# Xenotransplantation

# 24

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## Contents



**Abstract**

Transplantation is a major clinical means to replace damaged or failed human organs to improve or save a patient's life. The demand for organ transplantation has increased dramatically worldwide. However, the current dilemma is that the more that human organ transplants are performed, the less transplantable organs are available; the shortage of human organs is the major limiting factor for benefiting patients with organ dysfunction. Each year, thousands of patients are either removed from the waiting list due to death or become too sick for treatment. To solve the problem of organ shortages, several possible approaches have been considered and are under intensive investigations. These include artificial organs, tissue-engineered organs, and xenotransplantation (cross-species transplantation). While the former two hold hopes for the future, but with much higher social costs, xenotransplantation appears to have the potential to meet the current need of transplantation by providing adequate transplantable organs. However, several important issues, including immunological rejections, physiological incompatibilities, and safety, must be addressed before this approach can be a clinical reality. This review summarizes recent progress made in this field, the hurdles to be overcome, and the possible solutions for them.

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## Introduction

Allotransplantation (organs being transplanted from one person to another person) is currently the most effective surgical procedure for curing human end-stage organ failure, but a dramatically large number of patients who need a functioning organ to save their life do not have a chance to receive this therapy due to the shortage of human organs. The imbalance between organ demand and supply is a global problem despite the fact that significant efforts have been made to increase the donor pool. This discrepancy is becoming even larger as organ donations have actually gone down in recent years, while the number of patients being added to the waiting lists has increased dramatically. According to the United Network for Organ Sharing (UNOS), there are more than 110,000 people on the waiting list for an organ transplant in the USA alone, and at least 17,000 patients among them are waiting for liver donors (Ekser et al. 2011; Tisato and Cozzi 2012). In 2010, approximately 4,000 patients were removed from the waiting list due to death, organ deterioration preventing a surgical procedure, or some other unknown reasons (Ekser et al. 2011).

The fact that the demand for organs has far outpaced the supply opens a new multidisciplinary research field aimed at providing alternative solutions to fill this gap, with the aim of finding a practical solution to the shortage of human organs. Currently, there are three strategies available: the use of artificial organs, the development of tissue-engineered organs through regenerative medicine, and xenotransplantation, a process where animal organs are transplanted into humans for replacement of dysfunctional human organs.

The use of artificial organs seems to be a promising alternative to transplantation with regard to the shortage of human organs. However, this strategy is mainly applied to heart and kidney diseases. An artificial heart is a man-made device that is used to replace the heart or bridge the time until heart transplantation is possible. However, the cost is very high and the effects are limited due to biological incompatibilities. Hemodialysis is usually referred to as an artificial kidney that removes wastes from blood when the kidney is dysfunctional. Again, this treatment cannot permanently relieve the pain relating to the patient's renal failure, and the medical cost for this treatment is huge with a long-term burden on society. Thus, artificial organs deserve more investigation to improve the quality of patients' lives and to reduce costs.

Another alternative to transplantation is tissue-engineered organs. Regenerative medicine has opened a new and promising option by providing needed organs for transplantation. One example is the use of cardiac patches seeded with cardiac cells to make the artificial heart more biocompatible (Ott et al. 2008). The same method of repopulating the decellularized organ matrix with appropriate cell lines has been used to generate transplantable liver and lung in order to reconstitute the original structure and features of these organs (Uygun et al. 2010; Petersen et al. 2010). However, although these experimental results are encouraging, the clinical use of such bioartificial organs becoming a reality in today's hospitals remains a difficult challenge as many important issues such as organ quality and function are yet to be addressed.

To meet the current and future needs of organ transplantation, the most important issue is to find a supply of donor organs with sufficient quantity and transplantable quality. Xenotransplantation, as compared with artificial organs and tissue-engineered organs, has the potential to meet these requirements. Xenotransplantation utilizes nonhuman animals as donor organ sources, which makes this approach more practical as a stable supply of organs. Of course, there are still several barriers to be overcome before this therapeutic approach can be a reality. These barriers

and possible solutions are reviewed and discussed below.

## Brief History of Xenotransplantation

Although the use of organ transplantation between humans (allotransplantation) was reported more than 2,000 years ago, real xenotransplantation as we know it began in the seventeenth century, first with cells and tissues and later organs.

The first documented description of xenotransplantation is a transfusion of blood cells from a lamb to a 15-year-old boy to cure his severe fever by French doctors Jean-Baptiste Denis and Paul Emmerez on 15 June 1667 (Farr 1980). In the same year, several other xenotransfusions were also performed, with disappointing outcomes. The failure of xenotransfusion was later found to be due to incompatibility with heterologous blood. Inter-human transfusion was recommended, although at that time the need for blood type matching was not known.

