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The resurgent landscape of xenotransplantation of pig organs in nonhuman primates

Xuan Zhang[1†], Quancheng Wang[1†], Jingjun Zhao[2†], Xiao Li[1], Wei Peng[1], Zhaoxu Yang[1], Zhibin Lin[1], Long Yang[1], Rui Ding[1], Kaishan Tao[1*] & Kefeng Dou[1*]

[1]*Department of Hepatobiliary Surgery, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032, China;*
[2]*Institute of Nutrition and Food Safety, Shaanxi Provincial Center for Disease Control and Prevention, Xi'an 710054, China*

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Organ shortage is a major bottleneck in allotransplantation and causes many wait-listed patients to die or become too sick for transplantation. Genetically engineered pigs have been discussed as a potential alternative to allogeneic donor organs. Although xenotransplantation of pig-derived organs in nonhuman primates (NHPs) has shown sequential advances in recent years, there are still underlying problems that need to be completely addressed before clinical applications, including (i) acute humoral xenograft rejection; (ii) acute cellular rejection; (iii) dysregulation of coagulation and inflammation; (iv) physiological incompatibility; and (v) cross-species infection. Moreover, various genetic modifications to the pig donor need to be fully characterized, with the aim of identifying the ideal transgene combination for upcoming clinical trials. In addition, suitable pretransplant screening methods need to be confirmed for optimal donor-recipient matching, ensuring a good outcome from xenotransplantation. Herein, we summarize the understanding of organ xenotransplantation in pigs-to-NHPs and highlight the current status and recent progress in extending the survival time of pig xenografts and recipients. We also discuss practical strategies for overcoming the obstacles to xenotransplantation mentioned above to further advance transplantation of pig organs in the clinic.

organ, xenotransplantation, pig, non-human primate, genetical engineering

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Introduction

Currently, for patients with terminal organ failure, transplantation is the most practical and effective life-saving method worldwide, and recipients show less morbidity and a superior quality of life. However, many wait-listed patients die or become too sick for transplantation due to the lack of suitable donor organs. According to the latest literature (Wang et al., 2019), over 300,000 patients are on the waiting list in China, but only approximately 10,000 of them can receive organ transplantation annually, thus highlighting the urgent need to expand the number of available donor organs.

Several approaches have been proposed and explored to address this issue (Abouna, 2008); of these, genetically modified pigs have been discussed as a potential alternative of allogeneic donor organs, as they show many anatomical and physiological similarities to human beings (Whyte and Prather, 2011). Based on current preclinical progress, transplant scientists have proposed that clinical xenotransplantation would be the next medical revolution (Ekser et al., 2012a). Notably, many donor materials can be derived from pigs: (i) structural tissues with viable cells (e.g., heart valve,

†Contributed equally to this work
*Corresponding authors (Kaishan Tao, email: taokaishan0686@163.com; Kefeng Dou, email: doukef@fmmu.edu.cn)

small intestinal submucosa); (ii) viable cells or tissues with preserved biological functions (e.g., hepatocytes, neurocytes, blood cells, islets, cornea, skin); and (iii) solid organs (e.g., heart, liver, kidney, lung). Here, we will examine pig solid organ transplantation, representing a cutting-edge development of the xenotransplantation field.

Recently, ground-breaking advances have been achieved in pig-to-nonhuman primate (NHP) organ xenotransplantation, as shown by recipient survival of 499 days (Kim et al., 2019) and 195 days (Längin et al., 2018), respectively, for life-supporting renal and cardiac transplantation. Moreover, it was previously reported that porcine islets and heterotopic heart transplants survived for 965 days (Shin et al., 2016) and 945 days (Mohiuddin et al., 2016), respectively. Despite sequential advances in the field, there are still underlying problems that need to be overcome in xenotransplantation of pig organs, including (i) acute humoral xenograft rejection; (ii) acute cellular rejection; (iii) dysregulation of coagulation and inflammation; (iv) physiological incompatibility; and (v) cross-species infection. Moreover, various genetic modifications to the pig donor need to be fully determined, with the aim of identifying the ideal transgene combination for clinical trials. In addition, suitable pretransplant screening methods need to be confirmed for optimal donor-recipient matching, ensuring a good outcome from xenotransplantation. In this review, we summarize the understanding of organ xenotransplantation in pig-to-NHPs and highlight the current status and recent progress in extending the survival time of pig xenografts and recipients. We also discuss practical strategies for overcoming the xenotransplant obstacles mentioned above to further advance pig organ transplantation in the clinic.

Brief history of clinical xenotransplantation

Cross-species blood transfusions in the 17th century could represent an embryonic stage of xenotransplantation (Cooper, 2012). The first serious attempts at xenotransplantation appeared in the scientific literature in 1905, when slices of rabbit kidney were transplanted into a child with renal insufficiency (Reemtsma, 1995). In the first two decades of the 20th century, several subsequent efforts to use organs from lambs, pigs and primates were carried out; however, the results were disappointing (Reemtsma, 1995). Notably, in 1964, Reemtsma K first utilized azathioprine, hormones, and whole-body irradiation as immunosuppressive treatments and successfully prolonged chimpanzee kidney function for nearly 9 months in a patient with renal failure (Reemtsma, 1989). Between 1966 and 1974, Thomas E. Starzl carried out orthotopic liver transplants using chimpanzees as liver donors, with graft survival less than 14 days. Then, in 1992–1993, his team performed baboon liver transplantation in patients with cirrhosis from hepatitis B infection, and recipient survival reached approximately 70 days (Starzl et al., 1994).

