Xenotransplantation: how close are we?

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A group of experts reflects on what was learned from the first human transplants of genetically engineered pig organs and what the future of xenotransplantation may hold.

Xenotransplantation research marked a singular milestone last year with the first human transplants of kidneys and a heart from genetically engineered pigs. The genetic modifications were designed to improve transplantation outcomes by lessening immune rejection, controlling organ size and regulating complement, coagulation and inflammation. These pioneering surgeries were motivated by deficiencies of the current donor organ system, which have led to long waiting lists for organs (Fig. 1) and the deaths of thousands of patients in need of organs each year. Pig kidneys were transplanted into three brain-dead recipients — one at Legacy of Hope, University of Alabama[1] and two at New York University Langone Hospital[2]. A pig heart was transplanted into a living recipient at University of Maryland School of Medicine[3]. The donor pigs, supplied by Revivicor, had either one gene knockout or a set of ten gene knockouts and transgenes (Table 1). What have we learned from these experiences, and how will they guide future research and surgical practice in the field? Are phase 1 clinical trials on the horizon? Experts in transplantation medicine, immunology and virology discuss the present state and future prospects of xenotransplantation.

Joachim Denner is at the Free University Berlin in Germany; **Jayme Locke** is at the University of Alabama at Birmingham, AL; **Chung-Gyu Park** is at Seoul National University College of Medicine in South Korea; **Richard Pierson** is at Massachusetts General Hospital in Boston, MA; **Jeffrey Platt** is at the University of Michigan in Ann Arbor, MI; **Angelika Schnieke** is at the Technical University of Munich in Germany; and **Linda Scobie** is at Glasgow Caledonian University in the UK.

What was learned from the pig kidney and heart transplants?

RP: The heart xenograft case demonstrated unequivocally that a pig heart can support the
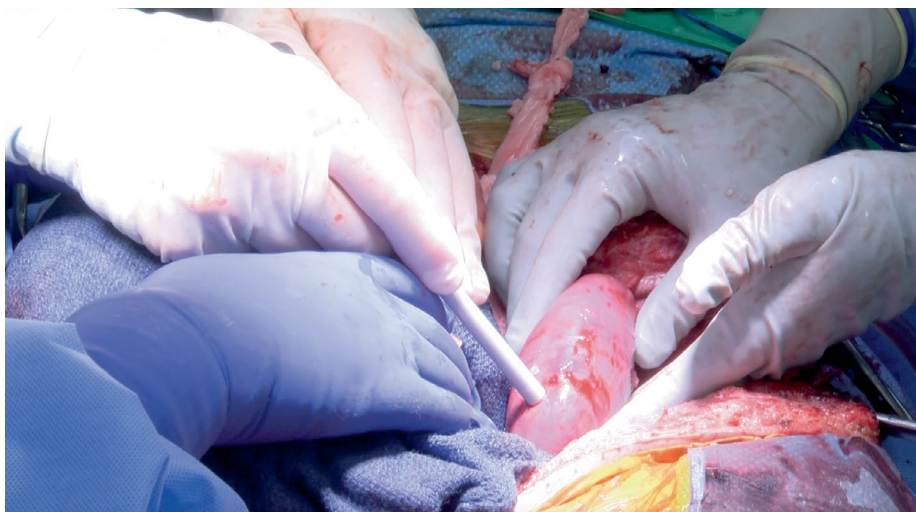
Surgeons at the University of Alabama's Comprehensive Transplant Institute transplant a genetically modified pig kidney into a human recipient. This photo was taken after reperfusion.

Richard Pierson, Massachusetts General Hospital

life of a human being for over a month. That operation was performed in a critically ill patient who was judged unsuitable for a heart allograft. Because of the patient's profound leukopenia, thrombocytopenia and recurrent infectious complications, the immunosuppressive drug dosing was minimized relative to the regimen shown to be effective in baboons.

Porcine cytomegalovirus (pCMV) replication was detected in the pig heart around the time that myocardial hypertrophy and diastolic dysfunction became manifest and likely contributed to cardiac dysfunction. Graft dysfunction in association with pCMV expression recapitulates an observation in baboon xenograft models, where thrombotic microangiopathy, consumptive coagulopathy and graft failure occurred around the time that pCMV replication was detected in pig xenografts. How pCMV escaped detection by preoperative screening

before the human xenotransplantation is uncertain. This safety signal has redoubled efforts to reliably exclude pCMV from pig donors.

Around the same time that pCMV was found, anti-pig antibody was detected in the patient's circulation. Although it may have been transmitted in human intravenous immunoglobulin preparations, the kinetics are more consistent with an elicited immune response, suggesting that the immunosuppressive regimen was insufficient. Whether anti-pig antibody contributed to graft hypertrophy or dysfunction is unknown.

In the kidney xenografts, two or three days of follow-up was too short to see infectious or immunologic safety signals. The two New York University kidney transplants were performed in subjects with retained native kidneys, obscuring assessment of xenograft function. In the bilaterally nephrectomized University of Alabama subject, the xenograft did not clear blood urea nitrogen and creatinine. Follow-up was too short to ascertain in all three cases whether the xenograft was life-supporting with or without dialysis and, in the University of Alabama case, whether the kidney might have recovered from the initial thrombotic microangiopathy.

Q&A

AS: We must first understand why human trials are needed at such an early stage in xenotransplantation. Immune reactions to pig epitopes in humans differ from those in non-human primates (NHPs). This limits the predictive value of NHP studies and makes assessment in humans necessary. The heart transplant taught us that the xeno-organ functioned physiologically and provided evidence that the genetic modifications could suppress hyperacute and acute vascular rejection. Detailed analysis will hopefully reveal the presence or lack of novel xenoreactive epitopes or other incompatibilities and indicate additional targets for genetic modification. We also learned that highly sensitive detection methods for infectious agents, such as latent pCMV, are needed.

