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XENOTRANSPLANTATION: PAST, PRESENT, AND FUTURE

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Abstract

Purpose of review—To review the progress in the field of xenotransplantation with special attention to most recent encouraging findings which will eventually bring xenotransplantation to the clinic in the near future.

Recent findings—Starting from early 2000, with the introduction of Gal-knockout pigs, prolonged survival especially in heart and kidney xenotransplantation was recorded. However, remaining antibody barriers to nonGal antigens continue to be the hurdle to overcome. The production of genetically-engineered pigs was difficult requiring prolonged time. However, advances in gene editing, such as zinc finger nucleases, transcription activator-like effector nucleases, and most recently CRISPR technology made the production of genetically-engineered pigs easier and available to more researchers. Today, the survival of pig-to-nonhuman primate heterotopic heart, kidney, and islet xenotransplantation reached >900 days, >400 days, and >600 day, respectively. The availability of multiple-gene pigs (5 or 6 genetic modifications) and/or newer costimulation blockade agents significantly contributed to this success. Now, the field is getting ready for clinical trials with an international consensus.

Summary—Clinical trials in cellular or solid organ xenotransplantation are getting closer with convincing preclinical data from many centers. The next decade will show us new achievements and additional barriers in clinical xenotransplantation.

Keywords

clinical; CRISPR; experimental; genetic engineering; xenotransplantation

Introduction

Outcomes of organ and cell allotransplantation continue to improve. However, the shortage of transplantable organs remains as the major hurdle in the field of transplantation despite the use of marginal deceased donors and living donors [1]. Xenotransplantation (i.e., cross-

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Conflict of interest

The authors declare no conflict of interest.

species transplantation between pig and humans) could offer an unlimited and prompt supply of transplantable organs, when needed [2]. In addition to organ transplantation, many disorders could be treated by xenotransplantation (Figure 1).

In this review, we (i) briefly mention the past experience with xenotransplantation (mainly by referring to seminal review articles), (ii) provide a review of the most recent (within the last 24 months) advances in the field (present), and suggest future applications in the clinic (future).

Past

The concept of xenotransplantation is not new, and there have been numerous clinical attempts during the past 300 years or more [3]. Clinical blood xenotransfusion was attempted in the 17th century by Jean Baptiste Denis, corneal xenotransplantation from pig-to-human followed in the early 19th century, and attempts were made at nonhuman primate (NHP) kidney xenotransplantation in the 1960s by Reemtsma [3,4] and others [5]. The world experience in pig-to-NHP models of xenotransplantation (until 1997) was reviewed by Lambrijs et al. [6], and a comprehensive review regarding progress in pig-to-NHP since then (1998–2013) was published in 2014 [7].

Present

Xenotransplantation research was stimulated by the production of pigs in which the important antigen, galactose- α 1,3-galactose (Gal), had been deleted by gene-knockout (GTKO pigs) in 2003 [8]. More recently, the identification of other xenoantigens has also been important.

Techniques for making genetically-engineered pigs have become easier and faster. Rapid improvement in the results of preclinical studies has made the field more hopeful of the initiation of clinical trials [8–11]. Recent papers have discussed the selection of patients for initial clinical trials for solid organ xenotransplantation [12] and islet xenotransplantation. We here briefly review progress in pig-to-NHP models.

Heart xenotransplantation—Mohiuddin et al [13] demonstrated that long-term survival of genetically-engineered pig heterotopic heart grafts could be achieved in NHPs. Genetic modifications in the pig (GTKO.hCD46.hThrombomodulin) combined with a successful treatment regimen based on a chimeric anti-CD40 monoclonal antibody (mAb), consistently prevented humoral rejection and systemic coagulation pathway dysregulation, sustaining cardiac xenograft survival in one case beyond 900 days (Figure 2) [13].