Early xenotransplantation of tissues was attempted using animal bone, skin, tooth, and pancreas to humans. The first bone xenotransplantation was made by a Russian using a portion of a dog skull to repair a nobleman's skull. The surgery was claimed a success; unfortunately, under threat of being excommunicated by the Russian Church, the nobleman was forced to have the graft removed (Rodriguez Umana 1995). A relatively successful tissue xenotransplantation was achieved through the transplantation of testicles from animals to humans in order to rejuvenate men. Early attempts were made by using an extract of crushed testicles from dogs or guinea pigs. Serge Voronoff, a French Russian whose interest was in reversing aging, carried out a significant number of chimpanzee or baboon testicular transplants in human male recipients. Slices of testicles from chimpanzees or baboons were inserted into elderly men's testicles (Schultheiss et al. 1997). Several hundreds of these operations were performed without significant inflammatory or infectious complications. This can be explained by the fact that testicles are isolated glands that are relatively more resistant to human immune responses.

With the development of techniques to control bleeding after resection of a sick organ and to restore circulation after transplantation, solid-organ xenotransplantation became accessible in the twentieth century for repairing failed human organs. Two Frenchmen, Alexis Carrel and Mathieu Jaboulay, pioneered a key technique called anastomosis that can restore the vascularization of a transplanted organ, which enabled the first solid-organ transplantation to be carried out successfully. Alexis Carrel was thus awarded the Nobel Prize in Physiology or Medicine in 1912 "in recognition of his work on vascular suture and the transplantation of blood vessels and organs." Mathieu Jaboulay used this technique to carry out two kidney xenotransplantations from a pig and a goat to humans on 24 January 1906 and 9 April 1906, respectively. Although these transplanted kidneys had to be removed after 3 days due to thrombosis, the transplantation itself is reported as being the first true transplantation and, of course, the first true xenotransplantation.

The disappointing outcomes of early transplantation experiments were found to be caused by human immune responses to xenografts. With the availability of immunosuppressive drugs, modern xenotransplantation experiments started in the 1960s. Keith Reemtsma, an American surgeon, suggested that organs from nonhuman primates, due to their close evolutionary relationship to humans, may function in humans. On 13 January 1964, he carried out a kidney xenotransplantation from a chimpanzee to a 23-year-old schoolteacher (Reemtsma et al. 1964). Although the recipient died 9 months later, it marked the longest survival record ever for the xenotransplantation of an organ. Surprisingly, an autopsy was conducted, and the cause of death was found to be acute electrolyte imbalance. The 9-month survival without rejection of the chimpanzee kidney provided evidence of the feasibility of xenotransplantation. Thomas Starzl, one of the greatest pioneers in the field of kidney and liver transplantation, carried out three liver xenotransplantations between chimpanzees and children in Colorado in the 1960s without lasting success. In

the 1990s, with the addition of the immunosuppressive drug tacrolimus, he and his team in Pittsburgh achieved 26 and 70 days of survival using baboon livers in two adult patients with chronic liver failure (Starzl et al. 1993). One of the advantages of using baboon over human liver was found to be its resistance to infection by the hepatitis B virus. Thomas Starzl also participated in xenotransplantation of kidney from baboons to humans in the early 1960s.

The first heart xenotransplantation was carried out by James Hardy in 1964. He used a chimpanzee heart to replace a patient's dying heart. Unfortunately, the patient died a couple of hours after the transplantation. It was found that the baboon heart was not large enough to support the circulation of a human. However, this experiment helped a later attempt to save a 12-day-old infant girl named Baby Fae in 1984. Leonard Bailey carried out this cardiac xenotransplantation as Hardy did by using a baboon heart, which should be comparable to a human baby heart. Although the surgery itself was claimed a success, the baboon heart in Baby Fae underwent acute rejection, most likely due to blood incompatibility. Baby Fae died 20 days after the surgery (Bailey et al. 1985). This attempt became well known and drew both public and medical specialist attention to the necessity of organ donation.

The first islet xenotransplantation was conducted from pigs to human patients with diabetes mellitus by Carl Groth in 1993. Pig insulin has only one amino acid different from its human counterpart and was used for the treatment of diabetic patients for decades before recombinant human insulin was available. The patient did not obtain any clinical benefit from this xenotransplantation, but one encouraging point was that the pig islet was found to survive in some patients (Groth et al. 1994).

In summary, most of the early xenotransplantation attempts used nonhuman primate species as sources of the organ; a few attempts used other non-primate mammals such as dogs and goats, but the outcomes showed no significant success. Thus, the choice of suitable animals for xenotransplantation remains a challenge.