Angelika Schnieke, Technical University of Munich

LS: In the heart study, the duration was sufficient to flag some concerns regarding the donor status and the initial pathogen analysis. The kidney xenotransplants were not long enough to fully evaluate pathogen transmission, but they demonstrated a lack of acute rejection and some efficacy. Informed by these results, regulators may require additional information for similar studies in the future. This could include, for the heart, assurances on pathogen testing and an analysis of contributors to mortality and, for the kidney, extended time points to assess safety outcomes, which may be challenging in a decedent.

Linda Scobie, Glasgow Caledonian University

JP: The clinical cardiac xenograft has had an enormous, mostly beneficial impact on the xenotransplantation field. A recipient deemed unsuitable for allotransplantation is obviously not ideally suited to xenotransplantation, and the genetic modifications and immunosuppression that optimize outcomes in NHPs will probably not be optimal in humans. But no rational alternative to these choices could be proposed. Still, the choices made and the outcome will improve the design of future clinical trials. Perhaps the most important lesson to be drawn is that opportunities for innovation and discovery remain wide open. Once clinical trials are undertaken, I think insights we can hardly imagine today will generate new commercial opportunities to engineer pigs with different and perhaps fewer mutations and to develop immune modifiers that target immune pathways unique to xenotransplantation.

Jeffrey Platt, University of Michigan at Ann Arbor

JL: Both the xeno-heart and xeno-kidney transplants demonstrated that genetic engineering successfully prevented hyperacute and early xenograft rejection. The xeno-kidney study[1] that I participated in also validated a flow crossmatch specific for pig-to-human transplantation. Determining tissue compatibility via crossmatch testing is standard-of-care in allotransplantation, and therefore this was a major advance for xenotransplantation.

Jayme Locke, University of Alabama at Birmingham

JD: Survival of the heart transplant recipient for almost two months is a great success considering that the recipient of the first heart allotransplant by Christian Barnard in South Africa lived for only 18 days and that of the first heart allotransplant in Germany for only 27 hours.

Joachim Denner, Free University Berlin

Why were kidneys and hearts prioritized for xenotransplantation?

JP: There were two compelling factors: surgical challenge and expense on the one hand and the epidemiology of organ failure on the other. Basic research on xenotransplantation three decades ago revealed that modeling the incompatibility of innate immunity, complement and coagulation between pigs and humans required the use of certain NHP species as xenograft recipients. This made experimentation vastly more complex and expensive and discouraged inquiry into hurdles unique to liver or lungs. Continuing to focus on hearts and kidneys is still justified because these organs are most likely to suffer failure with aging, and aging will generate the greatest demand for transplantation.

AS: Decellularized porcine and bovine heart valves are already in clinical use. Other tissue types, such as porcine islets to treat type 1 diabetes, have entered clinical trials. Vascularized organs are more challenging than cellular transplants, and the longest survival times of xeno-organs in NHPs have indeed been obtained for heart and kidneys, making them the obvious choice [see Fig. 2]. Also, the greatest clinical need is for kidneys. Complex organs, such as the liver, require additional genetic modifications to improve compatibility with the human host − for example, to avoid thrombocytopenia (elimination of human platelets by porcine Kupffer cells and liver sinusoidal endothelial cells).

RP: The imbalance between the number of patients who could benefit from kidney transplants and the number of available kidneys is measured in the hundreds of thousands. In heart disease, tens of thousands of patients are treated with high-risk surgery or are declined for heart allotransplantation. In both kidney and heart disease, timely access to healthy organs as a 'bridge to recovery' or as definitive therapy would be transformative.

For pig lung and liver xenografts, life-supporting function in animal models has proven more difficult to achieve. These grafts are associated with profound inflammation in the recipient, perhaps caused by cells or cell fragments elaborated by the pig organ after exposure to baboon or human blood.

Pancreas and intestine xenografts have not been studied in large animals. Pig islets are considered a more practical approach than pancreas transplantation to treat diabetes. Supply and demand for intestinal allografts is better matched than for other organs, limiting enthusiasm for intestinal xenografts. Intestinal xenografts are also presumed to be more difficult because of the large population of resident immunocytes and the need to maintain barrier function.

Q&A

JL: Heart was prioritized as its main function is muscular contraction. It is not required to produce physiologically necessary proteins or to eliminate waste, whereas the other solid organs are. Of those, kidney has received the most focus as in the event of failure there is dialysis.

What determines whether a xenotransplant is better suited to a brain-dead or living recipient?

AS: As kidney patients can survive on dialysis, any kidney transplant into a living patient has to provide an advantage over available treatment options. Testing xeno-kidneys in brain-dead recipients is therefore a sensible first step. For patients whose survival depends on obtaining a replacement organ, taking a higher risk might be acceptable. A xeno-transplant could prolong life or bridge the gap until a human organ becomes available; in the worst case, it could help improve xenotransplantation procedures to benefit future patients.

RP: Since experimentation after death is not legally regulated, a clinical center simply needs to identify a brain-dead recipient whose family or legal representative is willing to donate the body for this purpose.

For studies in non-brain-dead patients at the end of life from a disease or after catastrophic but non-lethal brain injury, an institutional review board might approve an experiment or a trial that takes into account such special circumstances. Altruistic participation is ethically defensible even when the outcome is unknown, participation could accelerate the participant's demise and there is no plausible benefit to the subject. However, the usefulness of such short-term studies is limited, although they may be more physiologically and clinically impactful than studies in decedents. If the procedure is intended to be therapeutic, standard regulatory, ethical and legal considerations should obtain.

Is matching organ size and age more complex with xenografts than with allografts?