Iwase et al. tested three different costimulation blockade-based immunosuppressive regimens in the pig-to-baboon heterotopic heart xenotransplantation model, and demonstrated that the combination of anti-CD40mAb+belatacept proved effective in preventing a T cell response [14]. Despite significant progress on the survival of heterotopic pig heart xenotransplantation, orthotopic heart xenotransplantation experiments were limited and the longest survival recorded to date was <60 days. Murthy et al recently reviewed the historical background, experimental progress, and clinical prospects in heart xenotransplantation [15].

Kidney xenotransplantation—The last 2 years have shown us that we are close to clinical trials of genetically-engineered pig kidney xenotransplantation. Two groups separately showed prolonged survival of life-supporting renal xenografts compared with historical 90-day survival [2,6] in different pig-to-NHP models [16,17]. The Emory group performed pre-transplant antibody screening in recipient monkeys and showed that the combination of low titer antibody and anti-CD154mAb costimulation blockade promoted long-term renal xenograft survival [16]. The Pittsburgh group showed that specific genetic modifications of the pig are important in achieving prolonged survival [17]. Most recently, Kim et al reported the longest survival (405 days) of a life-supporting pig kidney xenograft in a preclinical model, emphasizing the importance of CD4⁺ T cell depletion (Figure 2) [18].

Tanabe et al. studied the role of intrinsic (graft) versus extrinsic (host) factors in the growth of renal xenografts in GTKO pig-to-baboon model and identified that not only the size-mismatch (extrinsic – host factors), but also the intrinsic (graft) factors are responsible for growth of donor organs with a threshold for renal xenograft volume of 25cm³/kg of recipient body weight at which cortical ischemia was induced [19]. Iwase et al reported the immunological and physiological observations in baboons with life-supporting genetically-engineered pig kidney grafts with particular attention to the use of multiple-gene pigs, an effective costimulation blockade-based immunosuppressive regimen, and anti-inflammatory therapy in preventing immune injury [20]. In a recent review, Wijkstrom et al discussed the experimental progress and clinical prospects in renal xenotransplantation [21].

Lung xenotransplantation—Most recently, only the Maryland group has been active in exploring lung xenotransplantation. Burdorf et al. showed that platelet sequestration and activation during GTKO.hCD46 pig lung perfusion by human blood was primarily mediated by GPIb, GPIIb/IIIa, and von Willebrand Factor [22]. Laird et al showed that transgenic expression of human leukocyte antigen (HLA)-E attenuates GTKO.hCD46 pig lung xenograft injury [23]. A recent review from the same group concluded that genetic modification of pigs coupled with drugs targeting complement activation, coagulation, and inflammation have significantly increased duration of pig lung function in ex vivo human blood perfusion models, and life-supporting lung xenograft survival in vivo [24]. However, lung xenotransplantation is still measured in days rather than weeks or months.

Liver xenotransplantation—Although limited, fairly consistent 7–9 days' survival has been reported by different groups using GTKO and GTKO.hCD46 pig liver xenografts in NHPs after orthotopic pig liver xenotransplantation [25]. The Boston group increased survival to 29 days by the exogenous administration of human coagulation factors using the same model [26]. They reported two GTKO pig liver xenografts that survived >25 days (longest 29 days) (Figure 2), with immunosuppressive therapy consisting of anti-CD40mAb or belatacept [27]. Although there remain problems with this regimen, clinical trials of bridging to allotransplantation with a pig liver graft might become a possibility [28].

Islet xenotransplantation—In 2016, the International Xenotransplantation Association (IXA) published the first update on its consensus statement on conditions for undertaking clinical trials of porcine islet products in patients with type 1 diabetes [29–36]. This comprehensive report included (i) an update on national regulatory frameworks pertinent to

clinical islet xenotransplantation [30], (ii) evaluation of the source of pigs in order to prevent zoonoses [31], (iii) genetically-modified pigs as the source of islets [32], (iv) production and manufacturing of porcine islets [33], (v) requirement and efficacy of the pre-clinical data to justify a clinical trial [34], (vi) recipient monitoring and response plan for preventing disease transmission [35], and, finally, (vii) patient selection for pilot clinical trials of pig islet xenotransplantation [36].

