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# Pig heart and lung xenotransplantation: Present status

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## Abstract

The recent pig heart transplant in a patient at the University of Maryland Medical Center has stimulated renewed interest in the xenotransplantation of organs from genetically engineered pigs. The barriers to the use of pigs as sources of organs have largely been overcome by 2 approaches – (1) the deletion of expression of the three known pig carbohydrate xenoantigens against which humans have preformed antibodies, and (2) the transgenic introduction of human 'protective' proteins, such as complement-regulatory proteins. These gene modifications, coupled with immunosuppressive therapy based on blockade of the CD40/CD154 costimulation pathway, have resulted in survival of baboons with life-supporting pig heart grafts for almost 9 months. The initial clinical success at the University of Maryland reinforces encouraging preclinical results. It suggests that pig hearts are likely to provide an effective bridge to an allotransplant, but their utility for destination therapy remains uncertain. Because of additional complex immunobiological problems, the same approach has been less successful in preclinical lung xenograft transplantation, where survival is still measured in days or weeks. The first formal clinical trials of pig heart transplantation may include patients who do not have access to an allotransplant, those with contraindications for mechanical circulatory support, those in need of retransplantation or with a high level of panel-reactive antibodies. Infants with complex congenital heart disease, should also be considered.

### Keywords

clinical; heart; lung; nonhuman primate; pig; xenotransplantation

Xenotransplantation using organs from genetically modified nonprimate mammals offers a potentially unlimited supply of replacement organs. However, clinical translation has been impeded by vigorous innate and adaptive immune responses when organs from pigs

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– the species being developed for clinical xenotransplant indications – are transplanted into primates. Additional barriers include incompatibilities in thromboregulatory and 'self-recognition' molecular interactions between pigs and humans, and the risk of potentially contagious infection being transmitted from the pig to the xenograft recipient, and then to other humans.[1,2] In response to the recent report of a clinical pig heart transplant in Maryland, we here review the current status of heart and lung xenotransplantation, and their prospects as viable therapies for end-stage cardiopulmonary diseases.

## Thoracic organ xenotransplantation: brief history and recent experience

Even before the famous first human heart allotransplant by Barnard in 1967,[3] in 1964 Hardy performed the world's first clinical cardiac transplant using a chimpanzee heart that proved inadequate to support the circulation of the recipient.[4] Subsequent attempts, involving nonhuman primate, sheep, and pig hearts, were also unsuccessful (Table 1). However, Bailey's transplant of a baboon heart into an infant girl[5] did much to stimulate the successful development of pediatric heart allotransplantation. Clinical lung xenotransplantation has not been reported.

On January 7, 2022, the first clinical xenotransplant of a heart from a genetically-engineered pig (with 10 genetic modifications) was carried out at the University of Maryland, based to some extent on preclinical studies in Munich.[6] The 57 years old recipient experienced good heart function for 2 months before succumbing in the context of graft dysfunction. This clinical experiment surprised and energized the thoracic transplant and xenotransplantation communities.

## Developing 'biocompatible' pigs

### Rationale for development

The historic use of organs from primate species raised ethical concerns,[7] faced formidable logistical barriers, and posed potential risks of the transfer of infectious microorganisms.[8] Focus therefore shifted to pigs as the organ source, which have advantages in (1) better size-matching with humans, (2) favorable breeding characteristics (faster sexual maturity, shorter pregnancy, greater number of offspring, and lower costs for husbandry and propagation), and (3) the feasibility of genetic modification.[9] The short reproductive cycle of pigs and the development of in vitro fertilization and cloning technologies for this species enabled application of gene-editing to address the biologic obstacles of' pig-to-human transplantation (summarized below).