## Suitable Animals for Xenotransplantation

Since humans are primates, the obvious choice of donor animal for xenotransplantation would be another member of the primate family such as chimpanzees and baboons, because these animals have a close evolutionary relationship and physiological similarity to humans. However, nonhuman primates have been ruled out as human organ donors for practical and ethical reasons.

Primates, our closest cousins in the animal kingdom, are more likely than other animals to carry viruses capable of infecting humans. One example is HIV, which originates in chimpanzees. In addition, it is hard to breed enough primates to provide a sufficient number of organs to meet the increasing demand for donor organs. Furthermore, the close evolutionary relationship between primates and humans also poses ethical problems, as people are more reluctant to exploit animals that share many features with humans.

Pigs, on the other hand, seem to meet most of the requirements as a suitable animal for the donor source of organs (Fig. 1). First, pigs can be raised in a clean environment to reduce the risk of infection. The pig herd for transplantation can be housed under ideal conditions and be monitored at regular intervals for infectious agents, which almost guarantees that the donor animal would be free of all known pathogenic organisms that the average deceased human donor may have. Second, pigs are easy to breed and are already widely used in the food industry, so it is not hard to imagine that there would be an unlimited supply of donor organs, resolving the supply issue and ethical dilemma. Third, organs could be excised from a healthy pig under anesthesia, which avoids the problem of organ injury or no function that may be the case with a deceased human. Moreover, organs from a pig could be obtained whenever a patient needed it, helping improve survival. Fourth, pig organs have a similar size to human organs, so the transplants have the potential to match the human organs and function. Fifth, evidence obtained from animal models suggests that

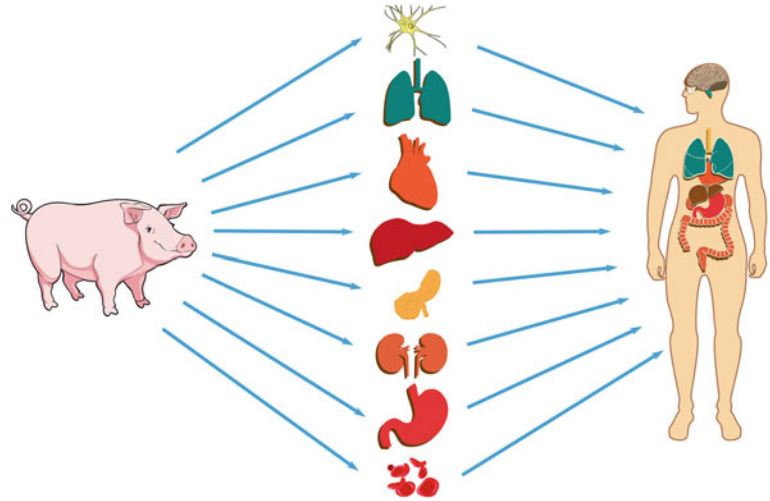

**Fig. 1** Pig-to-human xenotransplantation as a potential solution to the organ shortage for human end-stage organ diseases

most pig organs would work properly in human recipients. In fact, material from pigs has been routinely and safely used for medical purposes for decades, with the best known example being heart valves.

Despite the above advantages of using pigs as a donor organ source, there are also disadvantages. Pigs have a shorter life span than humans, so the organ transplant will have to be performed more than once since the pig organs have the potential to deteriorate at a much faster pace than an actual human organ. In addition, as pigs have a distant evolutionary relationship with humans, the human immune system would mount a very strong response to pig organs, leading to the organ transplants being rejected quickly, even when the immunosuppressive drugs that are supposed to prevent rejection of human transplants are used. It seems that drugs are simply not powerful enough to prevent rejection when pig organs are transplanted to humans.

To make pigs more suitable as the organ source for xenotransplantation, the problem of xenograft-induced immunological rejection needs to be solved. One solution for this is to take advantage of genetic engineering methods to modify pigs so that their organs will appear to be a part of the human body and will not be recognized as "non-self" when transplanted into humans. Genetically modified (GM) pigs have thus been produced for xenotransplantation research around the world. Although these GM pigs are still in the laboratory, progress made in the last decade suggests that the move to the clinic is not too far away, with cell xenotransplantation probably more feasible in the near future.

In order for GM pigs to serve well as an organ source for xenotransplantation, we need to know what the immunological challenges are and how to prevent them.

## Immunological Challenges

The most profound obstacle to a successful pig-to-primate xenotransplantation is the rejection of the organ transplant by a cascade of immune responses commonly known as hyperacute rejection, acute humoral xenograft rejection, cell-mediated immune rejection, and chronic rejection.