RP: In preclinical xenograft studies, hearts and kidneys are size-matched to the recipient similarly to what is done in clinical allotransplantation. In NHPs, juvenile pig hearts and kidneys grow in a physiologically appropriate manner, tracking the size that the organ would have achieved during normal growth and maturation in the donor pig. Growth of

the xeno-organ has been controlled by genetic means (growth hormone receptor knockout in commercial pig strains or selection of pig strains that are naturally smaller at maturity) or by pharmacologically suppressing proliferation using mammalian target of rapamycin inhibitors. Beyond matching the current and projected full-grown size of the pig xenograft to the body cavity size of the recipient, age will likely not be directly considered in the design of clinical xenograft trials.

How well do we understand hyperacute, acute and chronic rejection of a porcine graft and their prevention?

AS: Our understanding of the rejection mechanism, incompatibilities between the porcine and human coagulation system, and molecular mechanisms of endothelium activation have improved considerably. Hyperacute rejection has been overcome by eliminating the major xeno-epitope galactose-α-1,3-galactose (gal). Acute vascular rejection has been minimized by inactivating non-gal epitopes and overexpressing one or more human complement regulator genes. Expressing human coagulation factors suppresses the thrombosis risk, and human heme oxygenase 1 (HO-1) counteracts endothelium activation. Attack of the xeno-organ by macrophages or natural killer cells can be halted by expression of human CD47 and HLA-E. Chronic rejection should be controllable with immunosuppressive drugs, supported if necessary by expression of immunosuppressive genes in the xeno-organ.

JP: We have learned much about the immune and physiological pathways underlying hyperacute and acute rejection both in allografts and in porcine xenografts. Much less is known about pathways underlying chronic rejection of allografts, and practically nothing is known about chronic rejection of xenografts. Our work and the work of others showed that organ grafts are not simply passive targets of immunity but can actively mount changes that lead to characteristic features of rejection or limit the impact of rejection or even prevent it. We call this latter process accommodation. Because few experimental xenografts and no clinical xenografts have survived long enough to explore chronic rejection, we do not know whether factors implicated in chronic rejection of allografts will affect xenografts. However, a xenograft damaged by chronic rejection could be replaced, whereas replacing a failing allotransplant with a donated human organ could be more difficult.

Chung-Gyu Park, Seoul National University College of Medicine

C-GP: Although triple-knockout (TKO) pigs were produced to remove major porcine glycans, anti-TKO pig antibody is still detected in some human serum. In addition, although transgenic pigs expressing human complement-regulatory proteins (CD46, CD55 and CD59) and human coagulation regulatory proteins (for example, thrombomodulin, endothelial protein C receptor, tissue factor pathway inhibitor, CD39 and CD73) were developed and substantially reduced complement and coagulation activation, complement was still deposited and coagulation activated in rejected grafts in NHPs. How much these leaky reactions contribute to graft rejection in a clinical setting should be further elucidated. T cell-mediated rejection can be fairly well controlled by a combination of conventional immunosuppressants and costimulation blockade (CD40–CD154 pathway) in solid organs (heart and kidney), as well as pancreatic islets and corneas. In the cardiac xenotransplantation at the University of Maryland, acute cell-mediated and antibody-mediated rejection was not observed.

How different are the immune responses to allo- and xenografts − and the corresponding immunosuppressive regimens?

JP: Differences between the immune responses to xenogeneic and allogeneic stimuli have been studied for more than 50 years. But how these differences influence rejection is still uncertain. Often, but not invariably, immunosuppressive drugs and regimens that prevent rejection of allografts do not prevent rejection of xenografts, and long-term survival of xenografts has required more intrusive, toxic immunosuppression. The greater degree of immunosuppression needed to sustain xenografts could be taken to indicate that immunity to xenografts is more severe. But this conclusion is premature and might be incorrect. Immunosuppressive regimens in allotransplantation have been optimized and validated by observations in more than a million recipients over six decades. In contrast, there has been scarce opportunity to do the same for xenotransplantation in NHP models and no opportunity in humans. Furthermore,
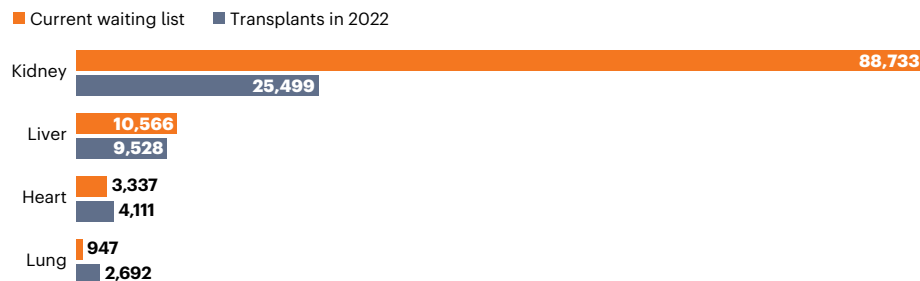
Q&A

■ Current waiting list ■ Transplants in 2022

Kidney
88,733
25,499

Liver
10,566
9,528

Heart
3,337
4,111

Lung
947
2,692

Fig. 1 | Transplantation figures in the United States. Number of people on waiting lists for four organs as of 27 February 2023. Also shown are the numbers of transplants performed in 2022. Source: Organ Procurement & Transplantation Network.

if the severity of rejection results from species differences in the regulation of complement, coagulation or inflammation, then more intrusive immunosuppression might not be the best solution.

The genetic background of donors and recipients is known to affect the frequency, severity and characteristics of rejection. In allotransplantation we cannot control those characteristics; usually, we cannot even match major histocompatibility complex (MHC). But in xenotransplantation we can choose or manipulate the graft's genetic background beyond the mutations already made. This will be an important subject of future research, along with strategies for tuning immunosuppression regimens to limit toxicity. Indeed, some basic studies suggest that immunosuppression for xenotransplantation might eventually be more focused and less intrusive than for allotransplantation.