### Anti-pig antibodies

When wild-type (i.e., genetically unmodified) pig organs are exposed to human or NHP blood, antibody-mediated endothelial injury occurs within minutes, driven largely by binding of preformed 'natural' antibodies to the pig vascular endothelial cells, leading to complement activation and graft loss from hyperacute rejection.[1] The majority of these natural human anti-pig antibodies are targeted at 3 carbohydrate antigens (Table 2), of which galactose-$\alpha$ 1,3-galactose (Gal)[10] appears to be the most important. Work in the 1990s focused on elimination or neutralization of these antibodies utilizing plasmapheresis

or immunoadsorption by immunoaffinity columns, which delayed, but did not prevent, rejection.[11–13]

In 2003, the first pigs that did not express Gal were produced by knockout of the a1,3-galactosyltransferase gene (GTKO pigs)[14] (Table 2). At the time, gene-editing techniques were less site-specific and very complex. Subsequent development of many gene editing technologies, like ZFNs, TALENS, and CRISPR-Cas9 (Table 3), enabled more precise and complex gene modification.[15] In this regard, reengineering of Cas9 by Charpentier and Doudna made CRISPR-Cas9 a capable cost-effective technology and enabled high-precision targeting and editing of genomic loci, which has had a revolutionary impact on the life sciences. Those techniques facilitated additional deletion of expression of the remaining xenoantigens, yielding double[16] and triple (TKO) knockout pigs.[17]

Complicating preclinical assessment of TKO organs, the TKO modification has unveiled or activated a ‘fourth’ xenoantigen that may be associated with antibody-mediated rejection in NHPs, but not in humans.[18]

## Complement activation

Complement activation, physiologically triggered in circumstances where the innate immune system senses ‘foreign invasion’ or ‘self-injury,’ is regulated by negative feedback mechanisms to avoid over-activation and collateral damage to healthy host tissues.[19] This is achieved by the expression of several ‘protective’ complement pathway regulatory proteins (CPRPs) on the vascular endothelial cells,[20] for example, decay accelerating factor (CD55); membrane cofactor protein (CD46), and membrane-attack-complex-inhibitory protein (CD59). CPRPs are relatively species-specific,[21] and pig CPRPs are less effective in controlling human complement-mediated injury than are human CPRPs. The expression of human CPRPs on pig cells reduces complement-mediated cell injury,[22] and has been associated with prolonged pig graft survival in a variety of NHP models.[23–25]

## Coagulation pathway dysregulation

Even in the absence of detectable antipig antibodies, diffuse microvascular thrombosis in the graft (thrombotic microangiopathy) and consumptive coagulopathy in the recipient were observed after transplanting GTKO pig organs that expressed human CPRPs.[26] This observation suggested that incompatibilities between pig and human thromboregulatory molecules might be physiologically consequential.[27,28] Pig thrombomodulin (TBM) binds human thrombin, but is less potent in activating protein C. Its cofactor, pig endothelial protein C receptor (EPCR), is also less efficient in activating protein C, and hence in regulating the coagulation process.[29] Pig tissue factor pathway inhibitor (TFPI) may be inefficient in regulating human tissue factor-initiated coagulation,[30] although this view has been questioned.[31]

Coagulation dysfunction is more problematic in regard to pig lung transplantation. Relatedly, pig von Willebrand factor (vWF) aggregates and activates human platelets spontaneously through aberrant non-physiologic interaction with human glycoprotein (GP)Ib,[32,33] a platelet surface membrane glycoprotein that functions as a receptor for vWF. Blocking the GPIb-binding site for vWF[34] or ‘humanizing’ vWF reduces platelet

sequestration during ex vivo perfusion of lungs with human blood, and after pig-to-baboon lung transplantation.[35]

Once activated, platelets propagate platelet and leukocyte aggregation through interactions mediated by GPIIb/IIIa, ADP- P2Y12, P- and E-selectins, integrins, and galectins, among others, leading to progressive thrombosis and necrotic destruction of the graft[1,36–38] (Figure 1). As a consequence, each of these procoagulant and proadhesive interactions are logical targets to reduce physiologically inappropriate clotting and inflammation after cell and organ xenotransplantation.