Hyperacute rejection is a rapid process of powerful immune responses that lead to the rejection of xenografts within a few minutes or hours after the surgery of xenotransplantation. It mainly destroys the vasculature of the xenografts, with subsequent interstitial hemorrhage, edema, and thrombosis of the small vessels. The process is initiated by binding of the host antibodies to the xenograft antigens that trigger the complement activation, resulting in endothelial damage,

inflammation, and necrosis of the xenografts and leading to transplant failure. It is widely accepted that the host xenograft antigen's active IgM or IgG initiates the hyperacute rejection process. Thus, hyperacute rejection represents the first barrier to clinical solid-organ xenotransplantation.

The main target antigen of the pig organs recognized by the primate immune system is α-1,3-galactose (α1,3Gal), an oligosaccharide that is produced by an enzyme called α-1,3-galactosyl transferase (α1,3GalT) (Galili et al. 1988). As most non-primate animals, including pigs, have this enzyme, the α1,3Gal is naturally expressed in endothelial cells and becomes the target of the host immune system. However, primates, including humans, actually lack α1,3GalT, so no α1,3Gal is expressed in the primates; however, primates have been exposed to α1,3Gal-similar epitopes derived from gut bacteria and have a high titer of anti-α1,3Gal antibodies in the body (spleen, lymph nodes, and bone marrow) already, which is why primates can mount an immune response to this antigen so quickly.

To reduce the frequency of hyperacute rejection, many approaches have been pursued to either remove the preexisting anti-α1,3Gal antibodies or control their effectors' functions by inhibiting complement cascade. Among them, the most well-known approach is to generate GM pigs by knocking out the gene that is responsible for α1,3GalT, so no α1,3Gal will be produced (Phelps et al. 2003).

Acute humoral xenograft rejection is a later immune response reaction that follows the hyperacute rejection. This delayed process is much more complex than hyperacute rejection and is mainly driven by interactions between the xenograft endothelial cells and host antibodies, leading to loss of the xenograft within days or weeks of transplantation (Crikis et al. 2006). An inflammatory infiltrate of mostly macrophages and natural killer (NK) cells, intravascular thrombosis, and fibrin deposition are involved in the rejection. The detailed mechanism of acute humoral xenograft rejection is currently not completely understood. Recent evidence demonstrates that anti-non-α1,3Gal antibodies directed against both carbohydrates and proteins play a critical role in the acute humoral xenograft rejection (Breimer 2011). Due to its multifactorial features, strategies to overcome acute humoral xenograft rejection require the combination of different approaches to generate synergistic effects. Thus, the use of immunosuppressive drugs along with GM pigs as organ donor should result in improved survival.

Cell-mediated immune rejection, which is different from hyperacute rejection and acute humoral xenograft rejection which are both xenograft antigen dependent, is mainly mediated by T cells. T cells play a role in the induction of anti-xenograft antibodies, but their role in direct involvement of rejection has not completely been clarified. However, it is clear that T cells can recognize xenograft antigens presented by major histocompatibility complex (MHC) molecules in direct and indirect pathways. Direct xenorecognition is via CD4 T cells, in which xenograft antigens are presented directly by MHC class II molecules on antigen-presenting cells from the xenograft; indirect xenorecognition is via CD8 T cells, in which xenograft antigens are first phagocytosed by the host antigen-presenting cells and then presented by MHC class I molecules on the host antigen-presenting cells. The indirect xenorecognition is thus expected to be stronger than its allogeneic counterpart, since the large number of antigens from xenografts is more readily recognized as foreign and elicits stronger immune responses.

Evidence obtained from pig-to-primate xenotransplantation experiments has demonstrated that CD8 T cells, monocytes/macrophages, B cells, and some NK cells are the predominant cells detected in the xenograft; CD4 T cells are only described in a limited number of cases (Ashton-Chess et al. 2003; Hisashi et al. 2008). It is generally believed that the cell-mediated immune rejection can be controlled by using the current immunosuppressive regimens.

Chronic rejection usually occurs in the xenografts after the initial acute antibody-based and cellular rejections. It is relatively slow and progressive. Knowledge in this area is poor as most xenografts rarely survive long enough for it to be studied. However, it is known that fibrosis of the xenograft vessel wall is the major cause of chronic

rejection. Arteriosclerosis occurs as a result of the combinatorial effects of T cells, macrophages, cytokines, and healing, leading to the hardening and narrowing of the vessels within the xenograft. Chronic rejection is believed to be more aggressive in xenografts than in allografts. As chronic rejection causes pathological changes in the organ, the xenograft will have to be replaced after several years.