None of these trials of clinical xenotransplantation have been entirely successful due to the many obstacles arising from the xenogeneic response of the recipient's immune system. Apparently, incompatibility between different species and lack of effective immunosuppressive treatment both contributed to these disappointing and inadequate outcomes. Even so, we still consider these experiences in organ xenotransplantation with humans as recipients valuable and meaningful for the subsequent development of preclinical pig organ transplantation.

Genetic modification in pig donors

NHPs showed similar anatomical and physiological features to humans, and a previous limited number of clinical trials also revealed good efficacy in using NHPs as donors. However, with the advent of genetic engineering and cloning technologies, pigs present more advantages as a potential source of donor organs or cells for humans than NHPs (Hryhorowicz et al., 2017; Pan et al., 2019).

The interspecies incompatibility between pigs and primates has been minimized through genome editing (e.g., clustered regularly interspaced short palindromic repeats and the associated protein 9, CRISPR/Cas9), which is an important factor in the recent progress of xenotransplantation of pig organs. Briefly, genome-edited pigs were generated by (i) deletion of pig genes and (ii) insertion of human transgenes (Table 1). Of these, three representative pig genes (e.g., α-1,3-galactosyltransferase, GT; β-1,4N-acetylgalactosaminyltransferase 2, β4GalNT2; cytidine monophospho-N-acetylneuraminic acid hydroxylase, CMAH) need to be deleted first, with the aim of reducing human preformed antibody-mediated severe humoral rejection (Adams et al., 2018) (Figure 1A). Moreover, the purpose of insertion of human transgenes is to provide protection from human complement activation (e.g., transgenic hCD46, hCD55 and hCD59), dysregulation of coagulation (e.g., transgenic hTFPI, hTM, hCD39, hCD73, hEPCR, etc.), inflammation and apoptosis (e.g., transgenic hHO-1, hA20 and shTNFRI-Fc, etc.), and/or cellular immunity (e.g., hCD47, hCTLA4-Ig, hPD-L1, HLA-E, etc.). In addition, genome editing may address issues of physiological incompatibility and potential cross-species transmission of zoonotic infections (see below). Numerous genetic modifications have already been introduced (Cooper et al., 2016a; Zhang et al., 2019), with some pigs even carrying PERV inactivation, knockout of three main xenoantigens and nine effective human transgenes (abbreviated to PERVKO·3KO·9TG) (Yue et al., 2019).

Despite the capability of CRISPR/Cas9 to generate pig

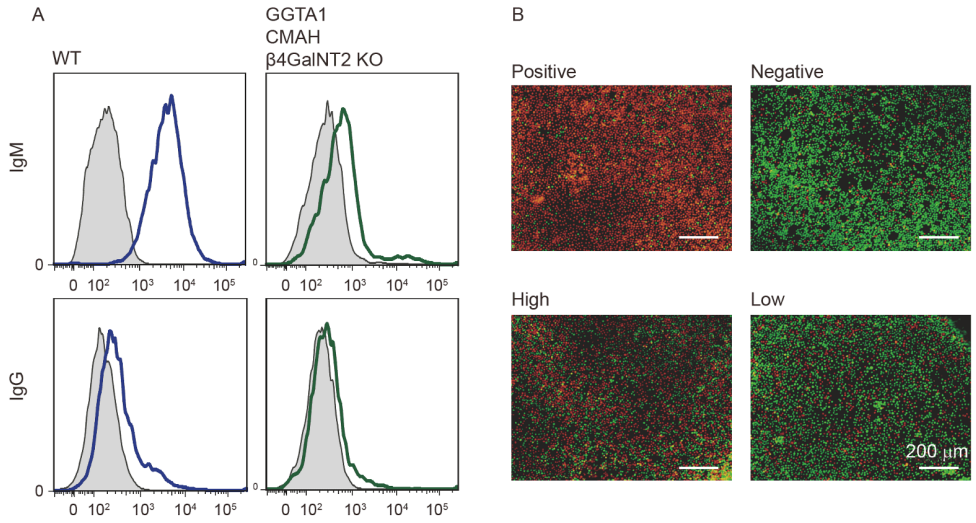

Figure 1 Xenoantibodies and complement activity determinations in pig-to-NHP organ xenotransplantation. A, Endothelial cells (ECs) were obtained from wild-type (WT) and GGTA1/CMAH/β4GalNT2-KO donor pigs and then cultured with the same rhesus macaque-derived serum. GGTA1/CMAH/β4GalNT2-KO ECs showed obviously lower IgM and IgG binding than WT ECs. B, Complement-dependent cytotoxicity (CDC) reaction for serum antibody screening in different rhesus macaque recipients. Optimal donor-recipient matching results will show a lower cell death rate. Scale bar, 200 μm.

donors with many genetic modifications, several concerns still need to be noted in clinical application: (i) the side effects of CRISPR/Cas9 technology, e.g., off-target effects (Tadić et al., 2019), p-53-mediated DNA damage response (Haapaniemi et al., 2018), megabase-scale chromosomal truncations (Cullot et al., 2019), and p53-inactivating mutations (Enache et al., 2020). These side effects might result in potential risks in extensive mammalian germline genome engineering. (ii) The pre-existing humoral and cell-mediated adaptive immune response to Cas9 in humans (Charlesworth et al., 2019). Scientists assessed human serum for the presence of antibodies against both SaCas9 and SpCas9 (orthologs of Cas9) in 78% and 58% of donors, respectively, and found anti-SaCas9 T cells in 78% and anti-SpCas9 T cells in 67% of donors. Both of these T cells were confirmed to be Cas9-specific (Charlesworth et al., 2019). (iii) The uncertainty about the effectiveness of multigene modifications. Indeed, the longest survival time for pig grafts in NHPs thus far was achieved using donors with relatively few genetic modifications. Every genetic modification carries with it the potential to produce neoantigens that may be immunogenic (Sykes and Sachs, 2019). "The 2018 Changsha Communique" suggested that pig donors with a minimum number of essential genetic modifications should be preferred in clinical trials (Hawthorne et al., 2019).