Coagulation dysfunction, together with an associated inflammatory response, has proved a major hurdle in achieving prolonged pig lung survival in NHPs.[39]

## Self-identification

Cluster of differentiation molecule 47 (CD47) is a self-recognition marker that inhibits phagocytosis of CD47+ cells by macrophages and other innate immune ‘scavenging’ cell populations.[40,41] When CD47 was introduced into GalT-KO pig cells by genetically engineering, bone marrow cells remained detectable in the circulation for days rather than minutes.[42] Recent reports describe improved results associated with GTKO.hCPRP lungs and kidneys that additionally express CD47.[43,44] While perhaps less essential than hCPRP or thromboregulatory genes, expression of human ‘self’ may prove advantageous for heart and lung xenografts.

## Rapid pig organ growth after transplantation

Rapid growth and hypertrophy have been documented in pig hearts (and kidneys) implanted into NHPs, likely associated with differences in the rates of growth between pigs and NHPs.[45,46] Cardiac xenograft hypertrophy can be reduced by hypotensive therapy and mTOR inhibition.[46] It might be avoided by using pigs that grow slowly, for example, miniature swine, or have been genetically engineered to reach smaller size at maturity, for example, by knock-out of growth hormone receptors.[47]

## Potential infectious concerns

Pigs could potentially transmit to humans a wide range of bacteria, parasites, and viruses.[48,49] The FDA will require that the organ-source pigs be bred and housed in clean, biosecure conditions, and be certifiably free of pig organisms that are known pathogens for humans. Thus, the infections that are most likely to affect recipients of pig xenografts are the same as those that typically occur in immunosuppressed recipients of allografts (e.g., CMV reactivation, opportunistic bacterial infections), for which effective treatments are generally available.

In the 1990s, concerns were raised regarding the possibility of humans being infected by porcine endogenous retroviruses (PERVs) that are present within the genome of all pig cells.[50] At that time, the pandemic spread of the human immunodeficiency virus (HIV), an exogenous retrovirus, helped feed these fears, appropriately raising concerns for regulatory authorities, and depressing public (and investor) enthusiasm for xenotransplantation.[1,51]

Reassuringly, PERV transmission to primates has never been observed in preclinical or clinical studies.[52] In addition, (1) multiple innovative strategies have been developed to inhibit possible PERV transmission,[53] (2) PERV-deleted pigs have been produced utilizing CRISPR-based technology,[54,55] and (3) potent antiretroviral drugs that were developed to treat HIV are effective in vitro against PERV.[56] Consequently, the risk of PERV infection for a given xenograft recipient appears to be very low; infection or disease, if either occurs, will likely be treatable; and subsequent transmission or pandemic infection seems improbable.

In summary, harvesting organs from pigs bred and raised under strictly controlled 'designated pathogen-free' (DPF) conditions[57] could decrease the risk of infection to less than that seen in recipients of allografts, where the human donor might have been exposed to a variety of pathogens, and in whom the available screening strategies are time-limited. As long as a surveillance strategy designed to detect both known and 'unknown unknown' pathogens is consistently deployed, xenotransplantation may prove to be safer than contemporary allotransplantation with respect to the risk of donor-transmitted infection.

## Preclinical progress

### Cardiac xenotransplantation

Two valuable lessons have been learned from preclinical in vivo organ xenotransplantation studies. First, conventional immunosuppressive therapy (i.e., tacrolimus-based) is less effective in xenotransplantation than in allotransplantation.[58] Rather, blocking the CD154/CD40 costimulatory pathway, first introduced into the pig-to-NHP model by Buhler et al in 2000,[59] results in substantial improvement in graft survival. Blockade of the CD28/B7 costimulatory pathway is less successful.[60,61] Second, pig hearts seem to be more susceptible to ischemia-reperfusion injury than human hearts.[62] Längin et al. addressed this problem by using non-ischemic heart preservation by continuous perfusion with 8°C Steen solution.[63,64] Consistent survival of 3 to 6 months was reported using GTKO/hCD46/hTBM hearts and blockade of the CD40/CD154 pathway.[45]