In addition to immunological challenges, dysregulated coagulation remains another barrier to successful xenotransplantation, particularly pig-to-primate xenotransplantation. There is a difference in the coagulation dysfunction in different organ xenotransplants. Lung xenotransplant is the most rapidly damaged organ xenograft by coagulation dysfunction and rarely lasts more than a day in nonhuman primates. Kidney xenotransplants have a higher degree of coagulation dysfunction than that of the heart, while liver xenografts are more easily affected by thrombocytopenia. Coagulation dysfunction is one of the molecular incompatibilities in pig-to-primate xenotransplantation and represents the most problematic issue. Strategies to overcome coagulation dysfunction in xenotransplantation will include the combination of GM pigs to reduce the effects of clotting cascade in the xenograft donor and the systemic treatment of the recipient to aid ready acceptance of the xenograft.

Among these immunological rejections, hyperacute rejection, acute humoral xenograft rejection, and acute cellular rejection are generally controllable when an adequate immunosuppressive regimen is given, but chronic rejection in the form of xenograft vasculopathy has been documented in cardiac transplants that survive for several months. Xenograft vasculopathy could be increasingly delayed as the immunological challenges of xenotransplantation are overcome. One of the practical approaches is to use GM pigs.

## Genetically Modified Pigs

The ultimate goal of generating GM pigs is to reduce or eliminate immunological responses of the donor organs and make the xenografts more acceptable by the recipient.

As described above, the pig is the preferred species as an organ donor for xenotransplantation due to its comparable organ size, rapid growth rate, large litters, and a more manageable ethical profile in comparison with other species. However, it is well documented that the existence of xenoreactive natural antibodies (XNA) in the recipient can recognize the α1,3Gal epitope and triggers a hyperacute rejection, a very rapid immune response that results in irreversible xenograft damage and loss within minutes to hours after the transplantation. Nevertheless, selection of donor organs that do not express α1,3Gal would be a better strategy for xenotransplantation. Thanks to the development of modern molecular biology, this can be achieved by generating GM pigs that lack the expression of α1,3Gal through genetic engineering.

The production of α1,3Gal is catalyzed by an enzyme α1,3GalT, which is encoded by the gene *GGTA1*. This gene is expressed in fetal fibroblasts and the α1,3Gal is readily detectable on the cell surface. To make the cell lack α1,3Gal expression and, hence, the epitopes to XNA, inactivation of the *GGTA1* gene is needed. α1,3GalT, a 371 amino acid protein, is encoded by 4–9 exons of *GGTA1*. The gene's endogenous translation start codon ATG is in exon 4, while the major portion of the protein including the catalytic domain is in exon 9. Thus, both exons have become the targets for the functional inactivation of *GGTA1* in transgenic pigs. The first transgenic pig ($\alpha1,3GalT^{-/-}$) lacking the α1,3GalT was generated using a somatic cell nuclear transfer (SCNT) method in 2002 (Lai et al. 2002). Grafts from $\alpha1,3GalT^{-/-}$ pigs can generally achieve an extended graft survival time and allow the use of reduced levels of the immunosuppressive therapy. The associated rejection is not caused by the classical acute humoral xenograft rejection, but predominantly by the development of a thrombotic microangiopathy that can ultimately result in coagulopathy. Moreover, the level of antibodies against non-α1,3Gal epitopes has been found to be elevated at the time of rejection, indicating the importance of antibodies against non-α1,3Gal antigens in xenotransplantation. It appears that the anti-non-α1,3Gal antibodies represent a

major critical barrier for the successful clinical application of xenografts. Apparently, transgenic pigs will be used with a combination of not only the functional inactivation of *GGTA1* but also other genetic factors for the further reduction of the rejection process in xenotransplantation.

Another xenoantigen, *N*-glycolylneuraminic acid (Neu5Gc), has been identified recently (Song et al. 2010). Humans do not produce Neu5Gc as humans have a DNA mutation that can cause the functional inactivation of cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH), the enzyme responsible for Neu5Gc production, but CMAH is synthesized in pigs and other mammals, including nonhuman primates. It is therefore expected that deleting both the *GGTA1* and *CMAH* genes to create double-knockout (KO) pigs may further reduce the xenoantigenicity of pig organs in humans and thus further reduce the severity of humoral rejection as seen in the *GGTA1* single KO mentioned above.

To generate double-KO pigs, a zinc-finger nuclease (ZFN) technology has been used with sequential disruption of the *GGTA1* and *CMAH* genes in cultured cells followed by SCNT to yield viable GM pigs. Compared with the standard technique based on homologous recombination, ZFN technology is more efficient and is able to knock out more than one gene at a time, which should accelerate the development of GM donor pigs to evaluate for clinical xenotransplantation.