Pretransplant cross-matching for optimal donor-recipient pairs

Although αGal antibody-mediated xenogeneic rejection has been addressed, non-Gal antibodies still induce acute humoral rejection, resulting in severe xenograft damage (Zhang et al., 2019). Previous studies have demonstrated that xenotransplantation of pig organs with no prescreened NHP recipients could lead to disparate results ranging from rapid xenograft loss to long-term and excellent preclinical outcomes. These results suggested that even if the Sda and Neu5Gc antigens were knocked out in pig donors, non-Gal antibody-mediated activation of the xenogeneic humoral response could not be completely avoided. Accordingly, pretransplant cross-matching is accepted in preclinical trials for optimal donor-recipient screening, with the aim of reducing non-Gal antibody-mediated xenogeneic humoral immune rejection as much as possible.

Even so, different forms of cell-based approaches have been attempted to perform this serum antibody screening (Kim et al., 2019; Zhang et al., 2017), which encompass (i) direct detection of preformed xeno-reactive natural antibodies (XNAs), e.g., IgG and IgM. Briefly, donor-derived endothelial cells (ECs) or peripheral blood mononuclear cells (PBMCs) were obtained and cultured in complement inactivated serum from different recipients. Then, the binding of XNAs to ECs or PBMCs was detected by FACS analysis (Figure 1A). (ii) Indirect reaction of complement-dependent cytotoxicity (CDC). ECs or PBMCs were cultured in normal recipient-derived serum, and additional complement was added to this culture system to assess the cytolytic effect (Figure 1B).

Pharmacological immunosuppression in NHPs

Unlike genome editing in pig donors, regulation in recipients

Table 1 Obstacles in xenotransplantation and relevant genetic modifications in pig donors

Obstacle	Solution	Full name	Function
Hyperacute rejection	GalT-KO	α-1,3-galactosyltransferase knockout (GGTA1-KO)	Delete the xenoantigen galactose-α-1,3-galactose (α-Gal)
Non-Gal antibody mediated graft damage	β4GalNT2-KO	β-1,4N-acetylgalactosaminyltransferase 2 knock-out	Delete the xenoantigen DBA-reactive glycans (Sda)
	CMAH-KO	Cytidine monophosphate-N-acetylneuraminic acid hydroxylase knockout	Delete the xenoantigen N-glycolylneuraminic acid (Neu5Gc)
Complement-mediated xeno-graft injury	hCD46	Transgenic human membrane cofactor protein	Inactivation of C3b and C4b Block the formation of the membrane attack complex
	hCD55	Transgenic human decay-accelerating factor	
	hCD59	Transgenic human membrane inhibitor of reactive lysis	
Inflammatory injury	hHO-1	Transgenic human heme oxygenase-1	Inhibit inflammation and apoptosis
	hA20	Transgenic TNFAIP3	
	shTNFRI-Fc	Transgenic soluble human tumor necrosis factor-α receptor inhibitor-Fc	
Coagulation disorder	hTFPI	Transgenic human tissue factor pathway inhibitor	Correct imbalance between procoagulant and anticoagulant activity and reduce adhesion, aggregation, and phagocytosis of primate platelets exposed to xenograft
	hTM	Transgenic human thrombomodulin	
	hCD39	Transgenic human ectonucleoside triphosphate diphosphohydrolase	
	hCD73	Transgenic human ecto-5′-nucleotidase, E5′N	
	hEPCR	Transgenic human endothelial protein C receptor	
	vWF-KO	von Willebrand Factor gene knockout	
	ASGR1-KO	asialoglycoprotein receptor 1 knockout	
Cell-mediated rejection	hCD47	Transgenic human integrin-associated protein (IAP)	Regulate macrophage activation and phagocytosis
	hCTLA4-Ig	Transgenic human cytotoxic T-lymphocyte-associated protein 4-immunoglobulin	Interrupt T cell activation Induce T cell apoptosis
	hLEA29Y	Transgenic human variant of CTLA4-Ig	
	hTRAIL	Transgenic human tumor necrosis factor-related apoptosis-inducing ligand	
	hPD-L1	Transgenic human programmed cell death ligand 1	
	hCIITA-DN	Transgenic human class II transactivator dominant negative	Reduce xenoantigen expression and thus reduce the recipient's immune response
	SLA1-KO	swine leukocyte antigen class I knockout	
	HLA-E	Transgenic human leukocyte antigen-E	Inhibit NK cell cytotoxicity
	β2M-KO	β-2-microglobulin gene knockout	
	hFasL	Transgenic human Fas ligand	
Physiological incompatibility	GHR-KO	Growth hormone receptor knockout	Prevent xenograft overgrowth.
Cross-species infection	PERV-KO	Porcine endogenous retrovirus knockout	Elimination of the cross-species transmission risk of PERVs

