.00046.x>
 - 89 Tanabe T, Watanabe H, Shah JA, et al. Role of intrinsic (graft) versus extrinsic (host) factors in the growth of transplanted organs following allogeneic and xenogeneic transplantation. *Am J Transplant* 2017;17:1778-1790. <https://doi.org/10.1111/ajt.14210>
 - 90 Ma D, Hirose T, Lassiter G, et al. Kidney transplantation from triple-knockout pigs expressing multiple human proteins in cynomolgus macaques. *Am J Transplant* 2022;22:46-57. <https://doi.org/10.1111/ajt.16780>
 - 91 Fishman JA. Infectious disease risks in xenotransplantation. *Am J*

- Transplant 2018;18:1857-1864. <https://doi.org/10.1111/ajt.14725>
- ⁹² Fishman JA. Prevention of infection in xenotransplantation: designated pathogen-free swine in the safety equation. *Xenotransplantation* 2020;27:E12595. <https://doi.org/10.1111/xen.12595>
 - ⁹³ Denner J. Sensitive detection systems for infectious agents in xenotransplantation. *Xenotransplantation* 2020;E12594. <https://doi.org/10.1111/xen.12594>
 - ⁹⁴ Denner J, Längin M, Reichart B, et al. Impact of porcine cytomegalovirus on long-term orthotopic cardiac xenotransplant survival. *Sci Rep* 2020;10:17531. <https://doi.org/10.1038/s41598-020-73150-9>
 - ⁹⁵ Halecker S, Hansen S, Krabben L, et al. How, where and when to screen for porcine cytomegalovirus (PCMV) in donor pigs for xenotransplantation. *Sci Rep* 2022;12:21545. <https://doi.org/10.1038/s41598-022-25624-1>
 - ⁹⁶ Fischer N, Gulich B, Keßler B, et al. PCR and peptide based PCMV detection in pig – development and application of a combined testing procedure differentiating newly from latent infected pigs. *Xenotransplantation* 2023;E12803. <https://doi.org/10.1111/xen.12803>
 - ⁹⁷ Denner J, Tönjes RR. Infection barriers to successful xenotransplantation focusing on porcine endogenous retroviruses. *Clin Microbiol Rev* 2012;25:318-343. <https://doi.org/10.1128/cmr.05011-11>
 - ⁹⁸ Harrison I, Takeuchi Y, Bartosch B, et al. Determinants of high titer in recombinant porcine endogenous retroviruses. *J Virol* 2004;78:13871-13879. <https://doi.org/10.1128/jvi.78.24.13871-13879.2004>
 - ⁹⁹ Denner J, Specke V, Thiesen U, et al. Genetic alterations of the long terminal repeat of an ecotropic porcine endogenous retrovirus during passage in human cells. *Virology* 2003;314:125-133. [https://doi.org/10.1016/s0042-6822\(03\)00428-8](https://doi.org/10.1016/s0042-6822(03)00428-8)
 - ¹⁰⁰ Denner J. Why was PERV not transmitted during preclinical and clinical xenotransplantation trials and after inoculation of animals? *Retrovirology* 2018;15:28. <https://doi.org/10.1186/s12977-018-0411-8>
 - ¹⁰¹ Wynyrd S, Nathu D, Garkavenko O, et al. Microbiological safety of the first clinical pig islet xenotransplantation trial in New Zealand. *Xenotransplantation* 2014;21:309-323. <https://doi.org/10.1111/xen.12102>
 - ¹⁰² Morozov VA, Wynyrd S, Matsumoto S, et al. No PERV transmission during a clinical trial of pig islet cell transplantation. *Virus Res* 2017;227:34-40. <https://doi.org/10.1016/j.virusres.2016.08.012>
 - ¹⁰³ Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. *Nat Med* 1997;3:282-286. <https://doi.org/10.1038/nm0397-282>
 - ¹⁰⁴ Niu D, Wei HJ, Lin L, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science* 2017;357:1303-1307. <https://doi.org/10.1126/science.aan4187>
 - ¹⁰⁵ Fu Y, Foden JA, Khayter C, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 2013;31:822-826. <https://doi.org/10.1038/nbt.2623>
 - ¹⁰⁶ Thomas M, Burgio G, Adams DJ, et al. Collateral damage and CRISPR genome editing. *PLoS Genet* 2019;15:E1007994 <https://doi.org/10.1371/journal.pgen.1007994>
 - ¹⁰⁷ Marks P, Solomon S. Clarifying US regulations on xenotransplantation. *Nat Biotechnol* 2021;39:1500-1501. <https://doi.org/10.1038/s41587-021-01144-7>
 - ¹⁰⁸ Chaban R, Cooper DKC, Pierson RN, 3rd. Pig heart and lung xenotransplantation: present status. *J Heart Lung Transplant* 2022;41:1014-1022. <https://doi.org/10.1016/j.healun.2022.04.010>
 - ¹⁰⁹ John M, Bailey LL. Neonatal heart transplantation. *Ann Cardiothorac Surg* 2018;7:118-125. <https://doi.org/10.21037/acs.2018.01.05>
 - ¹¹⁰ Morales DLS, Rossano JW, VanderPluym C, et al. Third Annual Pediatric Interagency Registry for Mechanical Circulatory Support (Pedimacs) Report: preimplant characteristics and outcomes. *Ann Thorac Surg* 2019;107:993-1004. <https://doi.org/10.1016/j.athoracsur.2019.01.038>
 - ¹¹¹ Rohde S, Sandica E, Veen K, et al. Outcomes in small children on Berlin Heart EXCOR support: age and body surface area as clinical predictive factors. *European Journal of Cardio-Thoracic Surgery* 2022;63:Ezac516. <https://doi.org/10.1093/ejcts/ezac516>