Encouraging with respect to long-term viability of pig heart xenografts, in a heterotopic (non-life-supporting) model (Figure 2), GTKO/hCD46/hTBM heart xenografts consistently survived for as long as CD40-blocking was given, in one case up to 945 days.[65,66] In this context, the Langin and Brenner reports mark an important benchmark, reaching the prerequisite conditions for embarking on a clinical xenotransplantation trial that were proposed by the Advisory Committee to the International Society for Heart and Lung Transplantation (ISHLT) in 2000.[67]

Nevertheless, it should be noted that to date the longest period of life support by a pig heart in a NHP has been limited to <9 months.[68] Based on this data and the recent Maryland clinical experience, we suggest that at present pig heart xenotransplantation might most appropriately be considered as a bridge to allotransplantation. In our estimation, to justify heart xenotransplantation trials as 'destination therapy,' consistent success in preclinical models, similar to that recently reported[6,45] or demonstration of more durable success in a 'compassionate use' clinical application – will be necessary.

## Lung xenotransplantation

Lung xenotransplantation research to date has primarily employed ex vivo models incorporating human blood perfusion to evaluate the effect of genetic modification of the pig and/or drug treatment on the rise in pulmonary vascular resistance and loss of vascular barrier function characteristic of pig lung injury.[69] The knowledge gained, however, has translated into relatively limited improvement in life-supporting lung function and recipient survival in the in vivo pig-to-NHP lung xenograft model.[39,69] Probably because of the more extensive endothelial surface area, pro-inflammatory resident immune cells, and anatomic vulnerability to even localized injury flooding adjacent airways, lung injury is more difficult to prevent, and thus in vivo lung xenograft survival remains limited to days or weeks.[39,70]

Increasingly sophisticated genetically-engineered pigs have been produced with 10 or even 15 gene edits,[1–71] including those designed to address some of the mechanisms of lung injury identified in the ex vivo lung perfusion model and partially validated in vivo.[39] We speculate that adding a humanized vWF modification[35] in the context of these mechanism-directed gene edits will be necessary, and may be sufficient, to enable prolonged lung graft survival in our non-human primate model, so as to eventually justify an initial clinical trial.

## Prospects for clinical heart or lung xenotransplantation

What additional evidence is needed to justify regulatory approval for a definitive, ‘qualifying’ trial of clinical heart xenotransplantation? The pig sources of hearts in the Munich studies (with 3 genetic modifications) were suitable for transplantation into NHPs, but may not prove to be optimal for transplantation into human patients.[72] The University of Maryland team provided encouraging results following the transplantation in baboons of hearts from pigs with 10 genetic manipulations, designed to be fully ‘biocompatible’ with humans, thus enabling the US Food and Drug Administration (FDA) to approve a single initial ‘compassionate use’ clinical study.

Although the predictive value of the NHP model has never been tested and may introduce confounding immune barriers,[73] several important remaining questions can potentially be answered in preclinical models: (1) define a ‘necessary and sufficient’ pig phenotype, (2) validate a consistently effective method of heart preservation, (3) identify a consistently effective, safe immunosuppressive regimen, and (4) test candidate strategies to prevent or manage graft hypertrophy, when evident. Cautious clinical experimentation is justifiable in the hands of experienced investigators, not least because information gained will determine whether or not NHP preclinical studies are informative.

We gauge that additional advances in understanding and modulating the mechanisms of lung xenograft injury will be required before pig lungs can emerge as a viable clinical option. Hopefully, with further experience in the pig-to-NHP lung transplantation model and from experience with clinical xenotransplantation of the heart and other cells and organs, the remaining barriers to clinical pig lung transplantation will also be resolved.