RP: For both allografts and xenografts, in circumstances where anti-donor antibody is present in the recipient, grafts typically fail rapidly, with macroscopic ischemia and parenchymal hemorrhage and microscopic antibody binding, complement activation, neutrophil and monocyte infiltration, intravascular thrombosis, and loss of barrier function (interstitial hemorrhage or edema). In the absence of preformed antibody, allografts typically elicit a T cell-mediated immune response that manifests as acute cellular rejection, whereas xenografts rarely exhibit prominent lymphocytic infiltration. Xenograft injury and failure beyond the first few days ('delayed xenograft rejection') is typically associated with endothelial activation and loss of vascular barrier function. Although incompletely understood in primate models, it appears to be driven by some combination of low-level antibody binding and complement activation coupled with coagulation pathway

dysregulation, resulting in inappropriate initiation and propagation of intravascular clots.

In addition, early xenograft failure (within hours or days after transplant) involves innate immune activation, with prolific release of histamine, thromboxane and pro-inflammatory cytokines, particularly tumor necrosis factor, interleukin-6 and interleukin-8 − pathways not associated with allotransplantation. Consumptive coagulopathy in the recipient and thrombotic microangiopathy in the xenograft are typically associated with xenograft demise, whether within a few days or several months, but are very unusual in preclinical or clinical allografts.

What was the rationale for the ten genetic modifications in some donor pigs? Is further engineering needed?

JL: There were four knockouts: three carbohydrate antigens to avoid hyperacute rejection and growth hormone receptor to avoid organ overgrowth. There were also six human transgene knock-ins: two for immunomodulation, two for anticoagulation and two for complement inhibition (Table 1).

JP: The ten genetic modifications were previously found to improve the survival and function of porcine xenografts in NHPs without causing obvious toxicity. Each change (save perhaps one) is supported by quantitative tests of its impact on reactions between porcine cells and human serum, plasma or cells. Considering the limitations of these in vitro tests and the physiologic differences between NHPs and humans, clinical experience may reveal some modifications to be unnecessary or detrimental and new modifications to be beneficial. Only studies in patients will provide the information needed to optimize genetic mutations in donor animals − including, for example, changes in expression and

regulation of existing human transgenes; introduction of novel, engineered genes; and selection of porcine background genes.

AS: There is consensus that successful xenotransplants require inactivation of gal and non-gal epitopes and high, ubiquitous expression of one or more complement regulators (CD46, CD55, CD59). Any incompatibilities between human and pig organs, which could vary between organ types, must be addressed. Local expression of co-stimulation inhibitors would be beneficial. The first human trials will instruct on whether further genetic changes are needed. It is unlikely that one type of xeno-pig will be a fit for all organs.

A strong immune response could also be avoided by genetically downregulating or inactivating the porcine MHC or by expressing inhibitors of T cell activation. Unlike human allotransplants, porcine organs can be genetically modified to express, for example, PD-L1, CTL4ig or its derivative LEA29Y, possibly in an inducible fashion when needed. The goal is to provide local rather than systemic immune suppression. Preclinical experiments have shown the efficacy of this approach for porcine islet transplants.

RP: GalTKO heart xenografts that express the human thromboregulatory protein thrombomodulin (hTBM) and one complement regulatory protein, CD46, are protected from thrombodysregulation and graft failure. hTBM appears necessary and sufficient to facilitate long-term life-supporting function of heart xenografts in baboons. Additional expression of other complement regulatory (CD55, CD59) and thromboregulatory (hEPCR, hTFPI, CD39/CD73) proteins, as well as anti-inflammatory proteins (HLA-E, CD47, HO-1, A-20), are mechanistically justifiable modifications, many of which are included in one or more pig constructs currently being advanced for possible clinical application.

Which innovations in immunosuppression are most promising?

JP: One may speculate about novel technologies and pathways, but the key issue is our lack of experience in observing xenografts in human recipients. NHPs cannot serve as surrogate recipients for optimizing immunosuppression regimens. The most important innovations will derive initially from clinical trials that are designed not to determine efficacy (although efficacy will inevitably be scrutinized) but to look for variables,

Table 1 | Genetic modifications of donor pigs used for xenotransplantation

Modification	Gene	Physiological effect
Donor pig used in ref. [2]		
Knockout of porcine gene	α1,3-Galactosyltransferase	Reduce immunogenicity by removing galactose-α1,3-galactose glycan antigen
Donor pigs used in refs. [1,3]		
Knockout of porcine gene	α-1,3-Galactosyltransferase	Reduce immunogenicity by removing galactose-α-1,3-galactose glycan antigen
	β-1,4-*N*-Acetylgalactosyltransferase	Reduce immunogenicity by removing SDa antigen
	CMP-*N*-acetylneuraminic acid hydroxylase	Reduce immunogenicity by removing *N*-glycolylneuraminic acid glycan antigen
	Growth hormone receptor	Reduce organ size
Addition of human transgene	CD46	Reduce complement activation
	Decay accelerating factor	Reduce complement activation
	Endothelial cell protein C receptor	Anti-coagulation
	Thrombomodulin	Anti-coagulation
	Heme oxygenase-1	Anti-inflammation
	CD47	Anti-inflammation

pathways, incompatibilities and toxicities that have been overlooked in the small number of NHP studies.

RP: Anti-donor antibody minimization, T and B cell depletion, CD154 or CD40 costimulation pathway blockade, complement inhibition, anti-inflammatory biologics, and anti-platelet or anticoagulation are all included in the most successful xenograft preclinical studies. Which among them are necessary, and which minimum subset is sufficient, may differ for different applications. Clinical experience is indispensable to understand whether additional innovations are needed.