Matsumoto et al published a clinical trial using encapsulated neonatal wild-type pig islets in patients with type 1 diabetes [37]. Their study showed that there was a clinical benefit of islet xenotransplantation with improved HbA1c, especially when a greater number of islets was transplanted [37]. Although recipients did not become normoglycemic, the study provided some hope for future clinical trials [38].

While progress of encapsulation (micro or macro) is still under investigation [39], studies have recently been published on the use of different materials, such as agarose encapsulation, the microbiological safety of porcine islets [40], and the anti-fibrotic effect of rapamycin-containing polyethylene glycol-coated alginate microcapsules [41]. New drugs, such as cell-penetrating tat-metallothionein for immunomodulation have been studied, together with xeno-islet encapsulation [42,43].

Although a recent pre-clinical study by Shin et al showed long-term control of diabetes in NHPs by the transplantation of wild-type adult porcine islets [44], a study by Kang et al showed that higher D-dimer levels negatively correlated with survival of porcine islet xenografts [45]. Despite more data becoming available on pig islet xenotransplantation in NHPs, the streptozotocin-induced diabetes model in NHP is still under debate [46].

The field is being advanced by the use of newly-available genetically-modified pigs and newer costimulation blockade agents. Lee et al used pig islets overexpressing human hemoxygenase-1 and soluble tumor necrosis factor-alpha receptor type 1 with human IgG1 Fc in order to suppress early apoptosis during engraftment of xeno-islets [47]. Arefanian et al showed that porcine islet-specific tolerance induced by the combination of anti-lymphocyte function-associated antigen-1 and anti-CD154mAb is dependent on PD-1 (programmed cell death protein-1) [48]. Two recent reviews by Hawthorne et al [49] on genetic strategies to bring islet xenotransplantation to the clinic, and Bottino et al [50] on the safe use of anti-CD154mab underline the importance of genetic engineering and costimulation blockade in islet xenotransplantation. Recently, a seminal review was published by Liu et al on the past, present, and future of pig-to-primate islet xenotransplantation [51].

Tissue (cornea, heart valve, skin) xenotransplantation—Porcine corneal xenotransplantation shows promising application in the clinic. Lee et al studied the impact of the expression of N-glycolylneuraminic acid on pig corneas, concluding that the absence of N-glycolylneuraminic acid expression on GTKO pig corneas may not prove an advantage over GTKO pig corneas [52,53]. Dong et al recently published their initial results of GTKO.hCD46 pig full-thickness corneal xenografts in NHPs, which were comparable to the survival of wild-type pig corneas [54]. Lee et al provided evidence that the limiting factor of

survival of pig corneas was the development of a retrocorneal membrane [55]. The Seoul group recently reported prolonged survival (>389 days) of porcine deep-lamellar corneal xenografts in NHPs under immunosuppressive therapy with anti-CD40mAb (Figure 2) [56]. The same group also published the biophysico-functional compatibility of their miniature pig corneas as grafts in clinical trials [57].

Reuven et al [58] and Lee et al [59] studied the impact of N-glycolylneuraminic acid expression in bioprosthetic pig heart valves on human antibody recognition and structural deterioration.

Tena et al demonstrated that pig cells expressing human CD47 are associated with an immune-modulating effect, which leads to markedly-prolonged survival of pig skin grafts in NHPs [60].

Cellular (hepatocyte, neuronal cell) xenotransplantation—Machaidze et al tested porcine hepatocytes in alginate-poly-l-lysine microspheres transplanted intraperitoneally immediately after hepatectomy in a model of fulminant liver failure in baboons [61]. The microencapsulated porcine hepatocytes provided temporary functional support [61]. Mahou et al. reviewed the contribution of polymeric materials in the xenotransplantation of microencapsulated cells (mainly hepatocytes and islets), and addressed the state-of-the-art in cell microencapsulation with special focus on the choice of materials, and the design and fabrication of the microspheres [62]. Iwase et al. transplanted genetically-engineered pig hepatocytes directly into the spleen and other sites in immunosuppressed baboons, and reported very early graft failure [63].