### Patient selection for clinical trials of pig heart transplantation

In our estimation, selection of patients for initial xenotransplantation trials should adhere to the general guidelines for heart allotransplant candidacy.[74] Until the actual risks and complications associated with receipt of a life-supporting pig heart are defined by clinical experience, basic ethical principles[75] will dictate first enrolling patients who are unlikely to have access to, or to benefit from, currently-available therapeutic alternatives. These patients include those with:

### Relative or absolute contraindications to mechanical circulatory support

Patients with restrictive or hypertrophic cardiomyopathies or severe right ventricular dysfunction experience a high morbidity and mortality after implantation of a left ventricular assist device (LVAD). Furthermore, the presence of a dysfunctional mechanical valve, or a degenerated bioprosthesis (e.g., associated with mitral or aortic stenosis, or aortic insufficiency), or an atrial or ventricular septal defect, greatly complicate bridging or destination therapy with any form of mechanical circulatory support (LVAD or BiVAD). These patients often exhibit rapid and unpredictable deterioration, and might benefit from timely access to a pig heart as a bridge to allotransplantation.

### High titers of broadly panel-reactive anti-HLA antibodies (PRA)

High PRA candidates (1) experience long waiting times for allotransplantation and a high wait-list mortality, (2) may require potentially high-risk and incompletely effective treatment to reduce anti-HLA titers, and (3) experience higher rates of rejection and early graft vasculopathy even after receipt of a ‘cross-match negative’ heart allograft. We caution to initially avoid patients with a high PRA who exhibit cross-reactivity with swine leukocyte antigens (SLAs) unless they are demonstrated to have a negative flow cytometric cross-match with cells from the ‘donor’ pig.[76]

### Chronic rejection after cardiac allotransplantation

Cardiac allograft recipients with graft vasculopathy have (1) a high risk of sudden death, (2) a low priority on the waiting list for a second deceased human donor organ, and (3) suboptimal support by a mechanical device. The risks of acute and/or chronic immune injury to a second or subsequent heart allograft are high.[77] Elective access to a crossmatch-negative pig heart (see above) might be attractive for patients with rapidly progressive graft vasculopathy or for those at high risk for fatal arrhythmia.

### Infants and children with complex congenital heart disease

Infants with complex congenital heart disease, especially those with single ventricle physiology, have limited access to allotransplantation due to the scarcity of size-matched deceased donor organs in that age group.[78–80] The results of mechanical support in these patients are poor,[81–82] and the results of multiple staged surgical reconstructive procedures for palliation remain mixed.[81] A genetically-engineered pig heart might be life-supporting as a bridge until a heart from a deceased human donor can be obtained. The relatively immature and ‘flexible’ immune system of infants may enable greater resistance to rejection

than in older patients.[83] Theoretically, a pig heart might grow proportionately with the infant, or could be electively replaced later if a size-mismatch develops.

### Future considerations

Traditional methods of monitoring for rejection (e.g., echocardiography, measurement of troponin and CK-MB levels[84] have been found to be helpful in determining whether graft atherosclerosis is developing. The utility of endomyocardial biopsy in patients with a pig heart transplant remains to be determined, but we are mindful that routine histologic surveillance of the graft was arguably pivotal to the current success of heart allotransplantation. Several novel, but largely unproven, methods of detecting rejection are worthy of exploration, for example, donor-derived cell-free DNA,[85–87] species-specific gene-expressing profiling,[84,88] or circulating organ-specific miRNAs.[89,90]

Success of pig heart transplantation in NHPs has been achieved to date only when experimental immunomodulatory drugs (not yet approved by US FDA or European Medicines Agency - EMA) have been used.[91] Fortunately, in published guidance[92] and by its approval on compassionate grounds of the Maryland patient, the FDA has demonstrated its willingness to consider an experimental drug as part of a clinical protocol when that approach is supported by preclinical data.

Maintaining ‘designated pathogen-free’ pigs in isolation will be costly, and, when and if clinically approved, cost may become a significant barrier to access. On the other hand, prolonged costly pre-transplant stays in the intensive care unit will become unnecessary, and high-cost, high-risk alternative medical or surgical therapies will become obsolete as the availability of a suitable pig organ will enable transplantation to be carried out timely when indicated and optimal for the recipient. Based on the initial success achieved in the University of Maryland case, this idealized vision for cardiac xenotransplantation now seems within reach.

