REVIEW

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Special Topic: Gene Editing towards Translation

Multiple gene modifications of pigs for overcoming obstacles of xenotransplantation

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Abstract: Xenotransplantation, involving animal organ transplantation into humans to address the human organ shortage, has been studied since the 17th century. Early attempts to obtain organs from animals such as goats, dogs, and non-human primates proved unsuccessful. In the 1990s, scientists agreed that pigs were the most suitable donor animals for xenotransplantation. However, immune rejection between pig and human has hindered the application. To overcome these challenges, researchers developed genetically modified pigs that deactivate xenoreactive antigen genes and express human protective genes. These advances extended xenograft survival from days to years in non-human primates, resulting in the first human heart xenotransplant trial. Using genetically engineered pigs for the organ shortage is promising. This review provides an overview of potential incompatibilities of immunogenicity and functional proteins related to xenotransplantation between humans and pigs. Furthermore, it elucidates possible approaches for multiplex gene modification to breed better-humanized pigs for clinical xenotransplantation.

Keywords: xenotransplantation, pig, immune rejection, genetic modification

Introduction

Patients with end-stage organ failure can only be saved life by accepting organ transplantation from brain-dead patients or volunteer donors. According to the World Health Organization, approximately two million people worldwide urgently need organ transplantation each year, but only one in 20 of them is lucky to get a donor organ. Although the government's legislation encourages organ donation, it still falls far short of the need. To overcome the imbalance of supply and demand, researchers have attempted to use animal organs to replace human ones, which is termed as xenotranspaltion.

Obtaining organs from many animals such as goats, dogs [1–3], and non-human primates [4,5] for xenotransplantation to support human life has been studied since the 17th century, but these attempts invariably

failed. In 1990s of last century, scientists in the organ transplation community turned thier efforts towards using pigs as the source of organ donor for xenotransplantation [6], given that pigs have many advantages over other animal species, including easy availability, low ethic and safety concern, as well as a close similarity to humans in anatomy, physiology and metabolism.

The primary barrier to using pigs as organ donors for humans is the incompatibility of the immune systems between the two species, which inevitably causes a series of immune rejections. These rejections can be classified into hyperacute rejection, delayed xenograft rejection, and chronic rejection, which occur as a continuous process and cannot be distinguished distinctly. Hyperacute rejection (HAR) is the first immune rejection, occurring within minutes or hours after pig organs are transplanted into humans or NHPs. This is defined as the binding of pre-existing antibodies in the recipient's serum to antigens expressed in vascular endothelial cells of the graft, which activates the complement system and leads to graft destruction [7]. The primary cause of HAR is α-1,3-galactose epitope, which is synthesized with the participation of α-1,3-galactotransferase (*GGTA1*) gene in pigs. Delayed xenograft rejection (DXR) is the second immune rejection, following HAR, and occurs within days or weeks after pig organs are transplanted into humans or NHPs, and it is mainly caused by the minor antigen epitopes and the recruitment of innate immune cells [8]. Two minor non-Gal epitopes, *CMAH* and *B4GalNT2*, which encode carbohydrate *N*-glycolyl neuraminic sialic acid (Neu5Gc) and glycan SDa [9], respectively, have been identified to cause antibody-mediated rejection. Innate immune cellular xenograft rejection is mainly caused by natural killer (NK) cells and macrophages. Coagulation disorders after transplantation are also an important component of DXR [10]. Graft rejection can cause damage to vascular endothelial cells, leading to exposure of collagen and tissue factors to the blood. Collagen can activate platelets, while tissue factors trigger the production of thrombin, which converts fibrinogen into fibrin. Both of these factors promote the formation of thrombus, leading to the development of thrombotic microangiopathy in grafts with the participation of inflammatory gene expression [11,12]. Chronic rejection (CR) usually appears within months and years [10]. The mechanism of chronic rejection has not been fully elucidated due to the rarity of xenotransplantation with long-term survival. Human lukemia antigen (HLA) and swine lukemia antigen (SLA) can share common epitopes, which may cause cross-reactivity between humans and pigs. Studies have shown that human CD8+T cells can recognize SLA-I and trigger an immune response [13]. Similar to allotransplantation, HLA-II recognizes porcine xenoantigens, presents them, and activates host's CD4+ cells to trigger an immune response. Persistent inflammation response may also play an important role in the process of CR. The role of pro-inflammatory cytokines, such as IL-6, TNF-α, and IL-17, still needs further investigation in xenotransplantation [14]. The histopathological features of CR are mainly associated with thrombotic microangiopathy, which is characterized by graft vascular endothelial cell proliferation, vascular stenosis, interstitial fibrosis, and ultimately graft failure [15].

