Transplanting organs from pigs to humans

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Abstract

The success of organ transplantation is limited by the complications of immunosuppression, by chronic rejection and by the insufficient organ supply and thousands of patients die every year while waiting for a transplant. With recent progress in xenotransplantation permitting porcine organ graft survival of months or even years in non-human primates, there is renewed interest in its potential to alleviate the organ shortage. Many of these advances are the result of our heightened capacity to modify pigs genetically, particularly with the development of CRISPR/Cas9-based gene editing methodologies. While this approach allows the engineering of pig organs that are less prone to rejection, the clinical application of xenotransplantation will require the ability to avoid the ravages of a multi-faceted attack on the immune system, while preserving the capacity to protect both the recipient and the graft from infectious microorganisms. In this review, we will discuss the potential and limitations of these modifications and how the engineering of the graft can be leveraged to alter the host immune response so that all types of immune attack are avoided.

One sentence summary.

Are genetically engineered pigs the answer to the organ shortage?

Introduction

In 2017 there were 114,000 patients in the United States on waiting lists for organ transplantation (http://optn.transplant.hrsa.gov). On average, 20 of these patients die every day because of failure to obtain a suitable organ. Disturbingly, the disparity between the number of patients on transplant waiting lists versus the number of available organs continues to increase (Figure 1).

Three possible approaches to solving the organ shortage have emerged: 1) Increasing deceased donor donation. Since only a very small fraction of deaths result in transplantable organs (i.e. brain deaths and donations after cardiac death, which account for less than 1% of

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mortalities), the potential success of this approach is limited. Even in countries with "presumed consent" laws for removal of organs from brain-dead individuals who have not specified their wishes regarding organ donation, the disparity between organ need and availability continues to increase[1]; 2) "Bioengineering" of transplantable organs. This approach often uses stem or progenitor cells to populate organ scaffolds produced either by 3-D printing or by decellularization of human or animal organs. This approach has not yet been successful in large animal models and is hampered by significant hurdles that will need to be overcome to produce an organ that can actually function as a replacement organ. Additionally, unless stem cells are obtained from the intended recipient, there will be antigenic differences requiring immunosuppression and the scaffolds themselves may be immunogenic; 3) The use of organs from animals, or xenotransplantation, the subject of this review. It is conceivable to have an essentially limitless supply of tissues and organs of lower mammalian species such as pigs. If successful, therefore, xenotransplantation could provide a near-term solution for the human organ shortage.

The field of xenotransplantation was jumpstarted by a series of chimpanzee to human renal transplants by Reemtsma et al. in the early 1960s[2]. One patient survived for nine months following transplantation of a chimpanzee kidney. However, it was clear even then that chimpanzees, as an endangered species, could not meet the growing need for organ transplants. A flurry of attempts to utilize much more readily available baboons as sources of kidneys and hearts met with dismal failure[3,4]. The use of lower mammalian species was not attempted because such transplants into nonhuman primates were uniformly rejected in minutes to hours, due to the high level of natural antibodies (NAbs) present across such discordant species barriers[5].

For the next 20 years, clinical needs were largely met by deceased donation. The enormous success of allogeneic transplantation following the introduction of much-improved immunosuppressive drugs in the late 1980s paradoxically led to a resurgence of interest in xenotransplantation. By this time, the use of chimpanzees as organ sources had been outlawed and the use of other nonhuman primates was plagued by problems of size, availability, viral pathogens and ethics. Nevertheless, several attempts to utilize baboon organs in humans were made with patient survivals ranging from just 20 to 70 days[6]. Collectively, these issues led the field of transplantation to seek another, less problematic xenograft source animal.

By the mid-1990s, most investigators agreed that the pig would be the most appropriate xenograft source animal for a number of reasons, including size, availability, breeding characteristics and physiologic similarities to human beings. The main impediment to use of the pig was the presence of a high level of NAbs to porcine antigens in old world primates and humans that leads to what has been termed hyperacute rejection (HAR) of xenotransplants[5] (Figure 2). However, with advances in genome editing and our understanding of immune rejection of porcine organs, pig to human organ transplantation might be a realistic option in the near future.

Successful xenotransplantation would have many advantages over allogeneic transplantation, including essentially unlimited availability and far better quality control for both function

and absence of infectious organisms in transplanted organs. At the moment elective transplants are only possible for living donation, but if xenotransplanation become a reality, transplants would become elective operations that could be offered before severe complications of organ failure arise. In this review, we summarize what is now known about the immunologic barriers to xenotransplantation and describe progress to date in overcoming these barriers, especially through genetic engineering of pigs.

1. Immune barriers to Xenotransplantation

Transplants from pigs to primates are subject to vigorous immunologic rejection involving both innate and adaptive immune responses. The innate barriers include monocytes and macrophages (reviewed in [7]), natural killer (NK) cells (reviewed in [8]); and complement and coagulation pathways (reviewed in [8]). In addition, natural antibodies (Nab), which are often classified as innate immune components, as they arise without exposure to their known ligands, present another formidable barrier. The most important of these Nabs recognize α-galactose-1,3-galactose (Gal). Gal is a carbohydrate moiety displayed on numerous cell surface glycoproteins and glycolipids of pigs and most other mammalian species, excepting humans and Old World monkeys, due to a frame shift mutation in α−1,3-galactosyltransferase (GalT) in our ancestors[9]. The high levels of NAbs to Gal in human serum likely reflect exposure to it on numerous microorganisms. Anti-Gal NAbs constitute about 1–4% of circulating human immunoglobulin (Ig)[10,11] and include both IgM and IgG. As is discussed below, anti-Gal NAbs were a major early obstacle to xenotransplantation. Antibodies to Gal are responsible for HAR, which occurs within hours following transplant. Attempts to absorb or neutralize these antibodies led to delayed rejection, but without long-term success[12] When HAR was successfully prevented by the removal of the Gal epitope from pigs using nuclear transfer technology in the early 2000's[13–15], it became evident that natural antibodies against other pig specificities[16] less prominent than Gal, can mediate a more delayed type of vascular xenograft rejection, termed "acute vascular rejection" or "delayed xenograft rejection (DXR)", which occurs over days to weeks, rather than minutes to hours, as is the case with HAR. Non-Gal NAbs showed binding to multiple cell surface antigens, and many recognize an SD[a] red blood cell antigen-like terminal carbohydrate produced by a β−1,4 N-acetylgalactosaminyl transferase 2 (B4GalNT2)[17] or a N-glycolylneuraminic acid (NeuGc) ligand that is expressed by glycoproteins and gangliosides. NeuGc is highly expressed on endothelial cells of all mammals except humans[18] and anti-NeuGc NAbs are present in the majority of human sera[19], reflecting a point mutation in the human gene for the CMP-NeuAc hydroxylase (CMAH) that produces the NeuGc epitope[20].