At present, the volume of preclinical work being done by any one group is not sufficient to rigorously assess the efficacy of any one component of a regimen or even to evaluate dose–response effects, and very few groups are working with more than one organ from individual pigs (we are at Massachusetts General Hospital). Some information has come from comparing results between groups that use different organs from the same source pigs and similar regimens (as we do with hearts and kidneys). In this line of work, small differences in what each pig actually expresses, 'invisible' details of experimental technique, and differences between the recipients (for example, in preformed antibody titers) can influence outcomes and confound interpretation. The innovation that would move the field forward fastest is the one that allows us to accelerate clinical studies.

Would immune-tolerizing approaches be helpful?

JP: Any method that induces immune tolerance to a xenograft would be invaluable. Although approaches to immune tolerance induction have not been widely embraced in allotransplantation, some of these approaches might be more successful in xenotransplantation. For example, while tolerance is eschewed in allotransplantation because of toxicity, the same regimens may lower the aggregate toxicity in xenotransplantation. More importantly, if the immune response to xenotransplantation proves less severe than commonly believed, the toxicity of a focused, xenograft-specific regimen may compare favorably to that of the corresponding allograft regimen. Evolution has made allorecognition and primary allogeneic reactions universal and more rapid and severe than any other immune responses. Humans and indeed all multicellular organisms strongly resist engraftment of allogeneic cells and tissues. By contrast, some xenografts (for example, unicellular and multicellular parasites) are accepted and may even confer benefit, reflecting tolerance and/or accommodation, which enables graft survival in the face of anti-graft immunity. Most clinical allografts may be accommodated to the host, and some cases of rejection may reflect disruption of accommodation. However, if immunity to xenotransplantation and allotransplantation are sufficiently distinct, some tolerizing approaches, including those that enhance

accommodation, could be more successful in xenotransplantation.

One specific concern is that long-term survival and function of xenografts in NHPs apparently requires long-term disruption of CD40–CD40 ligand interaction, presumably to thwart T cell-dependent B cell responses to the xenotransplant. If this requirement reflects responses of newly generated xeno-reactive B cells, long-term B cell tolerance could require repeated treatment or induction. But then we need to find out why de novo generated B cells do not develop tolerance spontaneously to the xenograft, as that could elucidate heretofore unrecognized pathways of tolerance.

RP: Tolerance induction strategies taking advantage of preexposure to donor antigens or induction of cross-species 'mixed hematopoetic chimerism' could eventually enable safer, more durable xenograft results.

Does the immune response to a xenograft depend on the organ type?

RP: The innate immune system is more efficiently activated by liver and lung than by heart or kidney xenografts, with deleterious consequences for the graft and the recipient. Rather than invoking mechanistically distinct pathways, my working hypothesis is that this is a quantitative difference mediated by the large endothelial surface areas of the liver and lung and the presence of monocytes and macrophages poised to clear pathogens. Strategies to overcome this phenomenon include depletion of resident mononuclear cells from the organ before transplant and 'humanizing' pig von Willebrand factor to diminish the endothelial activation and injury that promote inflammation.

JP: In allotransplantation, the recipient's genetic background beyond histocompatibility genes can have an important effect on the outcome, and the impact varies between lung, liver, heart and kidney. For example, variation in certain complement genes that have a modest impact on kidney transplants could well have a substantial impact on lung transplants, which are more susceptible to complement-mediated dysfunction. To my knowledge, the effects of these polymorphisms on lung transplants has not been explored. As another example, my laboratory has studied a highly polymorphic gene that governs B cell selection and maturation and production of natural and
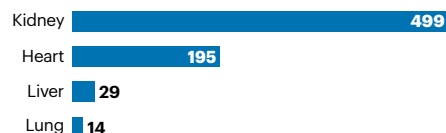
Q&A

Kidney — 499
Heart — 195
Liver — 29
Lung — 14

Fig. 2 | Longest survival times of pig xenografts in NHPs. Data on the longest survival times (in days) achieved in non-human primates of porcine kidney[12], heart[13], liver[14] and lung[15]. Data provided by Chung-Gyu Park.

elicited antibodies. This work revealed certain mutations that are especially common in kidney transplant recipients who develop antibody-mediated rejection and other mutations in recipients who remain free of rejection despite antigraft immunity. These mutations may have influenced the results of NHP experiments testing porcine genetic modifications and could prove important in xenotransplantation.

How important to xenotransplant outcomes are species differences in factors such as electrolytes, erythropoietin, renin and blood pressure?

AS: Pig and human kidneys are similar in structure, size, renal blood flow and general physiology. Still, some species-specific differences exist. Erythropoietin (EPO) in adult mammals is produced by peritubular kidney fibroblasts. Porcine EPO is not fully functional in NHPs, so after xeno-kidney transplantation, NHPs are treated with recombinant EPO to avoid anemia. If necessary, human xenotransplant patients could receive recombinant EPO. Alternatively, donor pigs could be engineered to express human EPO. Human and NHP angiotensinogen, important for fluid balance, cannot be efficiently cleaved by porcine renin. Again, expression of human renin might be a solution, but NHP recipients of a xeno-kidney maintain a normal fluid balance despite reduced renin activity. Values for most electrolytes are similar between pig and human, except for phosphorus, which is higher in pigs, but NHP recipients show only a transient increase in serum phosphate. Incompatibilities between human and porcine coagulations factors have been addressed by engineering xeno-pigs to express human thrombomodulin and/or endothelial cell protein C receptor.

In cardiac xenografts, the blood pressure difference between NHPs and pigs has contributed to xenograft overgrowth, and medication to adjust blood pressure can be administered.