Numerous of proteins are involved in incompatibility of the immune systems between pigs and humans. Therefore, multiple genetic modifications of the pig genome are necessary to reduce the molecular incompatibilities between pigs and humans. The first milestone of xenotransplantation from pigs to humans was achieved in 2002, in which pigs with a deficiencyof α-1,3-galactosyltransferase were generated [16], thereby resolving the issue of hyperacute rejection. With *GGTA1*-deficient pigs, successful long-term xenograft survival was achieved when pig hearts were transplanted into NHP [17]. With the programmable nucleases technologies for gene editing coming into use about a decade ago, breeding genetically modified

pigs become a easy practice. Subsequently, many more intensive efforts have been made to overcome the hurdle of immune rejections through genetic modification of pig genome, including the elimination of xenoreactive antigens in pigs and the overexpression of human protective proteins to protect pig xenografts [9]. As a result, the survival term of pig-derived xenografts in NHP has been subtantally extended. Furthermore, most recently, the first clinical trial of humanized pig heart xenotransplantation [18] and two cases of pig kidneys in brain-dead human recipients [19] have been particularly striking, which makes scientists see the dawn in the long journey of exploring the feasibility of transplanting pig organs into human [20–23].

Eilmination of xenoreactive antigens in pigs

The α-Gal epitope is synthesized on the cell surface of non-primate mammals, including pigs, through the action of α-1,3-galactosyltransferase (alpha1,3GT). However, it was inactivated in ancestral Old World primates due to a frameshift mutation [24]. It is the most detrimental antigen in xenotransplantation, as it causes hyperacute rejection within minutes to hours. The natural antibodis in primates' blood bind to α-Gal epitopes present on porcine endothelial cells, causing activation of the complement system, resulting in lysis of the endothelial cells and activation of the coagulation reaction [25]. The anti-Gal antibody is the most abundant antibody in human blood, including IgG and IgM antibody classes, and constitutes approximately 1%–4% of circulating human immunoglobulins [26]. The natural antibody against α-Gal epitopes in neonatal primates is absent, and it begins to develop after birth in response to gut bacteria that contain antigens on their cell walls or capsids. When an infant is 2 months old, the circulating natural anti-Gal antibody level is comparable to that in adults [27].

α-1,3-Galactosyltransferase knockout pigs (GalT-KO) were produced by nuclear transfer technology in the early 2000s [16] and successfully eliminated α-Gal epitopes. This achievement was a milestone in overcoming a major hurdle in xenotransplantation. Using organs from the GalT-KO pigs to perform xenotransplantation in NHPs, more xeno-antigens have been indentified later. After transplanting kidneys from alpha1,3-galactosyltransferase knockout pigs into baboons, non-Gal antibodies against GalT-KO porcine cells have begun to emerge, presenting an additional barrier to transplantation [8]. This phenomenon is known as delayed xenograft rejection (DXR). Two non-Gal antigens associated with DXR were identified after the pig *GGTA1* gene was knocked out. The first antigen is Neu5Gc, which is encoded by the cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH). CMAH is responsible for the hydroxylation of *N*-acetyl neuraminic acid (Neu5Ac) to *N*-glycolylneuraminic acid (Neu5Gc) on the cells of all mammals except humans [28]. The second antigen is SDa, encoded by the porcine beta-1,4-*N*-acetylgalactosaminyltransferase 2 (β*4GalNT2*) gene, which is involved in the transfer of *N*-acetylgalactosamine (GalNAc) to galactose present in the alpha-2,3-sialic acid chains [29]. These two non-Gal antigens are presented on multiple pig cell surfaces and are recognized by natural antibodies in human sera. Triple-knockout pig endogenous genes, including *GGTA1*, *CMAH*, and β*4GalNT2*, decrease the binding ratio of human natural antibody IgG/IgM to pig PBMCs, preventing natural antibody-mediated rejection [30,31].