B and T cells are the adaptive immune barriers in xenotransplantation.

B cell responses can be dampened by adequate T cell immunosuppression or tolerance induction, although some responses may be T cell-independent [21]. T cells can directly attack the graft and can also promote B and NK cell responses. T cell responses, including cytotoxicity, cytokine production, and recruitment and activation of innate cytotoxic cells, are not fully overcome by immunosuppressive therapy in pig-to-primate xenotransplantation[22,23]. While early studies of mouse anti-pig immune responses revealed very weak direct T cell xenoreactivity, this was due largely to the failure of multiple

accessory receptor/ligand interactions between species[24] and did not necessarily implicate poor TCR-ligand interactions in xenogeneic combinations. Indeed, the ability of highly disparate porcine MHC molecules to effectively mediate positive selection of murine T cells[25] (reviewed in [26]) and to select a diverse human T cell repertoire[27] suggests that these xenogeneic interactions are highly effective. Direct activation of human CD8+ and CD4+ T cells by porcine SLA class I and class II molecules, respectively, has been described, and are similar in magnitude to direct alloresponses[28], reflecting in part the demonstrated efficacy of a number of adhesive and costimulatory interactions between human T cells and porcine ligands. The inability of pig cytokine receptors to respond to human IFN-γ[29] may limit the upregulation of MHC and costimulatory molecules by pig cells in the presence of human T cells.

Indirect recognition, in which recipient APCs process and present donor antigens on recipient MHC molecules, is also potent in the human anti-pig direction[28,30] and is stronger than the indirect response to alloantigens[28], in keeping with the prediction of a greater number of protein, and hence peptide, polymorphisms between than within a species. Indirect activation of IFN-γ-producing recipient T cells was implicated in the rejection of porcine islet xenografts in monkeys, which was not prevented by a strong immunosuppressive regimen that prevented allograft rejection, highlighting the potency of xenogeneic responses[31]. Indirect memory T cell responses to porcine antigens in non-human primates (NHPs) have been reported to be more resistant to immunosuppression than direct xenoresponses[32]. Since induced antibody responses to transplanted organs largely reflect presentation of donor antigens by recipient B cells to peptide-reactive T helper cells, IgG responses in NHPs receiving porcine heart or kidney transplants[16,33,34] suggest a potent indirect T cell xenoresponse.

2.   Role of Genetic Engineering in Overcoming Immune Barriers to Xenotransplantation

Given the great evolutionary distance between pigs and primates (estimated to be 70 to 80 million years), it is remarkable how similar these two species have remained, both physiologically and immunologically[37]. Nevertheless, genetic disparities that occurred during evolution resulted in incompatibilities that need to be overcome in order for xenotransplantation to be successful. Fortunately, largely due to their favorable breeding characteristics, pigs are particularly amenable to engineering of their genome. Enormous progress has been made over the past two decades toward overcoming relevant inter-species incompatibilities through such genetic engineering (GE). Genetic modifications that have been introduced into pigs are summarized in Figure 3.

A.   Hyperacute Rejection:

As mentioned above, NAbs in primates reactive with porcine cell surface antigens led to HAR within minutes to hours following the completion of vascular anastomoses. Since the initiation of HAR required complement activation, the first genetic modifications (GMs) of pigs involved introduction of transgenic human complement regulatory proteins (CRPs) into the pig genome. CRPs are expressed on normal endothelial cells to prevent collateral

damage to host cells and modulate inflammation when complement is activated in the destruction of microorganisms. To enhance these protective mechanisms in pigs whose organs would be exposed to human antibodies and complement, DNA constructs for three such human CRPs (CD55, CD46 and CD59) were introduced into porcine fertilized ova via pronuclear injection[38]. Expression of human CRPs in pig endothelial cells mitigated, but did not eliminate HAR, prolonging organ graft survival for days to weeks when NAbs were removed by absorption procedures[39]. Unfortunately, NAbs returned after the transplants, often at even higher titers, causing DXR[12].

In the early 2000's, following the demonstration of cloning through nuclear transfer in mammalian species (i.e. the Dolly experiment[40]), several laboratories knocked out the GalT gene by homologous recombination in cultured fetal fibroblasts, followed by nuclear transfer into enucleated ova and implantation of the resulting modified embryos into receptive sows[13]. The process was repeated, to knock out the second allele for this enzyme, and the resulting offspring no longer expressed Gal. Pig organs were no longer affected by high titers of anti-Gal in primate recipients, thus largely eliminating the problem of HAR and markedly extending xenograft survival[33,41].

Further advances in techniques for genome editing, including the use of Zinc Finger nucleases, Talens (transcription activator-like effector nucleases) and most recently, CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats and their associated RNA-guided DNA endonuclease #9)[42], have made it increasingly feasible to change the pig genome in ways intended to improve the success of xenotransplantation. All of these techniques have been used to modify the genome through nuclear transfer of nuclei from gene-edited somatic cells.

B. Non-Gal-mediated DXR:

As discussed above, while the problem of HAR was overcome by genetic engineering, DXR mediated by non-Gal NAbs became the next major hurdle for xenotransplantation. The discovery of the B4Gal and NeuGc specificities led to the generation of pigs with the genes responsible for their production knocked out. Paradoxically, these developments highlighted a limitation of non-human primates as a model to study xenotransplanation of pig organs. While humans lack a functional gene for CMAH that is required for NeuGc synthesis[20] and often have Nabs against NeuGc in their sera[19,43,44], NHPs have a functional CMAH enzyme[45]. Given this difference between humans and NHPs, effectively modeling rejection due to NeuGc specificity in NHP recipients is impossible. Indeed, deletion of CMAH in addition to GalT in porcine cells increased the binding of baboon sera, suggesting that removal of NeuGc revealed another antibody target recognized by baboon sera[46]. A KO of the B4GalNT2 (1,4-N-acetylgalactosaminyltransferase) enzyme has also been produced by several groups. In fact "triple KO" pigs that lack GalT, B4GalNT2, CMAH have been generated and human sera show reduced levels of NAbs binding to these pig cells compared to cells lacking only Gal and NeuGc. The binding of sera from transplant waitlisted humans to triple KO (GalT KO, CMAH KO, B4GalNT2 KO) pig target cells is reportedly similar to their binding to allogeneic targets[47].