Transgenic pigs expressing human complement regulatory proteins have shown great promise in reducing the rejection of pig organs following transplantation into nonhuman primates. Various transgenic pigs expressing a single gene product of CD46, CD55, and CD59 have been produced. Double- and triple-transgenic pigs are also established. The gene expression levels vary in different transgenic pigs. In order to control the expression of transgenic genes, the tetracycline-regulated Tet-On and Tet-Off system is used. This system allows the transgene expression in a controllable way by exogenous stimuli.

Transgenic pigs expressing the enzyme α-1,2-FT can reduce expression of the major pig xenoantigen α-1,3-Gal by enzymatic competition between α-1,3-GalT and other terminal glycosyltransferases for the common acceptor substrate, resulting in a reduction in xenoreactive human natural antibody binding and complement activation. Therefore, pigs transgenic for the human *α-1,2-FT* gene can be comparable with their α-1,3-Gal-deficient counterparts.

It is expected that donor organs from GM pigs with a combination of some specific gene KOs and transgenes might even further reduce the immunological rejection rates for clinical xenotransplantation. Indeed, such multi-transgenic pigs with α-1,3-Gal KO and other transgenes such as CD55 or/and α-1,2-FT have been produced.

With regard to cell-mediated immune rejection, expression of human tumor necrosis factor (TNF)-α-related apoptosis-inducing ligand (TRAIL) in transgenic pigs has been used as a strategy to control post-hyperacute rejection mechanisms mediated by cellular components of the immune system. Another strategy is to inhibit the activity of NK cells by expressing HLA transgenes, mainly HLA-E. In addition to T cells and NK cells, macrophages play an essential role in both innate and adaptive immune responses. Signaling regulatory protein (SIRP)-α is expressed on macrophages that can recognize CD47, a cell surface protein expressed ubiquitously on most cells as a marker of "self." "Self" cells thus use this SIRP-α–CD47 interaction to avoid being phagocytosed by macrophages (Ide et al. 2007). Hence, transgenic pigs expressing human CD47 are likely to contribute to the xenotransplantation by inducing immune tolerance in xenografts.

With the availability of a plethora of GM pigs, it is expected that various immunological rejections can be reduced and xenotransplantation will likely become a clinical reality in the not-too-far future, at least as a bridge to allotransplantation.

## Cellular Xenotransplantation

The xenotransplantation of non-vascularized tissue, such as pancreatic islets, is not believed to be subject to classical hyperacute rejection. In

general, cellular xenotransplantation made in pig-to-nonhuman primate experiments has achieved much more encouraging results than in solid organs, and it appears much closer to clinical application than solid-organ xenotransplantation.

Currently, there are more than one million people in the USA with type 1 diabetes. Although islet allotransplantation has improved significantly in recent years, the need for islets from two deceased human donor pancreases for a single allotransplantation has greatly limited this procedure due to the very small number of suitable donors. In the past decade, it is estimated that fewer than 1,000 such procedures were carried out in the Western countries. It is therefore reasonable to consider islet xenotransplantation in order to meet the growing demand.

The first islet xenotransplantation was carried out in 1994 (Groth et al. 1994). In this attempt, pig islets were transplanted into ten type 1 diabetic patients who received kidney and islet double transplantation; four patients excreted detectable pig C-peptide in urine for 200–400 days, and there was insulin-positive staining in one patient. In several independent pig-to-nonhuman primate experiments that followed, a period of more than 6 months of normoglycemia and graft survival could be achieved. It has been found that an immunosuppressive regimen is needed to prevent immunological rejection when free pig islets are transplanted, while encapsulated islets can be transplanted in the absence of such immunosuppressive treatment. The latest approach is being tested in New Zealand with encapsulated pig islets transplanted into the peritoneal cavity to avoid the use of immunosuppressive treatment.

Despite the encouraging progress made in the field, successful clinical application of islet xenotransplantation is currently hampered by a number of barriers. These include the immediate loss of islets in an instant blood-mediated inflammatory reaction (IBMIR), T cell-mediated rejection, and the use of excessive immunosuppression.

IBMIR occurs with kinetics similar to hyperacute rejection in solid-organ xenotransplantation but with no antibody deposition on the graft, and the mechanisms behind it are poorly understood. Nevertheless, xenotransplantation of pig islets into the portal vein, the same site as used in allotransplantation, is associated with early graft loss, and IBMIR may account for the early loss of grafted islets and the consequent large tissue volume required to achieve a functional islet mass following transplantation via this route.