can only be realized through pharmacologic intervention, including immunosuppressive and/or adjunctive therapy, with the aim of reducing xenogeneic rejection and/or minimizing immune-independent xenograft injury. The experiences of immunosuppressive administration in xenotransplantation of pig organs were mainly derived from that in allotransplantation (Table 2), such as treatments with tacrolimus (FK506, FK), cyclosporine (CsA), mycophenolate mofetil (MMF), anti-thymocyte globulin (ATG) and corticosteroids (Cs), but the results were unsatisfactory. Therefore, additional monoclonal antibodies (mAbs) (e.g., CTLA-4Ig (abatacept or belatacept) (Shah et al., 2016), anti-CD20 mAb (Längin et al., 2018), anti-CD154 mAb (Ji et al., 2015), anti-CD40 mAb (Shah et al., 2017), anti-CD4 mAb and anti-CD8 mAb (Adams et al., 2018)) were successively introduced in the subsequent immunosuppressive therapy, and the acceptance of xenografts was substantially improved (Adams et al., 2018; Mohiuddin et al., 2016; Shah et al., 2017).

Several groups have reported excellent survival time of pig organ grafts in NHPs receiving experimental agents that block the CD40-CD154 costimulation pathway (Adams et

Table 2 Current attractive immunosuppressive treatment in preclinical trials of xenotransplantation[a)]

Drug	Mechanism of action	Application in xeno-Tx	Representative Ref.
Tac (FK506, FK)	Blocks T cell cytokine production by inhibiting the phosphatase calcineurin and thus blocking activation of the NFAT transcription factor	Liver	(Shah et al., 2017)
ALG/ATG	T-cell clearance from the circulation and modulation of T-cell activation, homing, and cytotoxic activities	Heart, liver	(Längin et al., 2018; Mohiuddin et al., 2016; Shah et al., 2017)
Abatacept/belatacept (CTLA4-Ig)	Inhibits T cell activation by blocking B7 costimulator binding to T cell CD28 (clinical trials)	Liver	(Shah et al., 2017)
Rituxan (aCD20 mAb)	Depletes B cells by binding to an epitope in the large extracellular loop of primate CD20	Heart	(Längin et al., 2018; Mohiuddin et al., 2016)
MMF	Blocks lymphocyte proliferation by inhibiting guanine nucleotide synthesis in lymphocyte	Heart, Liver	(Längin et al., 2018; Mohiuddin et al., 2016; Zhang et al., 2017)
CVF	Deplete complement (convert C3 to C3 fragments, C5 to C5a and SC5b-9)	Liver	(Shah et al., 2017; Zhang et al., 2017)
aCD40 mAb	Inhibits antigen-presenting cell activation by blocking CD40 costimulator binding to T cell CD40L	Heart, liver, kidney	(Kim et al., 2019; Längin et al., 2018; Mohiuddin et al., 2016; Shah et al., 2017)
aCD154 mAb	Inhibits T cell activation by blocking CD40L costimulator binding to antigen-presenting cell CD40	Kidney, Heart	(Kim et al., 2019; Längin et al., 2018)
aCD4 mAb	Depletes $CD4^+$ T cells by binding to primate CD4	Kidney	(Kim et al., 2019)
aCD8 mAb	Depletes $CD8^+$ T cells by binding to primate CD8	Kidney	(Kim et al., 2019)
Cs	Reduce inflammation by inhibiting macrophage cytokine secretion	All	

a) aCD20, anti-CD20; aCD40, anti-CD40; aCD154, anti-CD154; aCD4, anti-CD4; aCD8, anti-CD8; ALG/ATG, anti-thymocyte globulin; Cs, corticosteroids; CTLA4-Ig, cytotoxic T-lymphocyte-associated protein 4-immunoglobulin; CVF, cobra-venom factor; mAb, monoclonal antibody; MMF, mycophenolate mofetil; Tac, tacrolimus; Tx, transplantation.

al., 2018; Bühler et al., 2000; Iwase et al., 2017; Mohiuddin et al., 2016). Notably, the novel anti-CD154 domain antibody (BMS-986004) lacks the risk of platelet activation and resultant thromboembolism but can effectively block CD40-CD154 interactions and may synergize with conventional immunosuppressive therapy (Kim et al., 2017). The anti-CD40 mAb has shown ground-breaking efficacy in pig heart (Mohiuddin et al., 2016), kidney (Iwase and Kobayashi, 2015), and liver (Shah et al., 2017) xenotransplantation in NHPs.

Experiences of xenotransplantation in pigs-to-NHPs

The Old World NHPs (e.g., baboons, rhesus monkeys) have been proven to be similar to humans with regard to the immune system (Cooper et al., 2019) and can serve as suitable recipients in preclinical xenotransplantation trials. In this review, we highlight representative xenotransplantation cases in NHPs from extensive experimental pools and describe recent important advances (Figure 2).

Kidney

Because of the simple surgical procedure and physiological function, renal transplantation was performed earlier than that of other solid organs in xenotransplantation. Both experiences in human beings (Reemtsma, 1989) and NHPs (Adams et al., 2018; Iwase et al., 2018) suggested that renal xenotransplantation had relatively satisfactory outcomes. In 2015, GalT-KO/hCD55 background pig kidneys were transplanted into T-cell depleted rhesus macaques with administration of anti-CD154 mAb, and the resultant longest survival time of kidney graft was more than 125 days (Higginbotham et al., 2015). Notably, consumptive coagulopathy and proteinuria were both delayed in this successful case. Later, a GalT-KO/hCD46/hCD55/hTM/hEPCR/hCD39 pig kidney xenograft functioned for 136 days in a baboon recipient (Iwase et al., 2015). The anti-CD154 mAb was replaced by the anti-CD40 mAb in this report, and the results showed that these two kinds of immunosuppressants have equal benefits in kidney xenotransplant. Iwase et al. (2017) then transplanted a GalT-KO/hCD46/hCD55/hEPCR/hTFPI/hCD47 pig kidney into a baboon and prolonged xenograft survival up to 260 days.