A variety of additional GE approaches to overcoming the early antibody-mediated rejection of xenografts have been developed, including: 1) transgenic expression of human (h) hemoxygenase-1 (hHO-1), an enzyme that converts heme to bilirubin, carbon monoxide, and free iron. HO-1 helps cells to protect themselves from oxidative injury through anti-inflammatory, anti-apoptotic, and anti-proliferative effects through mechanisms that likely involve, at least in part, the production of CO[48]. Although this gene has now been included in several multi-gene edited swine donors, its effect on xenograft survival remains unclear; 2) transgenic expression of hA20, a human TNF-induced zinc finger protein enzyme that inhibits NF-kappa B activation and TNF-mediated apoptosis. Expression of this transgene in pigs was limited to the heart, where it appeared to partially inhibit ischemia-perfusion injury, although there was no demonstrable increase in organ survival[49]; 3) transgenic expression of human thrombomodulin (hTM) (CD141), an anticoagulant protein expressed on the surface of ECs. It was included among the transgenic modifications of pig hearts that was thought to be instrumental in allowing long-term survival of heterotopic heart xenografts to baboons[36,50]; 4) KO of porcine von Willebrand Factor (vWF): vWF is a glycoprotein that induces platelet adhesion at sites of vascular damage by binding to and stabilizing Factor VIII. It is hoped that the KO pig will mitigate induction of clotting at the interface of primate blood with porcine ECs. This protective function has been demonstrated *ex vivo* but its effect on xenograft survival has not yet been established[49]; 5) transgenic expression of hCD39 (ectonucleoside triphosphate diphosphohydrolase-1; ENTPD-1), an enzyme that hydrolyzes ATP and ADP to AMP, which is subsequently hydrolyzed to adenosine, providing anti-thrombotic and cardiovascular protective effects. The hCD39 transgene has been included in several pig-to-primate xenograft models[51] but without clear evidence for benefit.

Using serial nuclear transfer, pigs have been produced that have both GalT and CMAH knocked out, express multiple human CRPs and express transgenes encoding the anti-inflammatory/anti-apoptotic molecules HO-1 and A20[52].

C. T cell-mediated rejection:

The use of genetic engineering to subvert T cell immune responses and even make xenogeneic organs invisible to immune attack is appealing. Approaches that have been explored include the introduction of FasL[53], CTLA4Ig[54] and anti-CD2 monoclonal antibodies[55] into the source pig, aiming to achieve local immunosuppression following transplantation. Pigs expressing transgenic CTLA4Ig ubiquitously have been shown to be capable of reproduction despite global immunosuppression[54] and corneal transplants from them showed improved survival in NHPs[56]. Transgenic CTLA4Ig expression under control of the insulin promoter improved porcine islet survival in humanized mice[57]. However, immune evasion by a xenograft may impair immune protection from infection or viral reactivation. The absence of class I MHC could also make xenografts more susceptible to natural killer (NK) cell-mediated rejection, although this might be overcome by expression of transgenic HLA-E molecules[58–60]. Invisibility to direct immune attack would not obviate indirect recognition of xenoantigens with consequential antibody- and cytokine-mediated effector mechanisms.

Other transgenes such as hCD47 are discussed in more detail elsewhere in this review and additional KOs and transgenic modifications are undoubtedly in progress. However, it is important to note, as also described above for the CMAH KO, that unless a genetic modification can be demonstrated to be of value for the survival of xenografts in primates, it may be counterproductive to include it in a multi-gene construct, since every genetic modification carries with it the potential to affect pig health or produce neoantigens that may be immunogenic.

3. Recent advances in overcoming rejection in NHPs

A. Combined immunosuppression and genetic engineering:

The GE approaches discussed above, combined with advances in immunosuppressive therapies, especially costimulatory blockers, have permitted long-term organ and islet graft survival in NHPs, resulting in a resurgence in optimism about the potential for successful clinical xenotransplantation. Blockade of the B7-CD28[35] and the CD40/CD154 pathways[36] has played a significant role in these successes. The major advances in islet, kidney and heart transplantation are discussed here. Liver xenotransplantation has been much more challenging, as these grafts have not yet persisted beyond about one month in NHPs[61–63].

Islet xenotransplantation: Islet xenotransplantation is relatively non-invasive and is a non-life-supporting organ xenograft, leading to the notion that it may be quite readily applied in the clinic. Advances in porcine islet xenograft survival in NHPs have resulted from the use of immunosuppression that includes costimulatory blockade[22,64,65], but the grafts were ultimately rejected despite heavy immunosuppression. While prolonged survival of porcine islets expressing multiple human CRPs and CTLA4Ig has been reported, the role of GE in promoting islet xenograft survival in primates remains unclear[66]. The GalT knockout modification may be unnecessary for adult pig islet transplantation, as these do not express Gal[67]. However, human CRPs and anti-coagulation proteins may alleviate the immediate innate immune response that destroys islets immediately when they are injected into the portal circulation[68]. Given that diabetes can usually be controlled with insulin therapy, islet allotransplantation, which also requires chronic immunosuppression and usually only achieves a transient insulin-free period[69], is reserved for the small subset of patients with life-threatening diabetes complications like hypoglycemic unawareness. Islet xenotransplantation on a large scale could best be justified if the need for immunosuppression could be avoided, for example by islet encapsulation or tolerance induction.

Kidney xenotransplantation: Recently, with the use of powerful T cell-depleting and costimulatory blocking immunosuppression and selection of recipients with low non-Gal natural antibody levels, GalT knockout kidney graft survivals of >400 days have been reported in a few animals[70–73]. Immunosuppression included T and B cell depletion, steroids and costimulatory blockade, with rapamycin in one model and MMF in the other, and the grafts were eventually rejected. On the basis of these results, it has been suggested that kidney xenotransplantation could be offered in the near future to patients who are unlikely to receive an allograft due to high levels of alloantibodies, the existence of

underlying renal disease that is likely to recur rapidly in an allograft, or a lack of vascular access for dialysis[74]. The inability to predict outcomes in humans of GalT/B4/CMAH triple KO kidney transplants on the basis of NHP studies has led some to argue that clinical trials of porcine kidney transplantation are currently justified[75]. However, size incompatibility between humans and pigs could be another a significant problem if conventional-size pigs were used[76], as is discussed in more detail below.

It is likely that xenograft tolerance would broaden the potential applicability of porcine kidney xenotransplantation and accelerate progress. As is discussed below, a tolerance approach has led to graft acceptance >6 months in NHPs[76] without any rejection. Limitations to animal survival in this model have been consequences of nephrotic syndrome that may reflect physiologic differences between pigs and baboons. While this problem can be controlled by treatment with rituximab and CTLA4Ig[77–79], it is unknown whether this will be an issue in humans.