Overexpression of human proteins to protect pig xenografts

Although the removal of three known antigen genes reduces natural antibody-mediated rejection, the

molecular incompatibility between pigs and humans still makes xenografts vulnerable to other types of rejection, such as complement response, thrombosis, inflammation, and cellular killing [25]. To overcome these issues, additional genetic modifications have to be introduced into the pig genome to provide corresponding protection. Currently, more than 20 human genes have been confirmed to attenuate complement reactions, coagulation, anti-inflammatory responses, and cellular reactions [32].

Overexpression of human complement-regulating proteins

The complement system is part of the innate immune system and plays an important role in xenotransplantation immune rejection. It consists of approximately 50 proteins and protein fragments, including serum proteins and cell membrane receptors [33]. Antibodies binding to porcine cell surface antigens and the potential incompatibility of porcine complement regulatory proteins (CRPs) with the primate complement system could activate the complement system *via* three biochemical pathways. Three human CRPs, including CD55, CD46, and CD59, are reported to alleviate complement reactions through different mechanisms. Among them, decay-accelerating factor (DAF; CD55) inhibits the complement cascade by dissociating the multimolecular C3 [34], and membrane cofactor protein (MCP; CD46) is the key molecule involved in cell protection against autologous complement, thereby restricting the action of the complement system. As for CD59, it is an inhibitor of the complement system, found on erythrocytes and on many other cell types [35]. Genetically modified pigs that express human CD55, CD46, and CD59, separately or in combination, could significantly decrease susceptibility to the complement system [36]. Pigs that delete three xenoantigen genes and express CRPs simultaneously can significantly extend the survival of pig-to-NHP xenograft.

Overexpression of human coagulation-regulate proteins

Thrombotic microangiopathy and systemic consumptive coagulopathy are common pathological phenomena observed in pig-to-NHP xenotransplantation studies. These conditions are caused by several factors, including antibody-mediated complement response, inflammation, innate, humoral, and cellular immune responses [37]. Molecular incompatibilities between pigs and primates exacerbate these processes, leading to the destruction of the xenograft blood vessel endothelial cells and subsequent tissue infiltration by various immune cells. Key endothelial anticoagulant/antithrombotic proteins, such as thrombomodulin (TBM, CD141), endothelial protein C receptor (EPCR), tissue factor pathway inhibitor (TFPI), CD39, and von Willebrand Factor (vWF), have been reported to play a crucial role in regulating these processes.

In order to address coagulation dysfunction in pig-to-NHP xenotransplantation, donor pigs that express human clotting inhibitors have been produced. Pig thrombomodulin (pTBM) has been found to be unable to activate human anticoagulant protein C, leading to aberrant activation of coagulation [38]. However, the expression of human thrombomodulin in porcine aortic endothelial cells could inhibit this process. Studies have demonstrated that *GGTA1* knockout combined with hCD46 and hTBM expression in pigs could sustain long-term heterotopic cardiac xenograft survival beyond 900 days [39]. Furthermore, life-supporting xenografted hearts from donors with a similar genotype enabled baboon survival up to 195 days [40]. A comparative study also confirmed the critical role of human TBM in cardiac xenograft survival [41].

The endothelial protein C receptor (EPCR) is a transmembrane-anchored molecule that greatly enhances

the activity of human thrombomodulin (hTBM). To achieve the long-term survival, hEPCR and hTBM have been introduced into pig's genome, and *in vivo* studies have demonstrated that this approach can significantly extend kidney survival, with some cases extended up to 183 days [42]. While a recent study has suggested that pig EPCR protein may be compatible with the human TBM/thrombin complex [43]. However, it is important to note that human EPCR has additional beneficial effects beyond the regulation of clotting. These include anti-inflammatory, anti-apoptotic, and cytoprotective effects [44]. Therefore, the inclusion of hEPCR in transgenic strategies may still be desirable for maximzing the potential benefits of xeno-transplantation.