The European Consortium (Xenome Project) studied the feasibility of pig neuronal cell xenotransplantation in NHPs to cure Parkinson's disease [64]. Parkinsonian NHPs received wild-type or CTLA4-Ig-transgenic porcine xenografts and different durations of exogenous immunosuppressive therapy to test whether systemic plus graft-mediated local immunosuppression might avoid rejection. A striking recovery of spontaneous locomotion was observed in the NHPs that received systemic plus local immunosuppression for 6 months, which was also associated with restoration of dopaminergic activity [64]. However, some recipients developed post-transplant lymphoproliferative disease, probably due to over-immunosuppression [65].

Inflammation and coagulation—Further attention was directed to inflammation in xenotransplantation. Ezzelarab et al. showed that systemic inflammation in xenograft recipients precedes activation of coagulation [66]. Iwase et al. measured serum free triiodothyronine (thyroid hormone) as a marker of inflammation in healthy naïve baboons, healthy naïve monkeys, and after pig-to-baboon heterotopic heart xenotransplantation, orthotopic liver xenotransplantation, artery patch xenotransplantation, and in monkey heterotopic heart allotransplantation [67]. They showed that there is a dramatic fall in serum thyroid hormone levels following operative procedures. A persistent low level of thyroid hormone after pig heart and liver xenotransplantation may be associated with a continuing inflammatory state, which might be corrected with extraneous replacement of thyroid hormone [67]. Other inflammatory states and markers, particularly of extracellular histones,

have been discussed with their potential therapeutic regulation in xenotransplantation [68,69].

Zoonosis—The potential for the transmission of infection from animal-to-human has always been of concern. Therefore, several porcine viruses have been studied in regard to xenotransplantation. Denner et al. published seminal reviews on virological safety in xenotransplantation [70,71]. Particular attention has been directed to porcine endogenous retroviruses (PERV) [72], their susceptibility to retroviral inhibitors [73], and their genome-wide inactivation by genetic technology [74]. Morozov et al. showed that there was no PERV transmission during a clinical trial of pig islet xenotransplantation [75]. Similarly, no PERV transmission was shown by Choi et al. in pig-to-NHP corneal xenotransplantation [76]. Morozov et al. also published an extended characterization of porcine cytomegalovirus and other viruses in specially-bred pigs [77].

Porcine circoviruses (both type 1 and 2) were recently studied [78]. Whereas type 1 is not pathogenic in pigs, type 2 may induce severe disease. Although there is evidence that type 2 porcine circovirus does not infect (at least immunocompetent) humans, the recommendation is that pigs that will be sources of xenografts should be screened using sensitive methods to ensure virus elimination [78].

Ethics and regulatory aspects—As initial clinical trials draw closer, ethics [79], acceptance of xenotransplantation by hospital personnel [80], and by the general population with different cultural and religious backgrounds [81–83], are topics of importance. Schuurman has recently comprehensively reviewed regulatory aspects of xenotransplantation in Europe and in the United States in his seminal papers [84–85].

Genetic engineering—The introduction of CRISPR (clustered regularly interspaced short palindromic repeats) technology in xenotransplantation has increased the speed in which genetic manipulations can be achieved in pigs. In the early years, genetic engineering of pigs was performed by homologous recombination, which might take longer than 2 years from cell work to pregnancy [86]. Table 1 summarizes the timeline of the evolving genetic engineering techniques in xenotransplantation. Today, research groups can produce multiple gene knock-out or knock-in pigs using CRISPR technology [87–94], which can also be used to delete PERV expression [74,95]. Genetically-modified pigs using CRISPR technology have been used in several important studies relating to antibody binding [96–98] and coagulation dysfunction [99,100]. There are now more than 26 genetically-engineered pigs for xenotransplantation research (Table 2). Cooper recently published a review on carbohydrate antigen targets on pig cells [101]. Cowan et al also published a commentary on the importance of modifying the glycome in pigs for xenotransplantation [102].