Heart transplantation: Large animal models for orthotopic, life-sustaining heart transplantation are technically challenging and most heart xenotransplantation has been heterotopic, such that the graft is connected to the recipient great vessels in the abdominal cavity and serves as an accessory rather than a functioning heart. Heterotopic porcine GalT KO, human CRP and thrombomodulin transgenic heart grafts have survived in baboons for periods of several years with long-term immunosuppression[36]. Maintenance immunosuppression included CD40 blockade, upon which graft survival was critical, as dose reductions led to antibody-mediated rejection. More recently, advances have been made in orthotopic cardiac xenotransplantation, with life-sustaining graft survival >6 months of GalT KO, human CD46 and human thrombomodulin pig hearts transplanted into baboons treated with rituximab, ATG, anti-CD40/CD40L, mycophenolate mofetil and steroids[80]. Success was dependent on non-ischemic preservation of the heart prior to transplantation. Myocardial hypertrophy was a significant initial limitation to long-term survival, and this was controlled by maintaining low blood pressure in the recipient and using rapamycin. Therefore, miniature pigs may be needed as source animals for clinical cardiac xenotransplantation. Candidates for cardiac xenotransplantation who will otherwise die waiting for an allograft could include patients experiencing failure of and alloantibody formation while on left ventricular assist devices, failure of primary cardiac allografts, and complications of or contraindications to total artificial hearts[74].

B. Tolerance:

While the advances in GE and immunosuppression discussed above have greatly improved xenograft survival in primates, the eventual rejections seen in the presence of immunosuppressive therapy raise the concern that long-term rejection-free survival may not be reliably achieved with the use of clinically tolerable levels of immunosuppression. Induction of tolerance, defined here as the absence of a destructive response to the graft in the absence of global immunosuppression, is therefore an important goal for the long-term success of clinical xenotransplantation. Two approaches to tolerance induction that have shown promise in large animal models are mixed hematopoietic chimerism and porcine thymic transplantation.

Mixed hematopoietic chimerism: Mixed chimerism denotes a state in which hematopoietic elements of the donor and recipient co-exist. It can be achieved by transferring donor hematopoietic cells (e.g. bone marrow) to a recipient that has been immunosuppressed and conditioned in a way that does not eliminate host hematopoiesis (i.e. non-myeloablative) but is sufficient to make "space" for engraftment of the donor marrow. Mixed chimerism is associated with induction of tolerance to organ grafts from the same donor. However, the use of HC transplantation (HCT) for the purpose of tolerance induction is only acceptable if the conditioning regimen used to permit engraftment of donor hematopoietic cells does not ablate recipient hematopoiesis and has relatively low toxicity and if there is essentially no risk of graft-vs-host disease (GVHD). These criteria have been met in rodent models but have been more challenging to achieve in non-human primates and humans. Transient mixed allogeneic chimerism has recently led to successful renal allograft tolerance across HLA barriers in clinical studies (reviewed in[81]). Preclinical studies in rodents and humanized mice (reviewed in[82]) have indicated that non-myeloablative mixed chimerism induction can achieve xenograft tolerance, but the innate and adaptive immune barriers to xenogeneic HC engraftment are much stronger than those to allogeneic HCs (reviewed in [8,82]). Studies in the rat-to-mouse and pig-to-humanized mouse xenograft models have shown that this approach has the advantage of inducing xenogeneic tolerance not only in the T cell compartment, but also among Nab-producing B cells and NK cells[83] (reviewed in [8]). Furthermore, recipient HCs have a competitive advantage over xenogeneic HCs[85], likely due to homologous species preference for interactions with hematopoietic cytokines and other molecules such as adhesion molecules[86][87,88][89]. Genetic engineering approaches, such as expressing human hematopoietic cytokine receptors in pigs, are likely to enhance the establishment of porcine hematopoietic chimerism, as shown in pig cytokine transgenic (Tg) mice[86,90].

Once engraftment of xenogeneic HCs is achieved, pre-existing natural IgM antibodies against the donor disappear in mixed xenogeneic chimeras (reviewed in [91]). Using GalT-deficient mice, the loss of anti-donor Nabs in mixed chimeras was shown to be due to specific B cell tolerance induction, initially via anergy and later by a deletional mechanism (reviewed in [92]). Mixed chimerism prevented all types of rejection of primarily vascularized xenografts, including hyperacute, acute vascular, T cell-mediated and chronic rejection (reviewed in [92]). Thus, mixed chimerism has the combined ability to tolerize donor-reactive T cells robustly, also preventing induced antibody responses, as well as T cell-independent Nab-producing B cells.

Another innate immune barrier to xenogeneic and not to allogeneic HC engraftment is posed by macrophages (reviewed in[7]). While pig CD47 binds well to the human macrophage inhibitory receptor SIRPα, a signal leading to SIRPα phosphorylation fails to be transmitted, resulting in failure of the "don't eat me" signal and rapid destruction of the xenogeneic HCs. Introduction of human CD47 into porcine HCs reduces their phagocytosis by human macrophages[93]. Importantly, a human CD47 transgene on porcine HCs enhanced porcine chimerism in baboons receiving non-myeloablative conditioning, resulting in markedly prolonged survival of donor pig skin grafts compared to that in animals receiving non-CD47-expressing pig HCs, in the complete absence of immunosuppression[94].

Chimerism and tolerance induction in baboons may be further enhanced by injecting the hCD47-Tg transgenic HCs directly into the bones, an approach that enhances porcine HC survival[95].

NK cells pose a greater barrier to xenogeneic than to allogeneic HC engraftment. Once xenogeneic chimerism is achieved, however, NK cells demonstrate tolerance to the donor (reviewed in [82]). While the role of NK cells in the allogenic setting has been studied extensively, studies on NK cells in the context of pig-NHP transplants is still a relatively new area and much remains to be learned. It is likely that these studies will also influence genetic engineering of pigs for transplanation.

Thymic transplantation: An alternative to mixed chimerism as a means of inducing deletional T cell tolerance is transplantation of the donor thymus. In the thymus, potentially autoreactive T-cells are deleted or anergized by exposure to the appropriate self antigens presented by either bone marrow-derived cells or thymic stromal cells (i.e. negative selection), playing a key role in tolerance to self antigens. If one transplants an allogenic donor thymus into a recipient depleted of mature T cells but still having bone marrow stem cells that can migrate to the donor thymus, newly developing T cells are similarly exposed to negative selection by both host and donor elements (likely dendritic cells and thymic epithelial cells), leading to loss of reactivity to both host and donor[96,97]. Intrathymic self tolerance induction also involves the presentation of tissue-restricted antigens (TRAs) by medullary epithelial cells. These TRAs play a role in deleting thymocytes that recognize them and in generating Tregs with specificity for them.