Major progress in pig kidney xenotransplantation was reported in 2018; rhesus macaques receiving pretransplant T cell depletion were used as recipients this time, and anti-CD154 mAb and MMF were additionally administered. In the end, the longest survival of recipients with functional GalT-KO/β4GalNT2-KO xenografts was 435 days (Adams

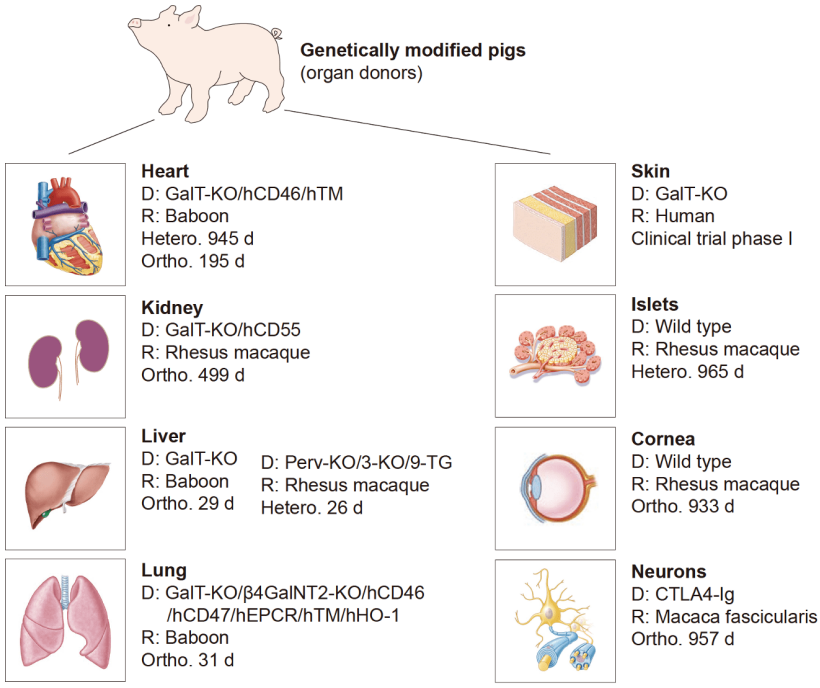

Figure 2 Genetically modified pigs as donors for xenotransplantation and representative survival times of xenografts in NHPs. Heterotopic hearts from GalT-KO/hCD46/hTM pigs survived for 945 days (Mohiuddin et al., 2016). Orthotopic hearts from GalT-KO/hCD46/hTM pigs survived for 195 days (Längin et al., 2018). Life-supporting kidney grafts from GalT-KO/hCD55 pigs survived for 499 days (Kim et al., 2019). Orthotopic liver from GalT-KO pigs survived for 29 days (Shah et al., 2017). Heterotopic livers from Perv-KO/3-KO/9-TG pigs survived for 26 days, as shown recently by our group (data not shown). Orthotopic lungs from GalT-KO/β4GalNT2-KO/hCD46/hCD47/hEPCR/hTM/hHO-1 pigs survived for 31 days (Burdorf et al., 2019). Skin from GalT-KO pigs was used in a clinical trial to provisionally treat extensive burns. Pancreatic islets from wild-type pigs survived for 965 days (Shin et al., 2016). Corneas from wild-type pigs survived for 933 days (Choi et al., 2015). Neurons from CTLA4-Ig pigs survived for 957 days (Aron Badin et al., 2016; Meier et al., 2017). D, donor; R, recipient; Hetero, heterotopic transplant; Ortho, orthotopic transplant; Perv-KO, porcine endogenous retrovirus knockout; 3-KO, GGTA1/β4GalNT2/CMAH knockout; 9-TG, human CD46, CD55, CD59, β2M, HLA-E, CD47, THBD, TFPI, and CD39 transgenes.

et al., 2018). The pathology indicated that the kidney graft finally succumbed to antibody-meditated rejection and dysregulation of coagulation, demonstrating the necessity of additional xenoantigen deletion and human anticoagulation gene insertion. More recently, life-supporting renal xenotransplantation extended recipient survival up to 499 days in the absence of hypoalbuminemia and consumptive coagulopathy, and renal grafts maintained normal function with minimal proteinuria (Kim et al., 2019). Considering that the pig donor had a GalT-KO/hCD55 background, it is unclear whether additional modification is still necessary. However, these results showed that pretransplant depletion of $CD4^+$ T cells in recipients is essential for a long-term outcome. Even so, scientists remain optimistic about genetically multimodified pig kidney xenotransplant and predict it will be the first to be applied in clinical trials (Cooper et al., 2018a).

Heart

Notably, preclinical cardiac xenotransplantation is always divided into two kinds of procedures: orthotopic (life-supporting) and heterotopic (nonlife-supporting). Most performed experiments are heterotopic transplants in which a pig heart is implanted into the abdomen where the heart simply serves as a blood pipe (keep beating) and is easily monitored or biopsied. In 2012, a GalT-KO/hCD46 pig heart survived for 236 days in a baboon receiving anti-CD154 mAb-based immunosuppression and finally succumbed to thrombotic microangiopathy. Later, the same group used a pig heart with the GalT-KO/hCD46/hTM background and anti-CD40 mAb instead of anti-CD154 mAb to rescue dysregulation of coagulation in the heart xenograft. Xenograft survival generally extends for years and reaches up to 945 days in this model, and none of the subjects experienced consumptive coagulopathy or thrombocytopenia (Mohiuddin et al., 2016).