RP: At present, phosphorus wasting and low albumin seem to be the result of subclinical graft injury. When the graft is well protected in NHPs by genes and an effective immunosuppression regimen, these phenomena recede from view.

Blood pressure in heart xenograft recipients may be higher than the blood pressure of donor pigs. Whether correcting for this discrepancy will prevent graft hypertrophy remains to be tested. It is more likely that graft hypertrophy results from incompletely effective immunosuppression. With effective immunosuppression, I suspect that tight blood pressure control in the recipient will prove unnecessary.

JP: This question should remind us that current approaches to treatment, such as dialysis and ventricular support devices, introduce devices or therapeutics that depart profoundly from the physiology of the human kidney and heart. Perfect matching of physiology, even if desirable, is not essential.

Are porcine endogenous retroviruses (PERV) still a concern?

LS: In the early days, PERV was shown to infect a susceptible human embryonic kidney cell line but not primary cells, and there has been no evidence of transmission in many animal models. Indeed, a large number of xenotransplants in many clinical settings — such as extracorporeal porcine splenic, liver or kidney perfusions, skin grafts, islet cells or bioartificial liver perfusion with the HepatAssist device[4–6] — have shown no evidence of PERV transmission in human recipients. PERV is unlikely to be a major risk, although this must be proven formally in a clinical trial and will continue to be monitored in patients.

JD: Retroviruses can induce immunodeficiencies, tumors or both, as has been demonstrated for the closest relatives of PERVs[7] — the feline leukemia virus, the murine leukemia virus and the koala retrovirus. In the first clinical trials of encapsulated pig islet cells in humans in New Zealand and Argentina, PERVs were not transmitted. PERVs were also not transmitted in trials with NHPs and in infection experiments in small animals and NHPs, but as these animals lack PERV receptors, the experiments were not relevant for evaluating the risk to humans. At the moment, there are no approaches to test this other than clinical trials.

AS: Infections such as pCMV, which can compromise graft function and patient survival, are of utmost concern. In contrast, most human and porcine endogenous retroviruses are non-functional, non-infectious remnants in the mammalian germline of once-infectious viruses. Expressed *PERV-C* sequences might rearrange with *PERV-A* elements, but pigs naturally lacking *PERV-C* exist. If a pig has a single copy of *PERV-C*, it can be excised from the genome using genome editing, which also allows inactivation of multiple PERVs as shown previously[8]. Recombination between human and porcine endogenous retroviruses might present a long-term, theoretical risk for immune-suppressed patients, which could be controlled by patient monitoring. From a regulatory point of view, PERVs are not an exclusion criterion in xenotransplantation clinical trials.

RP: This remains a theoretical concern, one that can be taken off the table by engineering PERVs out of pigs (eGenesis) or using pigs with defective PERV-C, which should prevent recombination and the risk of viral escape. Several antiviral drugs are available to suppress PERV propagation if an infection occurred.

Are additional knockouts to address PERVs needed?

RP: The 62-site gene knockout accomplished by eGenesis appears to eliminate the possibility that PERV recombination can occur, a step that is necessary for PERV to become infectious for human cells. However, many experts believe that PERV knockout is probably not necessary to safe conduct of initial clinical trials, and it was not present in the Revivicor pigs that have been used clinically so far. Clinical trials using cell or organ xenografts from pigs without the PERV knockout will tell us whether PERV is likely to pose a significant risk to the xenograft recipient or their close contacts.

JD: Pig cells in which 62 PERV proviruses were inactivated still produced virus particles able to infect human cells, although the viral life cycle was stopped before genome integration. More importantly, using CRISPR, live piglets with inactivated PERVs were born. However, there is no information about the development, the adulthood and the breeding of these animals. It remains unclear whether off-target effects of CRISPR can harm the animals and how to obtain large pig herds from the founder animals by breeding. Since there

was no transmission of PERV to humans in the first clinical trials, it is unclear whether this strategy is needed.

Do pig organs present other types of infection risk to human recipients?

LS: Herpesviruses are concerning because, as latent viruses, they are difficult to detect in donors. We must also consider future emerging pathogens, which requires continued monitoring. To evaluate exposure in the donor, sensitive diagnostics are imperative. A latent virus in an organ is likely to be reactivated after transplantation. Whether the virus can then infect the recipient requires more research. Generally, herpesviruses do not cross species. The most important aspect is to ensure in advance that the donor is negative.

JD: At the moment there are two well-known zoonotic pig viruses: hepatitis E virus (genotype 3 or HEV-3) and pCMV, a herpesvirus, which is a porcine roseolovirus more closely related to the human roseoloviruses HHV-6 and HHV-7 than to human cytomegalovirus. pCMV drastically reduced the survival times of different NHP recipients that received pig kidney and heart (<30 days for baboons with virus-positive hearts compared with up to 195 days with virus-negative hearts). pCMV was also transmitted to the Baltimore patient who received the first pig heart and contributed to his death. His clinical features were the same as those seen in baboons with transplanted pCMV-positive pig hearts.

Preventing pCMV transmission requires sensitive PCR and immunological assays, as well as an excellent test strategy, since latent virus may be undetectable. pCMV can be easily eliminated from a pig facility by early weaning, as shown in Munich. There are additional tools to eliminate unwanted viruses, such as colostrum deprivation, Cesarean delivery, embryo transfer, vaccines and antiviral drugs. Virus-free animals should be kept in isolation to prevent re-entry of viruses. Some viruses can be transmitted by oocytes or follicular fluid when performing somatic cell nuclear transfer and cloning.