<u>Pig to mouse and humanized mouse models:</u> Early studies in a pig-to-mouse model demonstrated the capacity of grafted porcine fetal thymic tissue to reconstitute functional host T-cells in thymectomized, T cell-depleted immunocompetent mice[96,97]. Murine thymocyte populations in the porcine thymic grafts were phenotypically indistinguishable from normal host thymocytes and peripheral CD4$^+$ T-cells recovered, demonstrating tolerance to the donor and the recipient *in vivo* and *in vitro* that extended to acceptance of donor pig skin grafts without immunosuppression[96,97]. Intrathymic clonal deletion was shown to be a major mechanism of tolerance to the xenogeneic donor and the recipient. Tregs developing in the porcine thymus graft were also implicated in the suppression of residual mouse anti-pig responses. Despite the exclusive role of porcine MHC in thymic positive selection, these T cells responded to protein antigens presented by murine MHC molecules and protected the mice from an opportunistic pathogen. These and other results in the model are consistent with the generation in the pig thymus of a sufficiently diverse TCR repertoire to permit recognition of foreign antigens on recipient MHC despite positive selection by porcine MHC (reviewed in[82]).

Proof that the porcine thymic transplantation approach could achieve human T cell tolerance has come from humanized mouse models in which human T cells develop normally from hematopoietic progenitors and are centrally tolerized to porcine xenoantigens as well as human "self" antigens (due to the presence of human APCs in the porcine thymic grafts) in pig thymic grafts in immunodeficient mice[98,99].

Pig to primate transplants: Fetal pig thymic tissue transplanted to baboons resulted in donor-specific hyporesponsiveness *in vitro* and prolongation of porcine skin graft survival. However, only a small amount of thymic epithelium remained at the implantation site by day 60[102]. We reasoned that the lack of permanent tolerance in grafted baboons might reflect incomplete T-cell depletion, which needed to be exhaustive in mice in order to prevent eventual rejection of the thymus graft[103].

Given earlier successes of vascularized thymus grafts in the form of composite thymokidney (TK) and vascularized thymic lobe (VTL) transplantation for induction of tolerance across allogeneic barriers in pigs (reviewed in[100]), this approach was tested across the xenogeneic pig-to-primate barrier. It was first applied using hDAF transgenic kidney donors, immunoabsorption of natural anti-Gal antibodies on Gal columns and immunosuppression including thymectomy, cobra venom factor (CVF) and T-cell depletion[104]. The vascularized thymic grafts were found to support reconstitution of recipient-type T-cells at early time points, as demonstrated by recovery of CD45RA high/CD4+ cells in the peripheral blood. In addition, the thymic grafts induced in vitro donor-specific unresponsiveness that was maintained for approximately 2 months. However, all grafts were lost thereafter to humoral rejection following the inexorable return of anti-Gal antibodies.

These TK and VTL experiments were subsequently repeated using GalT-KO swine donors, for which the presence or return of anti-Gal antibodies would no longer pose a threat. The improvement of results was remarkable, with renal xenograft survivals increasing to over 80 days and most animals expiring due to causes other than rejection[33,105]. While immunosuppression was not fully withdrawn before the demise of these animals, these results suggested that in the absence of rejection caused by returning anti-Gal antibodies, the TK and VTL tolerance-inducing regimens could prevent T cell-mediated responses as well as new, T-cell-dependent antibody responses, thereby permitting much longer survival of the xenografted organs. Recently, further modification of the induction regimen has facilitated survival of life-supporting thymokidneys for over 6 months, with loss due to cortical necrosis likely reflecting graft growth, without evidence of rejection, despite tapering of immunosuppression prior to sacrifice (Figure 4).

Several limitations associated with the generation of a human T cell repertoire in a xenogeneic porcine thymus deserve consideration. Human T cells positively selected by porcine MHC would preferentially recognize microbial antigens presented by porcine MHC, effectively protecting the graft from infection but perhaps less effectively protecting the host against microbial pathogens. Indeed, in humanized mice, human T cells that developed in a pig thymus demonstrated reduced responses to peptides presented by human APCs compared to those developing in a human thymus graft[99]. Additionally, porcine thymic epithelium might fail to completely negatively select conventional T cells or optimally positively select Tregs recognizing human TRAs. Therefore, we are exploring the idea of constructing a "hybrid thymus", in which recipient thymic epithelial cells obtained from human thymectomy specimens are injected into the porcine thymic tissue [106].

4. Requirements for a successful source animal:

As described above (see Introduction), the pig is generally considered to be the most suitable xenograft source animal, because of its size, favorable breeding characteristics and the similarity of many of its organ systems to those of humans[37]. In this section, we will consider these and other source animal characteristics that will likely be required for the success of xenotransplantation. We will also discuss several more subtle characteristics that may be of particular importance for transplantation of certain organs.

A) Availability:

Unlike the NHPs used as donors for the early clinical xenotransplantation efforts, swine are not an endangered species. Over 100 million pigs are slaughtered for food each year in the United States alone. Although there are animal rights groups that oppose the use of animals for any purpose, it seems unlikely that society would wish to limit the number of swine whose organs might be used to save the lives of human beings. However, not all swine would be appropriate for this purpose, since special breeding and maintenance would be needed to satisfy scientific and regulatory criteria.