In summary, in the initial clinical trials, we caution against including patients with poor prognosis based on medical or surgical risk factors not directly related to their heart pathology (e.g., advanced frailty, widespread peripheral atherosclerotic disease, a ‘hostile’ mediastinum), as this approach is likely to yield poor outcomes and undermine public and peer support for xenotransplantation. Similarly, we believe that enrolling subjects under emergency circumstances that do not allow thorough assessment of patient suitability should be avoided. Current or recently treated advanced malignancy or an active infection are likely to cause life-limiting complications under immunosuppressive therapy. Finally, adequate psychosocial support, a robust process of preprocedure education, and thorough informed consent for both the patient and his/her caregivers are important to encourage compliance with post-transplant treatment, monitoring, and public safety protocols.

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## Abbreviations:

| | |
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| **CRISPR** | clustered regularly interspaced short palindromic repeats |
| **CPRPs** | complement pathway regulatory proteins |
| **EPCR** | endothelial protein C receptor |
| **GTKO** | $\alpha$1,3-galactosyltransferase knockout |
| **hTBM** | human thrombomodulin |
| **LVAD** | left ventricular assist device |
| **mTOR** | mammalian target of rapamycin |
| **NHP** | nonhuman primates |
| **PERV** | porcine endogenous retrovirus |
| **PRA** | panel-reactive anti-HLA antibodies |
| **TFPI** | tissue factor pathway inhibitor |
| **TKO** | triple knockout |

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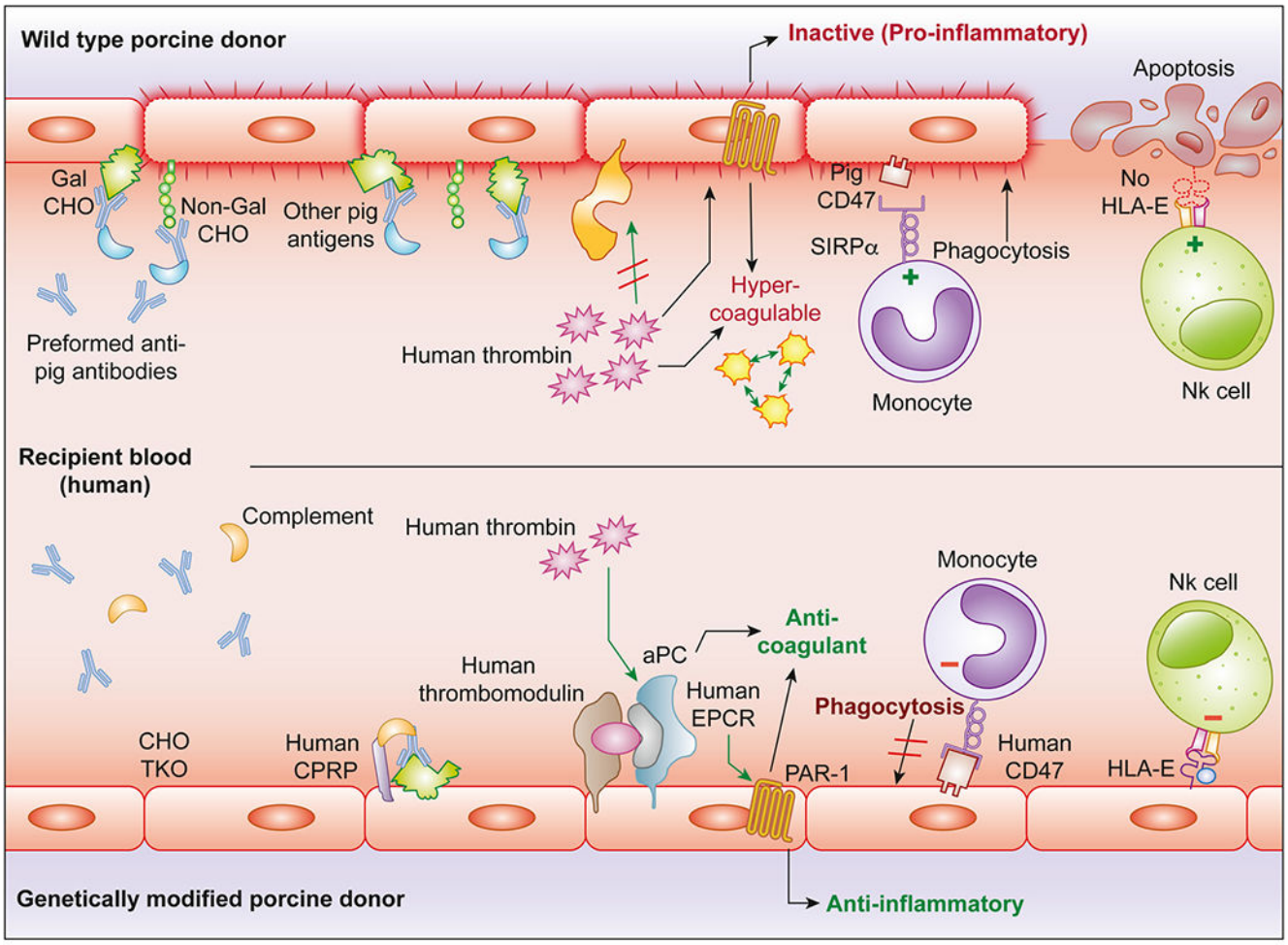