Conclusion

Future of xenotransplantation

With our accumulated experience [2,103] and recent achievements [13,18,86] in xenotransplantation, the stage may now be set for the first-in-human exploration [11]. Although a small clinical trial of microencapsulated wild-type pig islet xenotransplantation

is currently underway [37,38], the future is set for well-controlled trials of genetically-engineered pig islet xenotransplantation. The xenotransplantation research community needs to decide (i) whether successful orthotopic heart transplantation in the pig-to-NHP model is required before proceeding to a clinical trial [104], and (ii) whether the preclinical threshold for a clinical renal xenotransplantation trial can be reduced [105].

The resurgence of xenotransplantation is now obvious [9,10,106], with prolonged survival of cellular and solid organ xenografts (Figure 2) associated with the administration of newer costimulation blockade agents [107,108] and access to genetically-engineered pigs. Our increasing knowledge of the pig genome [109] will almost certainly lead to further genetic manipulations. The future of xenotransplantation is vibrant.

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Abbreviations

GTKO	α 1,3-galactosyltransferase gene-knockout
mAb	monoclonal antibody
NHP	nonhuman primate
PERV	porcine endogenous retrovirus

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KEY POINTS

- In the last 24 months, prolonged survivals were achieved in heart, kidney, liver, islet, and corneal xenotransplantation with the use of genetically-engineered pigs and/or newer costimulation blockade agents.
- Thanks to the CRISPR technology, the production of multiple-gene pigs is easier and faster and more genetically-engineered pigs are now available for xenotransplantation research.
- The International Xenotransplantation Association has recently published the first update of the consensus statement on conditions for undertaking clinical trials of porcine islet products.
- First-in-man explorations (in some organs), and/or clinical (solid organ, islet, or tissue) xenotransplantation trials might start sooner than expected.