B) Safety:

As in the case of allogeneic transplantation, the two most important safety concerns for xenotransplantation are the risk of failure due to rejection and the risk of side effects due to the immunosuppressive medications required to prevent rejection, most importantly infections, to which these medications lower resistance. During the late1990's, discussions around xenotransplantation became focused on the risk of infectious transmission from pigs to humans. This resulted in large part from the observation that a porcine kidney cell line was capable of transmitting c-type porcine endogenous retroviruses (PERVs) to human cell lines in vitro[107]. There was a theoretical concern that these porcine PERVs could infect human xenograft recipients and cause disease due to insertional effects, causing disruption of normal gene function or oncogenesis. Considerable research on the potential for human infection by PERV ensued, resulting in the realization that primary human cells are very difficult to infect due to the presence of APOBEC restriction factors and that it was primarily PERC A-C recombinants that infected cell lines in vitro (reviewed in [108]). An initial report that PERV infected human islets implanted in immunodeficient mice[109] was later found to reflect pseudotyping of PERV virions by murine endogenous retrovirus envelope proteins, that permitted infection of human cells living in the same mice as the porcine islet[110] or thymic[111] grafts. Most importantly, despite the development of sensitive assays for its detection[112], PERV infection has not been detected in any of over 200 patients who were exposed to porcine cells or xenografts in recent decades(reviewed in[108]). Hundreds of non-human primates receiving porcine xenografts have also failed to show evidence of PERV infection, but their cells are less readily infectable than human cells (reviewed in[108]). These developments have led to acceptance in the xenotransplantation community that the theoretical risk of PERV infection can be managed with appropriate surveillance and oversight from national health regulatory agencies[108,113]. Additionally, several groups have developed GE methodologies to further reduce this risk, by introducing small inhibitory RNA into the genome[114] or by using CRISPR-Cas9 technology to knockout

PERVs[115,116]. A concern with the latter approach is the potential for off-target genetic modifications that could have deleterious effects[117], especially when so many loci are targeted simultaneously.

While so much attention has been focused on the theoretical risk of PERV infection, it may have been underappreciated that porcine xenografts, when performed appropriately, can carry less infectious risk than human allografts[108,118]. Human deceased allograft donors are screened for a variety of known human viruses, but the time-urgency of utilizing organs from brain-dead human donors often makes it difficult to carry out sufficient safety testing. Currently, approximately 0.2% of transplant recipients experience unexpected infections transmitted from the allograft[119]. In contrast, raising of source pigs in a carefully controlled and monitored environment can virtually eliminate this concern. US regulatory agencies [120,121], the World Health Organization (WHO) and the International Xenotransplantation Association (IXA) have developed guidelines and recommendations for xenotransplantation clinical trials[122,123]. The recommendations include use of a "designated pathogen-free" pig that is housed in a closed facility where the risk of introducing new pathogens is minimized[108].

Besides the risk of transmission of porcine microbial infections to the immunocompromised host, porcine viruses that do not possess the ability to infect the human host may still pose a risk to the graft, particularly because the recipients typically will not have T cell immunity to porcine viruses that recognize these antigens in the context of porcine MHC (swine leukocyte antigens, SLA).

C) Size:

One of the attractive features of swine as potential xenograft sources is the fact that their organs could potentially be matched to the size of any potential human recipient. Which strain of swine will be most appropriate on this basis remains undecided. One criterion might be the age at which an animal might be anticipated to reach the size required for a particular recipient. Domestic swine, which are being used by most groups developing genetically engineered swine as potential xenograft donors, attain mature adult weights of over 1000 pounds, so that their organs would be too large for use in humans after the age of about one year. In contrast, miniature swine, that have been developed extensively for this purpose in the laboratory of the authors, achieve maximum adult weights of 200 to 300 pounds, similar to those of humans, so that their organs could be of potential use at any age. While it is not yet clear how much of the growth potential of a transplanted organ is intrinsic to the organ itself versus extrinsically controlled by the size of the recipient, transplantation of kidneys from conventional swine to miniature swine with a tolerance induction regimen was associated with persistent graft growth in the miniature swine, reaching volumes 3.7 times their initial volume over 3 months, versus 1.2 times their initial volume for miniature swine kidneys over the same time period[124]. Similarly increased growth ratios were seen for lung allografts, in this case leading to impaired function of the organs[124].Pig-to-baboon kidney xenografts have also been associated with rapid growth of the organs leading to cortical necrosis[124].

D) Breeding Characteristics:

In addition to making it possible to raise large numbers of animals quickly and relatively inexpensively, their breeding characteristics make swine one of the few large animal species in which it is possible to carry out a selective genetic breeding program. They have large litter sizes (5 – 10 offspring), early sexual maturity (5 months), short gestation time (114 days), and frequent estrus cycles (every three weeks). Thus, it has been possible to produce miniature swine homozygous for the MHC in a relatively short time [125], as well as one inbred line which has reached greater than 94% coefficient of inbreeding, and has been demonstrated to be histocompatible, allowing transplants within this line to be accepted without exogenous immunosuppression [126]. GE of appropriate xenograft donors is enhanced by the availability of such inbred animals. Thus, if one starts with a highly inbred animal, it is possible to combine any number of independently-introduced transgenes into the same genetic background by simple crossbreeding and selection for the desired transgenes among the offspring, which retain the same genetic background. Having inbred donor animals is of particular importance for approaches to xenotransplantation tolerance for two reasons: 1) cells (+/− gene modifications) capable of inducing tolerance can be used from one animal followed by an organ from another, perhaps with different gene modifications tailored for that organ's function; and 2) if, for any reason, the transplanted organ needs to be replaced, the recipient would remain tolerant to a second organ from an animal of the same line.

E. Structural and Physiologic Similarity to Humans:

The pig has been widely agreed upon as the optimal source animal for human transplantation in large part due to its physiologic similarities to humans[37]. Most tissues and organs of swine bear a remarkable resemblance to those of humans both in structure and physiology. This includes the heart and circulatory system, the kidney, the pancreas, the liver, the lungs and even the skin[127], which is almost indistinguishable histologically from skin of humans. The major difference that may have to be taken into account in choosing the optimal source animal is likely to be size, as discussed in detail above. This factor may be of more importance for thoracic organs, that are confined to a closed cavity, then for abdominal organs, for which additional room for growth might be possible. Nevertheless, excessive growth could lead to organs that would outgrow their blood supply, causing the kind of cortical necrosis already seen in the case of porcine kidneys transplanted between animals of markedly different eventual size[76].

However, organ-specific physiologic differences are to be expected, especially if the organ produces a species-specific product, such as erythropoietin produced in the kidney, or coagulation factors produced by the liver. As long as the number of such differences is limited for each organ, the use of selective genetic modifications or of biological replacement therapies may be able to overcome the incompatibility. As more prolonged pig organ xenograft survival is achieved in NHPs, previously-unknown physiologic incompatibilities may emerge that will require appropriate therapy or additional pig genetic modification. As an example, pig liver xenograft survival up to 28 days was recently achieved in baboons when it was recognized that liver-derived porcine coagulation factor VII was insufficient and needed augmentation from human prothrombin concentrate to prevent coagulopathy and thrombocytopenia[62,63]. Likewise, advances in pig-to-primate islet

xenotransplantation have revealed differing metabolic requirements between the species[128]. Recently, it has been suggested that differences in the intrinsic blood pressures of pigs vs baboons may contribute to myocardial hypertrophy in orthotopic heart transplantation[80]. While a detailed discussion of potential physiologic differences between pigs and humans is beyond the scope of this article, it is worth pointing out, in the context of the tolerance approach discussed above, that suboptimal xenogeneic hematopoietic cell engraftment partly reflects physiologic incompatibilities in the hematopoietic microenvironment in xenogeneic recipients[129].