The other zoonotic pig virus, hepatitis E virus genotype 3 (HEV-3), is already transmitted to humans by undercooked meat or contact with pigs. HEV-3 may be fatal in immunosuppressed humans and causes liver disease in patients with preexisting liver damage. For all other porcine viruses, it is unclear whether they pose a risk to the xenotransplant recipient. With the exception of HEV, it seems

unlikely that a pig virus would infect a transplanted pig organ.

AS: Through implementation of proper hygiene procedures (for example, separate housing for xeno-pigs in specific-pathogen-free or designated-pathogen-free facilities), most infectious agents, including pCMV, can be avoided. A prerequisite is establishment of a clean xeno-pig herd, as shown by a number of groups. Considering the high infection risk of human allotransplants, xenografts could even be a safer option.

RP: pCMV is known to be associated with graft failure and should be excluded from the source pig. Pig pathogens that cause disease in humans are quite well characterized. The presence of a xenograft wouldn't change the recipient's risk profile beyond that of any patient taking immunosuppressive drugs. Unless the pig-specific pathogen was introduced enterally and trafficked to the xenograft or was introduced parenterally (highly unlikely), pig-specific pathogens will not be problematic in xenograft recipients.

Are any animals other than pigs being considered as xeno-organ donors?

C-GP: During the early period of xenotransplantation (1960s to 1990s), attempts were made to transplant NHP kidney, heart and liver into humans. NHPs are no longer being considered, for several reasons, including a high risk of virus transmission, breeding difficulties and ethical issues stemming from their close phylogenetic relationship with humans. In addition, large primates are endangered species. The pig is considered the animal of choice because of its many advantages, such as human-like organ sizes, ease of breeding and genetic engineering, large litter size, and anatomical and physiological similarity to humans. Since millions of pigs are slaughtered for human consumption each year, there should be less ethical concern about using pig organs to treat human diseases.

LS: NHPs are not ethically or practically feasible but will continue to be the go-to preclinical model. Limitations of NHP models include the inability to evaluate PERVs given the lack of the PERV receptor. Old World NHPs (for example, baboons, rhesus macaques, cynomolgus macaques) do not express gal and so, like humans, produce anti-gal antibodies. Removing the main gal antigen from donor organs has revealed other xenoantigens, such

as Neu5GC[9]. Recent publications suggest that Neu5GC may have deleterious effects[10]. However, we have demonstrated that patients exposed to pig skin xenografts have antibodies up to 34 years post-treatment without adverse effects[5]. This does not preclude immune responses that may occur in, for example, solid organ xenotransplant recipients using gal knockout donors.

How well do NHP studies predict human outcomes?

C-GP: NHPs are probably the best in vivo models, especially for evaluating the immune response and the efficacy and safety of immunosuppressive regimens. NHPs mount an antibody response against pig cells in which all three major carbohydrate antigens – gal, Neu5GC and Sd[a] – have been knocked out, as deletion of Neu5GC exposes a fourth xeno-antigen. Neu5Gc antigen is expressed in pigs and all NHPs but not in humans. Thus, triple-knockout pig xenografts are less successful in NHPs than in humans and should be quite successful in humans.

Another issue is mismatch of organ size. For example, the chest and abdominal cavities of rhesus or cynomolgous monkey are not large enough to accommodate pig hearts and kidneys. This issue can be addressed by such strategies as deleting the growth hormone receptor in the donor pig. How well NHPs predict many other aspects of human xenotransplantation will not be known until clinical trials are performed.

RP: NHP models are challenging for testing triple-knockout organs because of the 'fourth antigen' problem. A regimen that is successful in NHPs is likely to overperform in humans. We can't predict how much we can safely peel back immunosuppression regimens developed in NHPs, but once we have achieved initial success in humans, reducing the immunosuppression intensity can be explored and will be a priority.

JD: NHPs are not a suitable model to study the PERV risk since PERV cannot replicate efficiently in NHP cells.

Are new in vitro systems for xenotransplantation research forthcoming?

AS: Ex vivo perfusion systems using human blood or serum are already used to assess rejection mechanisms and to analyze molecular changes in the endothelium when it

contacts human blood. Single-cell sequencing is being implemented to determine the specific cell types and pathways involved and to define effective adjustments.

C-GP: A model for ex vivo perfusion of pig organs such as heart, lung and liver is being used. It is valuable for evaluating immediate immune responses such as xenoreactive antibody binding, complement and NK cell activation, and activation of fibrinolytic and coagulation systems. However, it does not reflect the long-term effects of other immune responses on physiological function.

What are the main ethical issues for xenotransplantation?

RP: The absence of clinical data makes it difficult to provide informed consent. Although unknowns exist in any clinical trial, the consent process in xenotransplantation must accommodate novel unknowns, such as previously unidentified pig viruses that can cause disease in humans. Providing sufficient information to the subject and their close contacts to allow them to understand and reflect on unforeseen possible consequences of 'unknown unknown' risks is necessary in order to respect the ethical imperative underpinning exploratory biomedical research: to make every reasonable effort to protect the experimental subject from possible harms and to balance risks and possible benefits. Given the complexity of the science and the unanswered questions, providing appropriate education in the consent process is an important goal that will be difficult to achieve. Consent must also be obtained from family members and close contacts, who (unusually) will likely be required to consent to life-long monitoring.

Issues of sentience, intellectual capacity, and relational duties deserve ongoing conversation. Although some may object to the use of pig organs to save human lives, this is a minority view that is not generally supported by any major religion or social group. Society has chosen to use pigs as a food source, and most believe that their use for lifesaving organ transplants serves a morally higher purpose. A moral hierarchy that values human life over other life forms is admittedly anthropocentric, but most individuals will find it ethically acceptable to use pigs as a xenograft source if the animals receive compassionate, ethically defensible care.