Even so, life-supporting orthotopic heart transplantation has more applications than heterotopic transplantation, providing us with definitive answers to the questions we raised. Due to the associated surgical technical problems and idiosyncrasies of pig hearts, recipient survival of orthotopic heart xenotransplant was restricted to only 57 days until 2018 (Byrne et al., 2011). On the basis of the experience of heterotopic transplantation, Längin et al. (2018) performed (i) nonischemic preservation with continuous

perfusion and (ii) control of post-transplantation growth to cardiac xenografts, successfully extending the survival record up to 195 days. In their experiment, no major immunosuppression-related infection was observed, indicating a potential tolerant state of recipient to the immunosuppression protocol. This success represents exciting progress in cardiac xenotransplantation, predicting a closer distance from future clinical trials.

Liver

Xenotransplantation of pig liver appears to be more difficult than that of pig hear, as shown by the more severe dysregulation of coagulation and particularly thrombocytopenia, which often results in spontaneous hemorrhage (Zhang et al., 2019). The underlying mechanisms have been proposed and explored: (i) release of tissue factor overactivates the coagulation cascade; (ii) depletion of endothelial anticoagulants and anti-platelet capacity amplifies activation; (iii) interspecies incompatibilities of coagulation-regulatory proteins aggravate dysregulation; (iv) liver sinusoidal endothelial cells (LSECs) and macrophages mediate sequestration and phagocytosis of leukocytes, erythrocytes, and platelets.

In addition, pig liver xenografts need to provide more physiological functions, including synthesis, metabolism, and detoxication (Trefts et al., 2017). Thus, in contrast to that in renal or cardiac xenotransplantation, the problem of interspecies incompatibilities in functional proteins seems to be more prominent, which could also account for the short-term survival of recipients following xenotransplantation of pig liver (a maximum of 29 days) (Shah et al., 2017). Notably, there is no obvious T or B cell infiltration in the liver grafts (Shah et al., 2016; Shah et al., 2017), which shows that immunosuppressive regimens are effective at the present stage. However, considering the experiences in liver allotransplantation, we still believe that pathological insult caused by xenogeneic adaptive immunity will be inevitable and emerge soon over time.

Lung

The lung possesses a specific fragile structure and shows many issues in pig-to-NHP lung xenotransplantation. Similar to pig liver xenografts, dysfunctional coagulation is also a major obstacle in lung transplant experiments (Cooper et al., 2018b), and recipient survival can only be measured in days during the long-term development of pig lung xenotransplantation. Thus, most preclinical trials chose to perform *ex vivo* pig lung perfusion with human blood instead of *in vivo* lung xenotransplantation in NHPs. Studies have found that the predominant mechanism of failure of xenogeneic lung grafts is the inflammatory process, leading to vascular barrier function injury with interstitial and tracheal edema (Burdorf et al., 2018).

Subsequently, Watanabe's group made the first progress in *in vivo* xenotransplantation of pig lung. They extended the median graft survival from 3.5 days to 8.7 days in baboons with lung xenografts from human-CD47 (hCD47)-expressing pigs (Burdorf et al., 2018), demonstrating that hCD47 could attenuate the xenogeneic inflammatory response and dysregulation of coagulation to a certain extent. Soon after this report, GalT-KO/hCD47/hCD55 transgenic pig lungs were used in their *in vivo* experiments, and the survival of NHP recipients reached up to 14 days (Watanabe et al., 2020). Even so, the limited survival suggests that additional strategies will be needed; specifically, more genetic modifications in donor pigs are necessary. Thus, GalT-KO/β4GalNT2-KO/hCD46/hCD47/hEPCR/hTM/hHO-1 pigs were recently used as donors, and a baboon receiving lung xenotransplant survived for 31 days, representing major progress in clinical applications.

Pancreatic islets

Islet xenotransplantation is mainly applied for treating type 1 diabetes (van der Windt et al., 2012). Distinct from the features of the immune/inflammatory response of solid organ xenotransplantation, instant blood-mediated inflammatory reaction (IBMIR) (Bennet et al., 1999) and T-cell-mediated rejection are notable in islet xenotransplantation, which results in approximately 70% of the loss of portal vein transplanted islets (Liu et al., 2017). Delayed revascularization within xenogeneic islets may also contribute to early graft failure (Del Toro-Arreola et al., 2016). In an attempt to protect islets from IBMIR and xenogeneic immune damage and to increase porcine insulin production (Cooper et al., 2016b), researchers have developed various genetically modified pigs. Moreover, encapsulated islet transplantation has been explored (Cooper et al., 2016b), in which islets are encased in some form of protective capture or device, protecting they from immune/inflammatory insults from recipients, as well as allowing insulin to be released accordingly (note: This approach has been used in clinical trials (Shimoda and Matsumoto, 2017)). Currently, xenotransplantation of pig islets in diabetic NHPs has achieved relatively satisfactory success, with maintenance of insulin-independent normoglycemia for over 900 days (Shin et al., 2016).