5) Conclusions:

The remarkable clinical success of transplantation over the past several decades has paradoxically led to an increasing shortage of transplantable human organs, by increasing the waiting list of patients aware that transplants could save their lives. Although there are other, competing technologies also under development, xenotransplantation is likely to the best near-term solution for this organ shortage. Many of the obstacles that have previously inhibited progress in xenotransplantation have now been overcome through GE of pigs to make their organs more compatible with human immune systems and physiology. Even with these modifications, however, it is not yet clear that the use of clinically-acceptable levels of immunosuppression could permit xenograft survivals similar to those of allografts. To achieve this goal, tolerance may be required. Additional GE of pigs to improve the likelihood of tolerance induction to pigs is also underway. Judicious combinations of modifying host immunity and genetically engineering swine donors is likely to lead to clinical success of xenotransplantation in the near future.

Abbreviations

Gal	α1,3Gal
αGalT	α1,3Galactosyl transferase
APC	antigen-presenting cell
CMV	cytomegalovirus
ECs	endothelial cells
GVHD	graft-versus-host disease
HAR	hyperacute rejection
HCT	hematopoietic cell transplantation
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
IL-15	interleukin 15
MHC	major histocompatibility complex

NHP	non-human primate
NK cell	natural killer cells
TRA	tissue-restricted antigen

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The growing organ shortage
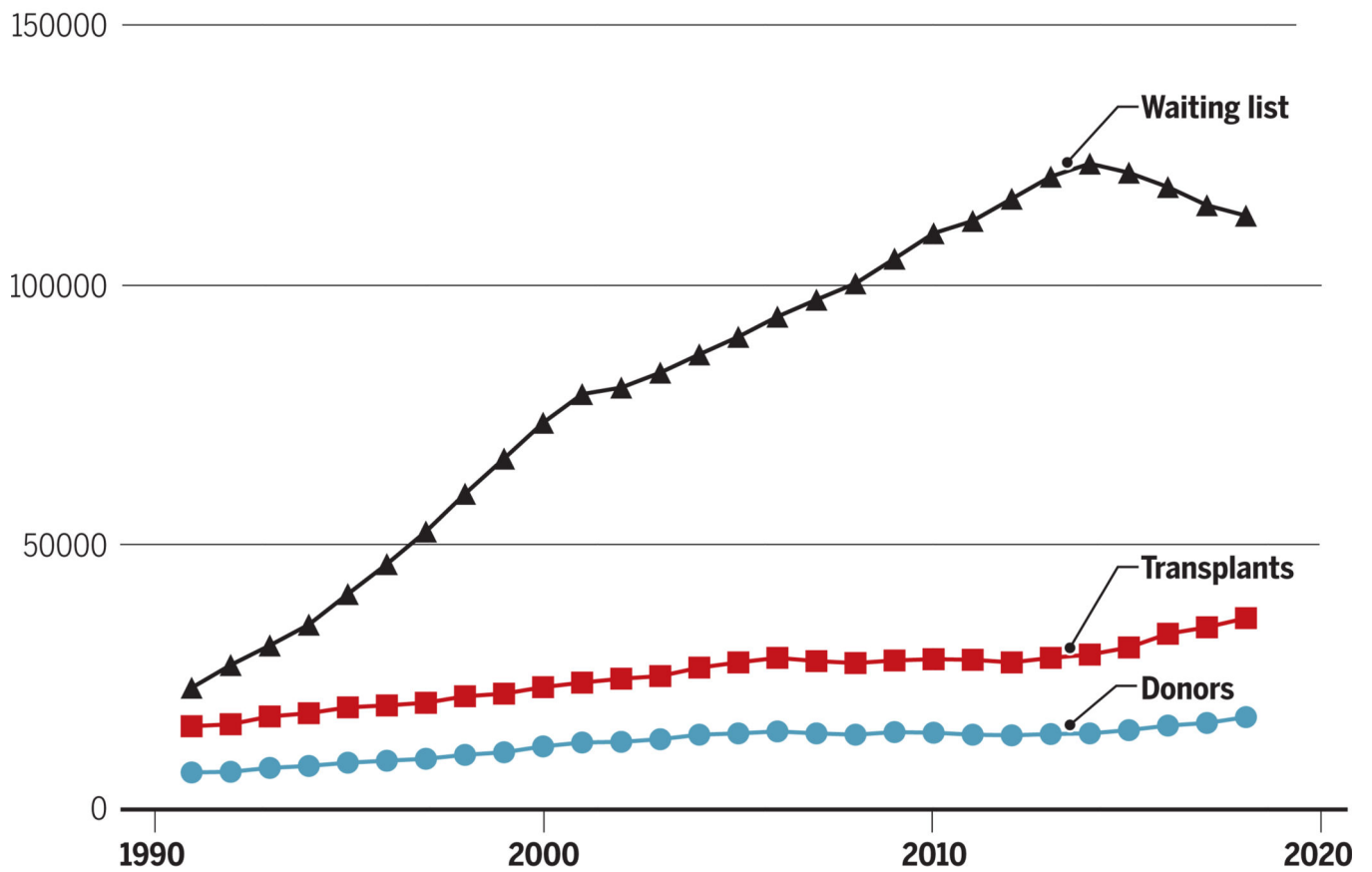
From: UNOS Database, 2019

Figure 1:
The growing allogeneic organ supply/demand imbalance has resulted in an expanding transplant waiting list.