Xenotransplanted pig islets that survive IBMIR may subsequently encounter strong cell-mediated rejection phenomena. Studies have demonstrated that pig islets, following transplantation to nonhuman primates in the absence of immunosuppression, are predominantly destroyed via the infiltration of immune cells, largely T cells, at the graft site, leading to localized graft destruction. However, with an immunosuppressive regimen containing various antibodies and drugs, pig islets xenotransplanted into nonhuman primates can achieve a survival of more than 180 days (Hering et al. 2006). Apparently, the use of novel immunosuppressive strategies designed to abrogate cell-mediated rejection, such as using T cell co-stimulatory pathway blocker cytotoxic T lymphocyte antigen 4 (CTLA4)-Ig, is likely to produce extended islet survival and a better outcome of the cellular xenotransplantation.

Encapsulation of pig islets as mentioned above is another approach to prevent cell-mediated rejection and has been demonstrated to be effective in nonhuman primates. Of course, transgenic pigs represent the most promising solution to the immune responses, with an aim of providing resistance to the effects elicited by IBMIR and cell-mediated rejection. Various transgenic pigs expressing CTLA4-Ig, hCD46, and TRAIL have been produced. Islets from these pigs are thus expected to have reduced immunological rejections. Overall, the combined use of the above immunosuppression strategies forms the basis for future clinical application of pig islet xenotransplantation.

Other cellular xenotransplantations have been attempted using pig red blood cells, pig neuronal cells, pig corneas, pig mesenchymal stem cells, and pig hepatocytes. Again, the progress made in cellular xenotransplantation is much more encouraging than that in solid-organ transplantation and holds the promise of not-too-far away future

clinical application to meet the ever-growing demand and benefit the patients.

## Pig Liver Xenotransplantation

Transplantation is currently the most efficient way to treat liver failure, but the wailing list is extremely long as the availability of transplantable donor livers is very limited worldwide. One of the potential solutions to the liver shortage is to take advantage of xenografts from pigs for liver xenotransplantation or at least as a bridge to allotransplantation.

The first pig liver xenotransplantation was carried out in 1968 by Calne's team. They performed seven pig-to-baboon liver transplantations: four died within a day from hemorrhage, and the other three lived no more than 4 days, even with the addition of human fibrinogen which stops xenograft bleeding (Calne et al. 1968). After this attempt, several other groups tried pig liver xenotransplantation in other nonhuman primates such as rhesus monkeys and chimpanzees. Different immunosuppressive regimens were attempted with an aim of reducing the host immunological rejection and prolonging the survival of the transplanted pig liver. Unfortunately, these efforts did not significantly help extend the pig liver survival.

The emergence of GM pigs makes it possible to genetically modify immune responses of the pig and enable its organs to be more compatible with that of human or nonhuman primates. Pigs transgenic for the human complement regulatory protein CD55 were first used in liver xenotransplantation. This experiment achieved 4 and 8 days survival in two baboons. It seems progress has been made by using GM pigs. However, a later attempt using CD55, CD59, and H-transferase triple-transgenic pigs did not obtain a better result. In addition, the use of $\alpha$1,3GalT$^{-/-}$ pigs as the liver donor in baboon xenotransplantation by the Pittsburgh group did not improve survival (Ekser et al. 2010). It was found that thrombocytopenia, which developed within 1 h after reperfusion of the xenograft, caused complications in the recipients, preventing prolonged survival.

The mechanisms underlying the rapid thrombocytopenia are still not clear, but evidence has shown that the main reason is that pig liver induced recipient platelet phagocytosis which leads to reduced platelet production. Several factors have been identified as causing this phenomenon to occur. These include the interaction between von Willebrand factor and endothelial cells, the interaction between von Willebrand factor and glycoprotein (GP) Ib, and the interaction between CD47 and SIRP-$\alpha$ (Burlak et al. 2010). Further investigations into the factors associated with the development of the rapid thrombocytopenia after pig liver xenotransplantation are still under way.

Nevertheless, the only clinical pig liver xenotransplantation was performed in an attempt to bridge a 26-year-old patient with fulminant hepatic failure to allotransplantation (Makowka et al. 1995). In this case, even though the majority of the circulating natural anti-pig antibodies were removed from the patient before the pig liver was transplanted, the xenograft failed to survive as it was damaged by a rapid return of the antibodies and the associated immunological rejection, suggesting that GM pigs with reduced immunological rejection may provide some benefits.

Taken together, it is now clear that the rapid development of thrombocytopenia remains the major obstacle in pig liver xenotransplantation. Strategies of preventing the development of thrombocytopenia are thus absolutely necessary before a clinical transplantation using a pig liver could be successful.

## Physiology and Safety

Although pigs are considered to be the most appropriate organ source for xenotransplantation due to their comparable organ size, rapid growth rate, a more manageable ethical profile, and the chance for genetic modification, physiological incompatibilities, mainly the molecular difference in the complement and coagulation system, have been detected between pigs and primates.