Skin

Skin transplantation is a standard of care for deep second- and third-degree massive burn wounds. Recently, through an Food and Drug Administration (FDA)-cleared phase one clinical trial, burn specialists successfully used live-cell, genetically engineered pig skin for the temporary closure of a

burn wound. In this case, the pig skin graft was placed next to the cadaveric skin graft, both of which were adherent to the underlying wound bed and appeared indistinguishable from each other. No adverse events were further observed, and the wound was then treated further with a skin graft harvested from the patient′s own thigh. Healing progressed as anticipated. This procedure marks the first-time tissue derived from genetically engineered pigs was transplanted directly onto a human wound (data available from URL: https://healthperiodical.com/2019/10/14/pig-skin-graft-can-treat-human-burn-wounds/).

Pathological obstacles in xenotransplantation

Conventionally, there are four major pathological obstacles preventing pig xenotransplantation in clinical applications: (i) hyperacute rejection (HAR); (ii) acute vascular rejection (AVR); (iii) acute cellular rejection; and (iv) dysfunction of coagulation. However, HAR has already been largely prevented by the deletion of expression of Gal antigens in the graft (in a GalT-KO pig) (Zhang et al., 2019). Thus, AVR is a prominent problem impeding the progress of xenotransplantation of pig organs. Although AVR is driven by interactions between graft ECs and recipient xenoantibodies, macrophages, and platelets (Candinas and Adams, 2000) and is characterized by an inflammatory infiltration of mostly macrophages and natural killer cells (with small numbers of T cells), intravascular thrombosis, and fibrinoid necrosis of vessel walls (Candinas and Adams, 2000), the exact process is much more complex and is currently not completely understood.

Briefly, the nonGal antibodies bind to the donor ECs to activate recipient macrophages as well as the ECs themselves. This activation ultimately leads to the development of a procoagulant state, the secretion of inflammatory cytokines and chemokines, and the expression of leukocyte adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (Platt et al., 1998). More importantly, this injurious response might be further perpetuated with molecular incompatibilities between pig donors and NHPs. Moreover, the sequestration of leukocytes, erythrocytes, and platelets occurs, associated with adhesion and capture mechanisms, and these processes could also be exacerbated by increased cytokine production (Zhang et al., 2017), cell desialylation (French et al., 2017), and interspecies incompatibilities (Barclay and Van den Berg, 2014). Of note, cross-species incompatibility between CD47 (in the donor organ) and signal regulatory protein-α (SIRP-α) (in the recipient) contributes to phagocytosis of recipient blood cells (Ide et al., 2007). Species discordance of negative regulatory proteins, such as HLA-E on porcine ECs, facilitates NK cell-mediated cytotoxicity in an antibody-dependent or antibody-independent manner (Lilienfeld et al., 2007).

Classic acute cellular rejection (predominantly mediated by lymphocytes) has been reported in relatively long-term survival (e.g., heterotopic heart xenotransplant), which might explain why the major focus has been directed at the inflammatory response and coagulation disorders. Nevertheless, suppression of the adaptive immune response by T cell signal 2 costimulation blockade (Bühler et al., 2000) has proven very successful after xenotransplantation of pig heart (Mohiuddin et al., 2014; Mohiuddin et al., 2016) and kidney (Adams et al., 2018; Higginbotham et al., 2015). In *in vitro* experiments, thrombin-activated pig ECs increased human T cell proliferation via upregulation of CD86 on pig ECs (Ezzelarab et al., 2012). Recent xenotransplantation of *in vivo* pig organ suggested that cellular rejection could be effectively inhibited by a combination of conventional immunosuppressive therapy and anti-CD40mAb (Längin et al., 2018; Shah et al., 2017), although the contribution of costimulation blockade to this regimen is probably more important.

Dysfunction of coagulation is represented by the formation of thrombotic microangiopathy (TMA) in the pig grafts and the occurrence of systemic consumptive coagulopathy in the recipients. Studies have shown that this dysregulation of coagulation appears to be more severe after pig liver transplantation than after pig heart or kidney transplantation (Knosalla et al., 2009) and ultimately results in lethal hemorrhage. Tissue factor (TF) derived from pig donors or recipients initiates the coagulation cascade (Ekser et al., 2012b), (Zelaya et al., 2018), which is intricately associated with inflammation and innate immunity (Long et al., 2016). Depletion of endothelial anticoagulant and antiplatelet capacity amplifies this activation (Yau et al., 2015), and the absence of expression of effective coagulation-regulatory proteins, e.g., human tissue factor pathway inhibitor (TFPI) or thrombomodulin, eventually results in hemorrhagic complications (Ji et al., 2015).

The coagulation cascade initiated by TF appears to be overactivated after xenotransplantation of pig organs and exacerbates the complex network of inflammation, xenogeneic immunity, and coagulation. Notably, inactivated ECs, monocytes, or platelets do not express TF until exposed to inflammatory molecules. Studies have revealed that xenogeneic insult can activate ECs to expose subendothelial TF, which eventually initiates the coagulation cascade (Ekser et al., 2012b). Importantly, this may be independent of the xenoreactive immune response.

TMA and consumptive coagulopathy can occur in the presence or absence of features of acute humoral xenograft rejection (Cowan et al., 2011; Ekser et al., 2012b), and dysregulated coagulation is associated with a failure to prevent the anti-nonGal antibody response (Lin et al., 2010).

Inefficient inhibition of activated clotting factors or other components contributes to the overactivated coagulation cascade, and interspecies molecular incompatibilities account for defective anticoagulant activity (Wang et al., 2018).