**Figure 1.**

Genetic modifications designed to address mechanisms of xenograft injury: Examples of genetic modifications designed to prevent known xenograft injury (top) include Gal a 1-3Gal and 2 other carbohydrates (triple knockout, TKO) and expression of human complement pathway regulatory proteins (hCPRPs) and coagulation pathway regulatory proteins, eg, thrombomodulin and endothelial protein C receptor (bottom). Absence of carbohydrate antigens and expression of human complement and coagulation pathway regulatory molecules reduce endothelial activation and injury.

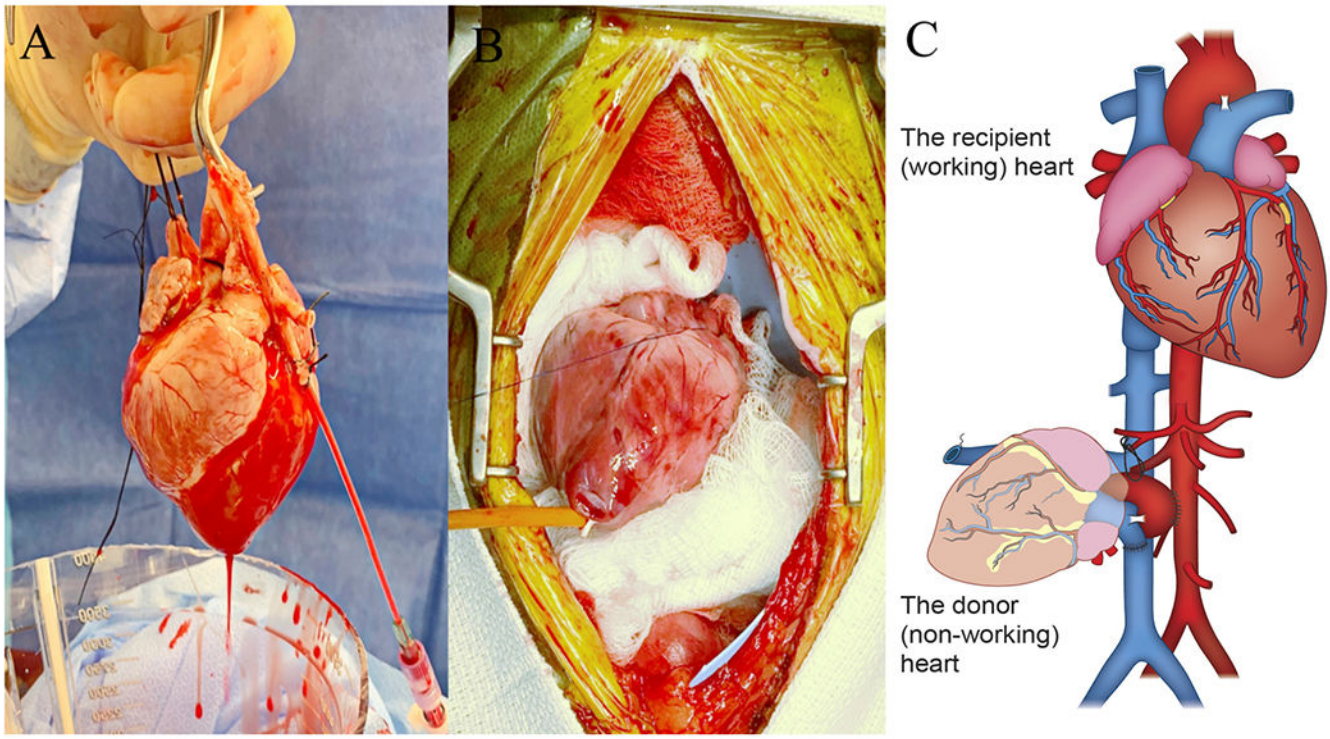


**Figure 2.**

Experimental heterotopic non-working heart transplantation: After harvesting the pig heart and perfusing it for 30 minutes in ex vivo (A), it is implanted in heterotopic position in baboon abdomen (B), by anastomosing the donor aorta to the recipient abdominal aorta and the donor pulmonary artery to the recipient inferior caval vein, after legating both donor caval veins and closing the left atrium (C).

**Table 1**

World Experience in Clinical Heart Xenotransplantation[93]

| Year | Surgeon | Donor | type | Patient survival (days) |
|---|---|---|---|---|
| 1964 | Hardy | Chimpanzee | O | <1 |
| 1968 | Cooley | Sheep | O | <2 |
| 1968 | Ross | Pig | H | <3 |
| 1968 | Ross | Pig | Perfused with human blood but not transplanted | <4 |