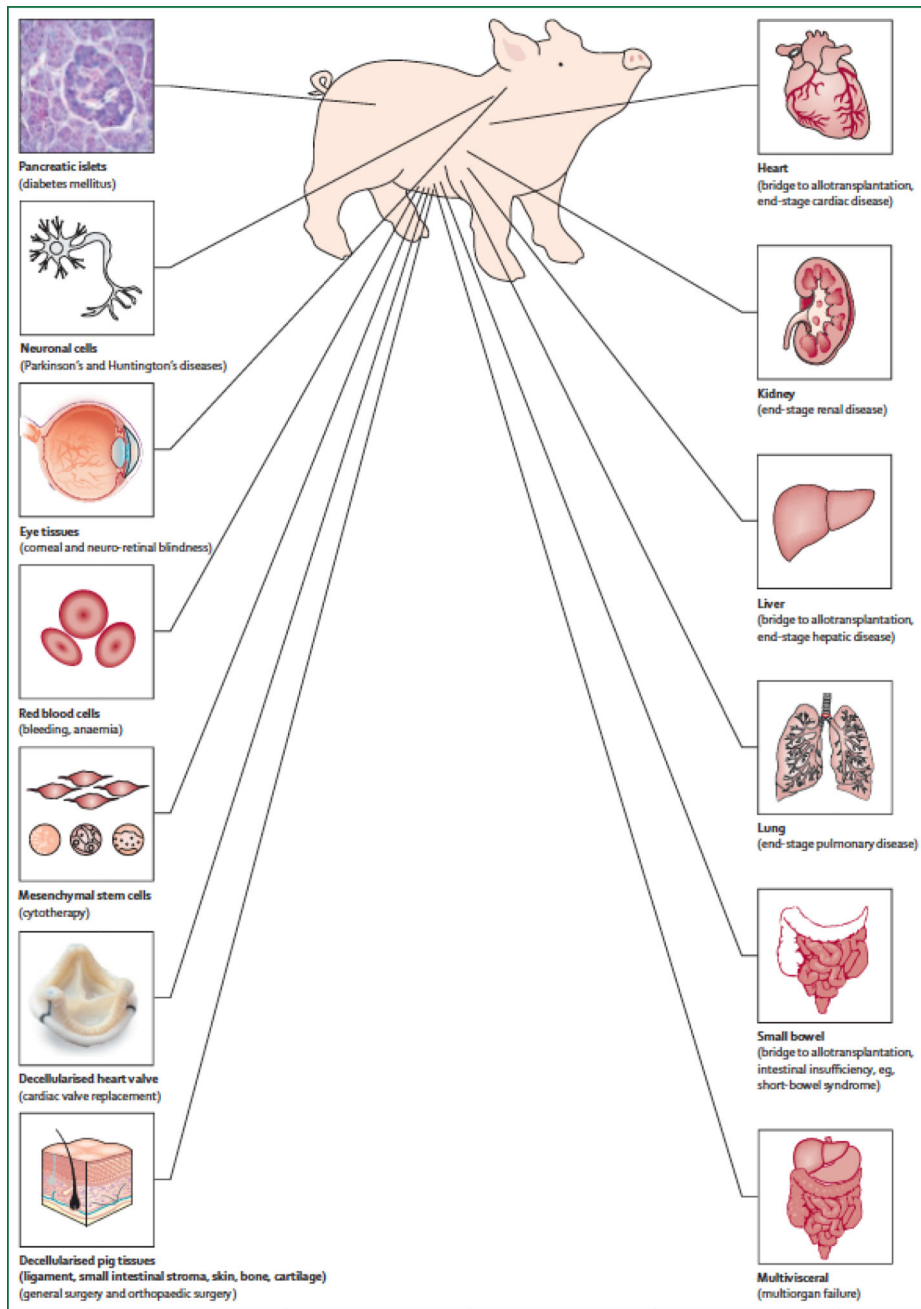


Figure 1. Disorders for which xenotransplantation is a potential therapy*

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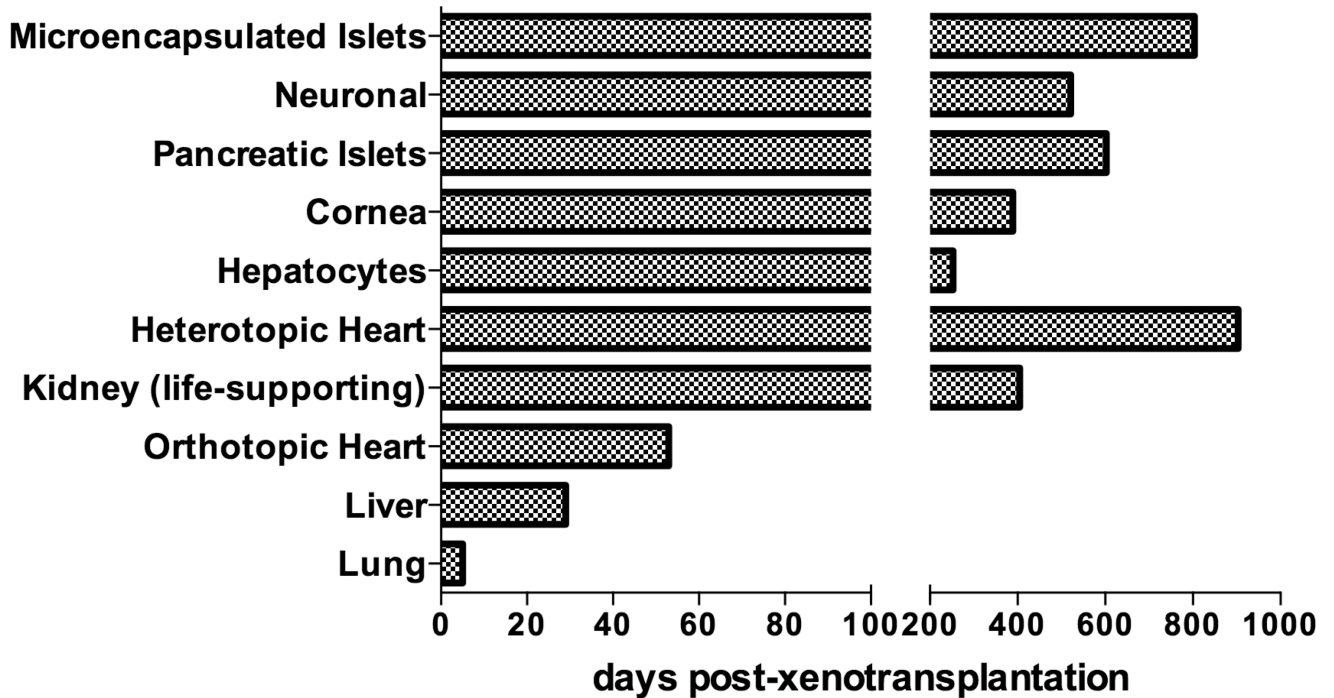