With regard to coagulation, it has been found that the pig von Willebrand factor is able to bind to

human platelet GPIb receptors with high affinity, leading to increased procoagulant activity. In addition, despite the fact that pig thrombomodulin has been shown to bind to human thrombin, the resulting hybrid complex is a weak activator of human protein C. Therefore, there is not sufficient production of activated protein C, causing an increased level of thrombin and eventually leading to the initiation of clotting. Moreover, as the pig tissue factor pathway inhibitor (TFPI) is unable to neutralize human factor Xa, pig TFPI could not inhibit the activation process of human prothrombin to thrombin, resulting in the accumulation of thrombin and thus clotting.

Approaches to reducing the physiological incompatibilities and prolonging the survival of xenografted organs have been proposed. These include the use of platelet fibrinogen receptor antagonist (GPIIb/IIIa), P-selectin inhibitor, soluble adenosine triphosphate (ATP) diphosphohydrolase, and, of course, GM pigs with several specific gene targets for either KO (e.g., procoagulant proteins) or transgenic overexpression (TFPI, thrombomodulin, CD39, etc.). Overall, thrombocytopenia appears to be a crucial barrier in xenotransplantation regarding physiological incompatibilities. The survival of xenografts can be extended if this problem can be overcome.

Besides physiological incompatibilities and thus the long-term xenograft survival, another important consideration in xenotransplantation with regard to a possible clinical application is safety. Viruses, such as cytomegalovirus and Epstein–Barr virus, are frequently transferred from an allograft to the recipient, and the same is true for other donor-derived microorganisms that can cause infectious complications in recipients. Pig organs or cells would not carry such microorganisms as the organ-source herd would be monitored at regular intervals to ensure that organs and cells are free of such infectious agents. However, endogenous retrovirus, which is integrated in the genome of pig cells, will be inevitably carried with the pig xenografts, even if the pigs are housed in a "clean" environment (Patience et al. 1997). Fortunately, humans who are exposed to pig tissues and cells have never been identified as having active replication of the pig endogenous retrovirus, and transfer of this virus is thus not currently considered a serious risk.

It has been pointed out that strategies aimed at reducing xenograft immunological rejections may have the potential to increase the risk of microorganism infections. These include the use of immunosuppressive regimens that decrease the antivirus immune responses, the application of an $\alpha$1,3GalT$^{-/-}$ pig which lacks $\alpha$1,3Gal expression and thus is less sensitive to complement-mediated inactivation, and the transgenic pig-expressing human complement regulators. Nevertheless, several approaches have been taken to relieve the above concerns. These include the use of currently available virus-sensitive antivirus agents, generation of GM pigs that inactivate the endogenous retrovirus replication, and the application of small interfering RNA (siRNA) to block the endogenous retrovirus transcription. Furthermore, novel techniques such as microarray-based technology and whole-genome DNA sequencing allow rapid identification of potential infectious agents and help ensure that infectious agent-free organs are used in xenotransplantation (Wang et al. 2002).

It is therefore anticipated that a high-safety profile of xenotransplantation will be ultimately achieved with the combined use of the above strategies.

## Conclusion

Xenotransplantation is a multidisciplinary science involving cell biology, immunology, developmental biology, regenerative medicine, and genetic engineering. Although remarkable progress has been made in the past decade, the clinical application of xenotransplantation to replace human organs is still not a reality in today's hospitals as several major obstacles remain. These are immunological rejections, the development of rapid thrombocytopenia, molecular incompatibilities, physiological discrepancies, microbiological safety issues, and ethical issues. However, results obtained from preclinical transplantation of pig cells – such as islets, neuronal cells,

hepatocytes, or corneas – are much more encouraging than those from solid-organ transplantation, with a general survival longer than 1 year in all cases. In addition, the risk of transferring an infectious microorganism to the recipient is much smaller in cellular xenotransplantation.

The development of genetic engineering technology has provided a powerful tool for genetic modifications of organ donor pigs, with the aim of overcoming the hurdles that are associated with pig-to-primate xenotransplantation. Thus, various GM pigs have been produced to try and achieve elimination of immunological rejections. Such GM pigs, when used in combination with other novel immunosuppressive drugs, provide hope for enabling safe and long-term xenograft survival. Because of the much easier protection from the recipient's immune system for cells than organs, it is expected that clinical xenotransplantation of pig cells will be a reality in the not-too-distant future.

## Cross-References


- ▶ Cell Therapy for Liver Failure: A New Horizon
- ▶ Downstaging Hepatocellular Carcinoma for Liver Transplantation
- ▶ History of Liver and Other Splanchnic Organ Transplantation
- ▶ Immunology of Liver Transplantation
- ▶ Systemic Chemotherapy in Orthotopic Liver Transplantation


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