Physiological incompatibility

In addition to immunological incompatibilities, physiological differences between pig organs and primate recipients deserve more attention. It is presumable that intrinsic growth regulatory properties of the transplanted pig heart lead to detrimental overgrowth of the xenograft, and inactivation of the growth hormone receptor (GHR) gene in donor pigs could reduce the sizes of both body weight and internal organs (Hinrichs et al., 2018). Recently, GHR-KO donor pigs with a GalT-KO/hCD46/hTM background have been generated to prevent orthotopic transplanted heart xenografts from overgrowth in baboons (Hinrichs et al., 2019). In kidney xenotransplantation, an early concern is post-transplant proteinuria, which has not been observed in more recent studies, suggesting that it is associated with the immune response and that there is no clinically significant physiological incompatibilities in renal function between pigs and primates (Wijkstrom et al., 2017), except for possible discrepancies between pig erythropoietin and the primate erythropoietin receptor (Iwase et al., 2018). In contrast to heart and kidney xenografts, liver xenografts show a more complicated situation. It is likely that not all of the 2000 proteins produced in the pig liver will function adequately in humans (Ibrahim et al., 2006). For example, scientists have examined species-specific differences between primary human and porcine hepatocytes in the regulation of coagulation protein expression and function (Ramackers et al., 2014). If this proves to be the case, pigs can be genetically engineered to produce the specific human protein required.

Cross-species infections

Most disease-causing organisms from pig donors can be eradicated through cesarean birth and/or designated pathogen-free (DPF) environment breeding (Scobie et al., 2018), except for porcine endogenous retrovirus (PERV), which is carried as part of the porcine genome (Fiebig et al., 2018). The potential risk of PERV infection for xenotransplant patients, clinicians, regulators and the general public is now the major concern ahead of clinical application (McGregor et al., 2018). The US FDA has renewed guidelines for clinical xenotransplantation and required establishing procedures and assays to monitor the potential for PERV cross-species transmission. Although current experiences suggest that PERV does not pose a substantial infectious risk to recipients (Denner, 2018), the "The 2018 Changsha Communique" (Hawthorne et al., 2019) recommended that the potential of cross-species transmission is minimal and manageable, and regulatory agencies enable the phase transition to clinical development under proper regulatory oversight. PERV knockout (PERV-KO) in pig donors is still the best choice to avoid this risk. Niu et al. (2017) successfully generated PERV-KO pigs with CRISPR-Cas9 gene editing technology. Nevertheless, dissenting voices about the necessity of PERV-KO soon arose in academic circles: (i) the frequency of karyotype anomalies in PERV engineered pigs raises the possibility of unforeseen genomic changes (McGregor et al., 2018); (ii) pigs with fully degenerate PERV sequences show

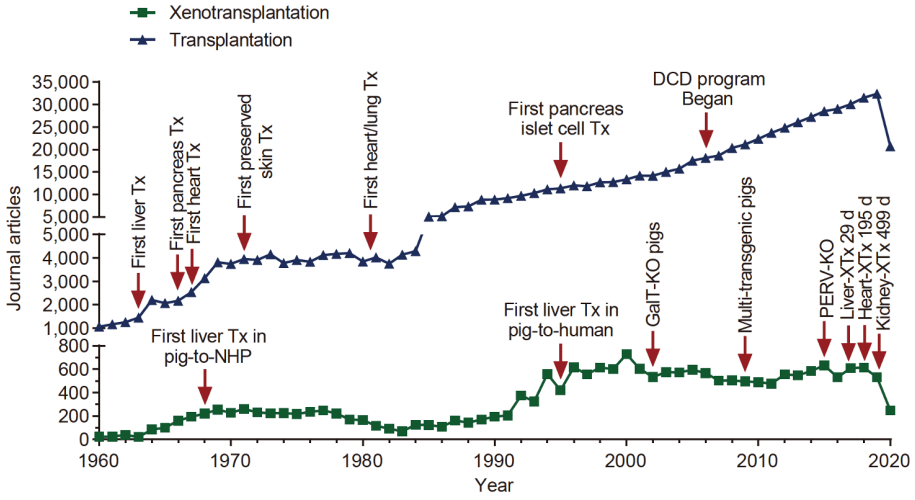

Figure 3 Published journal articles in the field of xenotransplantation (excluding tumor transplantation) have been increasing since the 1960s. A PubMed search of original articles for the term "xenotransplantation" excluding (AND NOT) "tumor" was performed for the years between 1960 and 2020. The exclusion criterion was aimed at removing tumor implant studies. In addition, a "Transplantation" AND NOT "tumor" search was also performed. Black arrows indicate probable events that positively impacted the field of transplantation and xenotransplantation. DCD, donated after cardiac death; GalT-KO, α-1,3-galactosyltransferase knockout; NHP, nonhuman primate; PERV, porcine endogenous retrovirus; Tx, transplantation.

a reduced *in vitro* frequency of infection and thus are expected to proportionately reduce the likelihood of *in vivo* infection (McGregor et al., 2018); (iii) human-tropic PERV is susceptible to antiviral therapies (Argaw et al., 2016).

Despite these considerations, PERV-inactivated pigs with additional multigene modification have been produced recently (Yue et al., 2019), providing the possibility of US FDA authorization for clinical xenotransplantation.

Concluding remarks

In the past ten years, sequential advances, including increasingly sophisticated genetically engineered pig donors and *de novo* immunosuppressive regimens, have significantly increased pig graft survival in NHPs. Accordingly, the function of pig xenografts can be more reliably assessed before clinical application. Our experience in the management of NHP recipients with pig xenografts as well as the range of investigations required in preclinical trials has increased (Figure 3). With the update of official guidelines for clinical pig xenotransplantation, we believe it will bring curative therapy to millions of patients with potentially life-threatening diseases or conditions that seriously impact quality of life.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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