| 1969 | Marion | Chimpanzee | ?O | <5 |
| 1977 | Barnard | Baboon | H | <6 |
| 1977 | Barnard | Chimpanzee | H | 4 |
| 1964 | Bailey | Baboon | O | 20 |
| 1992 | Religa | Pig | O | 1 |
| 1996 | Baruah | Pig | O | 7 |
| 2022 | Griffith | Pig | O | ~60 |

H heterotopic (auxiliary) heart transplantation, O orthotopic heart transplantation.

**Table 2**

Known Carbohydrate Xenoantigens Expressed on Pig Celts

| Carbohydrate antigen (abbreviation) | Responsible enzyme | Gene-knockout pig |
|---|---|---|
| Galactose-$\alpha$1,3-galactose (Gal) | $\alpha$1,3-galactosyltransferase | GTKO |
| N-glycolylneuraminic acid (Neu5Gc) | Cytidine monophosphate-N-acetylneuraminic acid hydroxylase | CMAH-KO |
| Sd[a] | $\beta$-1,4N-acetylgalactosaminyl-transferase 2 | $\beta$4GalNT2-KO |

**Table 3**

Timeline for Application of Evolving Techniques for Genetic Engineering of Pigs Employed in Xenotransplantation

| Year | Technique |
|---|---|
| 1992 | Microinjection of randomly-integrating transgenes |
| 2000 | Somatic cell nuclear transfer (SCNT) |
| 2002 | Homologous recombination |
| 2011 | Zinc finger nucleases (ZFNs) |
| 2013 | Transcription activator-like effector nucleases (TALENs) |
| 2014 | CRISPR-Cas9[a] |

[a]CRISPR-Cas9 = clustered randomly interspaced short palindromic repeats and the associated protein 9.