Figure 2. Longest survival times of organ and cell xenotransplantation from pigs to nonhuman primates

Microencapsulated pancreatic xeno-islets survived for 804 days with retransplantation, but 250 days without retransplantation. Neuronal xeno-cells survived for 521 days. Pancreatic xeno-islets survived for >603 days. Corneal (deep-lamellar) xenografts survived for >389 days. Xeno-hepatocytes survived for 243 days with retransplantation, but 80 days without retransplantation. Heterotopic xeno-heart survived for >900 days. Kidney xenograft (life-supporting) survived for 405 days. Orthotopic xeno-heart survived for 57 days. Liver xenograft survived for 29 days. Lung xenograft survived for 5 days.

Table 1

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

CRISPR/Cas9, clustered randomly interspaced short palindromic repeats and the associated protein 9. (Table adopted from Cooper et al.) [8]

Table 2

Selected genetically-modified pigs currently available for xenotransplantation research *

Complement regulation by human complement-regulatory gene expression
CD46 (membrane cofactor protein)
CD55 (decay-accelerating factor)
CD59 (protectin or membrane inhibitor of reactive lysis)
Gal or nonGal antigen 'masking' or deletion
Human H-transferase gene expression (expression of blood type O antigen)
Endo-beta-galactosidase C (reduction of Gal antigen expression)
α 1,3-galactosyltransferase gene-knockout (GTKO)
Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) gene-knockout (NeuGcKO)
β 4GalNT2 (β 1,4 N-acetylgalactosaminyltransferase) gene-knockout (β 4GalNT2KO)
Suppression of cellular immune response by gene expression or downregulation
CIITA-DN (MHC class II transactivator knockdown, resulting in swine leukocyte antigen class II knockdown)
Class I MHC-knockout (MHC-IKO)
HLA-E/human β 2-microglobulin (inhibits human natural killer cell cytotoxicity)
Human FAS ligand (CD95L)
Human GnT-III (N-acetylglucosaminyltransferase III) gene
Porcine CTLA4-Ig (Cytotoxic T-Lymphocyte Antigen 4 or CD152)
Human TRAIL (tumor necrosis factor-alpha-related apoptosis-inducing ligand)
Anticoagulation and anti-inflammatory gene expression or deletion
von Willebrand factor (vWF)-deficient (natural mutant)
Human tissue factor pathway inhibitor (TFPI)
Human thrombomodulin
Human endothelial protein C receptor (EPCR)
Human CD39 (ectonucleoside triphosphate diphosphohydrolase-1)
Anticoagulation, anti-inflammatory, and anti-apoptotic gene expression
Human A20 (tumor necrosis factor-alpha-induced protein 3)
Human heme oxygenase-1 (HO-1)
Human CD47 (species-specific interaction with SIRP- α inhibits phagocytosis)
Porcine asialoglycoprotein receptor 1 gene-knockout (ASGR1-KO) (decreases platelet phagocytosis)
Human signal regulatory protein α (SIRP α) (decreases platelet phagocytosis by 'self' recognition)
Prevention of porcine endogenous retrovirus (PERV) activation
PERV siRNA

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