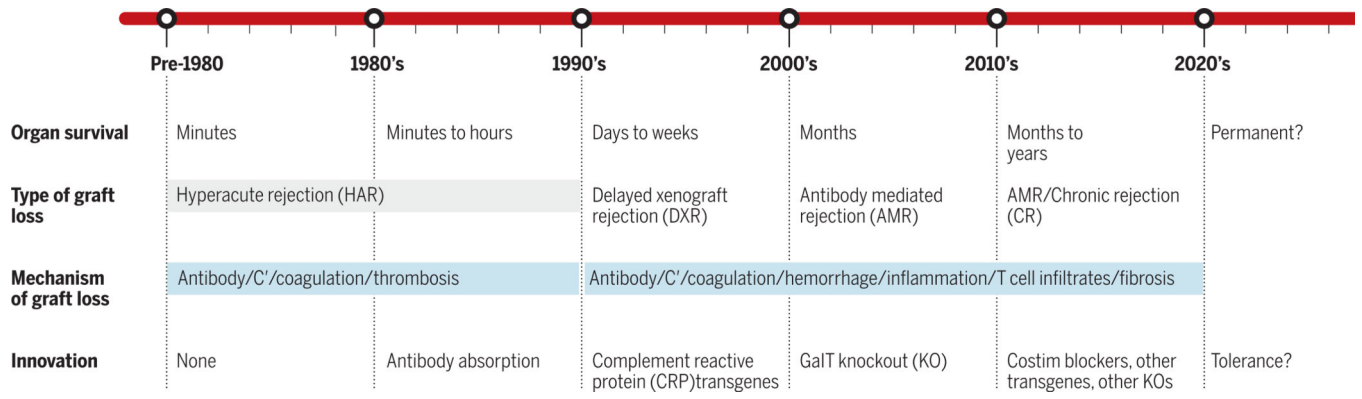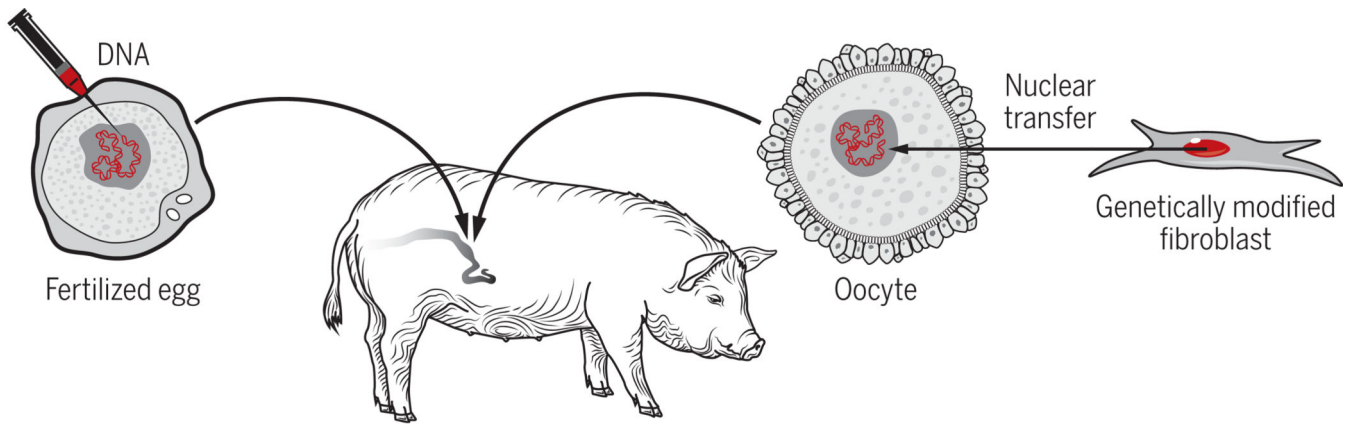|	Pre-1980	1980's	1990's	2000's	2010's	2020's
Organ survival	Minutes	Minutes to hours	Days to weeks	Months	Months to years	Permanent?
Type of graft loss	Hyperacute rejection (HAR)		Delayed xenograft rejection (DXR)	Antibody mediated rejection (AMR)	AMR/Chronic rejection (CR)	
Mechanism of graft loss	Antibody/C'/coagulation/thrombosis		Antibody/C'/coagulation/hemorrhage/inflammation/T cell infiltrates/fibrosis			
Innovation	None	Antibody absorption	Complement reactive protein (CRP)transgenes	GalT knockout (KO)	Costim blockers, other transgenes, other KOs	Tolerance?

Figure 2:
Schematic timeline showing advances in overcoming the immunologic challenges to xenograft survival and the impact on pig organ survival times in primates.

Transgenics

Complement inhibition	Immunosuppressive molecules
hDAF	Anti-CD2
hCD46	CTLA4Ig
hCD59	hCD47

Coagulation inhibition	PERV siRNA
hCD39	MHC genes
Human thrombomodulin	Class I (NK inhibition)

Anti-inflammatory genes
  HO-1
  A20

Knock-outs

α1,3-galactosyl transferase
CMAH
B4GalNT2
vWF
PERVs

Figure 3:
Genetic modifications that have been made in pigs to facilitate pig to human organ transplantation. See text for details.
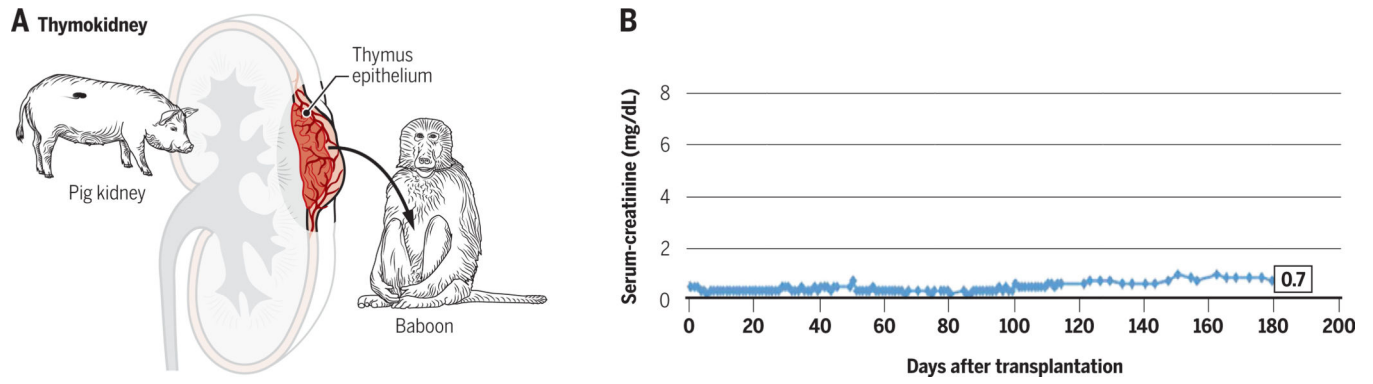
Figure 4:

A. Schematic of a thymokidney transplant from a pig to a baboon. B. Long-term survival of a GalT KO miniature swine thymokidney graft in a baboon with tapering immunosuppression. Serum creatinine was normal for >6 months and the graft grew markedly and was lost to cortical necrosis with no evidence of rejection at Day 193 post-transplant. Reprinted with permission from American Journal of Transplantation, 2017, 17:1778–1790.