LS: Many studies have investigated public perception of xenotransplantation. Differences may exist between the public and the scientific community, emphasizing the need

for scientists and clinicians to engage with the public to provide a balanced view. Adhering to ethical and regulatory requirements for animal welfare and for the consent process is essential and of utmost importance to the field.

Do clinical centers need to establish special processes for xenotransplantation work?

RP: To optimally protect the public from a highly unlikely but potentially catastrophic pandemic infection risk, clinical centers wishing to perform xenografts will need surveillance measures to detect known or unknown potential pathogens. Contingency planning and the informed consent process must anticipate the possibility of requiring isolation of an infected patient and their close contacts. Initial studies should only occur in jurisdictions with effective regulation and legal frameworks sufficient to protect the experimental subject, caregivers and the general public from predictable risks and to support safe conduct of informative clinical experiments. Ethical design and oversight of xenotransplant clinical trials will be enhanced by transparency and by regulatory and institutional review board access to international expertise.

LS: No clinical center would proceed without the relevant regulatory approval and the appropriate mitigating protocols in place. Ideally, a center requires a multi-skilled team and access to suitable donors, which is not always easy given the small number of facilities raising donor animals. The centers conducting these trials have substantial expertise in xenotransplantation and are already aware of the requirements through participation with the International Xenotransplantation Association, the Changsha Communique[11], and the development of consensus guidelines for the community.

AS: If transplantation is into a brain-dead recipient, duration limits might be required for ethical reasons or to respect the wishes of relatives. Scientifically, short-term experiments are of limited value, and if the condition of a brain-dead recipient permits, studying organ survival and function over months would be more informative for predicting outcomes in living patients. Safeguarding against zoonotic infections requires good animal husbandry, regular monitoring of donor pigs, and highly sensitive methods to identify latent infections (for example, pCMV). In most countries, oversight by trained regulatory and ethics committees is already in place.

Which organs will be attempted next?

C-GP: Pancreatic islets or cornea could be next because they have survived long-term in wild-type pig-to-NHP models with immunosuppression. They have met efficacy and safety outcomes in preclinical studies as required by the International Xenotransplantation Association. For pig lung and liver, the maximum survival time achieved in NHPs is 14 and 29 days [see Fig. 2], respectively, indicating that they are not ready for human transplantation. Xenotransplantation of porcine skin still presents several challenges as it is rich in tissue-resident immune cells such as antigen-presenting cells and memory T cells and evokes a strong immune response. Porcine skin from genetically modified pigs could be used to temporarily cover a burn wound, or a decellularized porcine skin, such as acellular dermal matrix, could be used to treat burns.

When can we expect the first phase 1 trials of genetically engineered pig organs?

RP: Regulators have set rather formidable benchmarks before allowing the field to proceed to clinical trials. These include putting in place a strategy and infrastructure for long-term monitoring of human recipients; defining criteria that specifically qualify the product for release at the individual donor or herd level; and achieving reproducible long-term preclinical results despite the 'fourth antigen' problem, the difficulty of working with NHPs compared to humans, and the likely biological differences between NHPs and humans. Meeting all these benchmarks will be expensive and might take a long time, which will be a challenge for commercial entities. Defining appropriate patient populations to go first will be challenging but feasible.

AS: In June 2022, the FDA signaled willingness to allow pig-to-human transplants in "small, focused" clinical trials with "appropriately selected patients."

C-GP: Human transplantation of wild-type pig pancreatic islets and corneas is imminent. Korea's xenotransplantation research team will begin pig islet xenotransplants in two patients with immunosuppression in 2023. Solid organs pose more immunological, functional and physiological obstacles. Single experiences in living patients through a 'compassionate use' emergency authorization or in decedents will accumulate knowledge to support small elective clinical trials, perhaps

in about 5 years. The heart xenograft case showed that the pig heart failed at 49 days, not from typical immune-mediated graft rejection but from an unknown cause. Such findings would be difficult to observe in NHPs and can guide clinical trial design.

Are there any other issues you would like to raise?

AS: Alternative technologies for producing transplantable organs are in development. These include differentiation of pluripotent stem cells, tissue engineering, and chimeric-animal methods to grow pigs with a human organ. Especially for complex organs, these approaches are still very far from the clinic, although this may change in the future. Producing organs from a patient's own cells would circumvent immune rejection but would be very costly, whereas maintaining a xeno-pig herd is relatively inexpensive.

JP: The most challenging issue in clinical xenotransplantation today is the greater intensity of immunosuppression compared with allotransplantation. Even in allotransplantation, immunosuppression has profound side effects, including heightened susceptibility to infection, sepsis, malignancy and cardiovascular disease. As long as xenotransplantation requires more toxic immunosuppression, allotransplantation will be the preferred treatment. My hope is that clinical experience will bring decreases in toxicity that expand the scope of xenotransplantation.

Research on complement, coagulation and inflammation has revealed more complexities than anyone could have imagined. Graft failure in both allo- and xenotransplantation may have less to do with absolute incompatibilities than with variation in immunity or deficient accommodation. All xenografts and probably most allografts generate C3b, thrombin and other reactive moieties that migrate to the host and exert systemic and local effects. In allotransplantation, accommodation enables long-term graft function despite ongoing interaction of natural antibodies with blood-group saccharides in the graft. In xenotransplantation, such interactions could be unavoidable but may not prevent success. Put another way, the barrier posed by some porcine glycans and other antigens may be more apparent than real. Further, before targeting glycosyltransferases, one should establish that absence of the saccharide does not impair graft function.

The demand for organ replacement is likely to grow substantially over time as a result of population growth, dissemination of technical expertise, biomedical advances that extend lifespan and increase the prevalence of diseases of aging, and concerns about healthcare costs. In this context, we must continue to revisit widely held assumptions about xenotransplantation.

This roundtable was conducted by Laura DeFrancesco and Kathryn Aschheim.

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