The pig von Willebrand Factor (pvWF) is another incompatible protein between pigs and primates. The presence of pvWF can lead to spontaneous platelet adhesion and activation. To address this issue, pigs with vWF gene knockout have been generated by zygote injection of Cas9 mRNA and sgRNA, and the FVIII level was decreased in the plasma of vWF-null pigs. However, the bleeding time of biallelic mutant pigs was much longer than that of wild-type pigs, potentially resulting in adverse effects on pig health [45]. To overcome this challenge, the Connolly group [46] has developed a strategy to replace the glycoprotein Ib-binding site of pvWF protein with the human's ortholog, which could prevent physiologically inappropriate activation of primate platelets while preserving platelet activation function.

CD39, also known as ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1), is an NTPDase that prevents platelet aggregation triggered by ADP [25]. CD39 hydrolyzes ADP to AMP and subsequently to adenosine, thereby inhibiting platelet aggregation. So, the expression of hCD39 is often used in combination with other genetic modifications within transgenic pigs. The Choi group [47] has developed transgenic pigs expression hCD55 and hCD39 within a GT-KO background (GT-KO/hCD55/hCD39), and an *in vitro* study showed that platelet aggregation was inhibited at ADP concentrations of 10 μM. This suggests that the expression of human CD39 in pigs can effectively prevent platelet aggregation. In another study, Professor David Cooper [42] reported that kidneys from GT-KO/hCD46/hCD55/hTBM/hEPCR/hCD39 pigs survived up to 136 days in the NHP model. Furthermore, the Wheeler group [48] confirmed that the expression of human CD39 in pigs could protect against myocardial injury.

Overexpression of anti-inflammatory proteins

The role of inflammation has become an increasingly important area of research in xenotransplantation research. Two human proteins have been identified as effective in mitigating inflammation. One of these proteins is human heme oxygenase-1 (hHO-1), which is capable of degrading heme to bilirubin, carbon monoxide, and free iron, thereby protecting pig cells from the pro-inflammatory and oxidative properties of free heme [49]. Genetic strategies for source pigs often involve hHO-1 expression [50,51], but the effect of hHO-1 alone has not been fully assessed.

Another protein that has been found to be effective in mitigating inflammation is human A20 (hA20). This protein is a tumor necrosis factor-alpha (TNF-a)-inducible gene with protective features against inflammatory and apoptotic stimuli in various cell types, including endothelial cells. Genetically modified pigs have been developed to express hA20 [52]. And *in vitro* studies have shown that hA20-transgenic pig cells are protected against TNF-α-mediated apoptosis and partially protected against CD95L(FasL)-mediated cell death. It has been reported that GT-KO/hHO-1/hA20 transgenic pig kidneys could alleviate rejection and

ischemia-reperfusion damage [53]. However, more research is needed to fully understand the potential benefits and limitations of the effect of hHO-1 and hA20 on xenograft survival in NHP models.

Overexpresssion of cellular-rejection regulating proteins

Macrophage-regulating protein

Macrophages are immune cells that can exert direct toxic effects on xenografts by producing pro-inflammatory cytokines such as TNFα, IL-1, and IL-6. CD47 is a transmembrane protein that can interact with SIRPα to inhibit macrophage activation [54]. It is a well-known "do not eat me" signal. However, interspecies incompatibilities between pig and primate render pig CD47 ineffective at suppressing human macrophage activity. As a result, some research groups have developed source pigs that express human CD47, which has been shown to prolong the survival of pig kidney grafts [55]. CD24 [56], like CD47, is another "do not eat me" signal that has been recently found to promote macrophage disability through interaction with the inhibitory receptor Siglec-10. Thus, overexpression of hCD24 may be a potential candidate for breeding genetically modified pigs. Another strategy involves expressing human IL-10 in a PK15 cell line which has been shown to decrease macrophage cytotoxicity *in vitro* [57], providing an alternative factor to overcome cytotoxic effects of macrophages.

NK cell-regulating proteins

Human natural killer (NK) cells are a critical effector component of cellular rejection, which remains a significant hurdle for xenograft survival. Activated NK cells can destroy xenografts by releasing cytotoxic granules which could directly lysis porcine endothelial cells, and recruiting other immune cells through cytokine secretion [58]. To mitigate NK cell-mediated rejection, various strategies have been proposed, including eliminating potential porcine ligands for activating NK cell receptors, expressing human MHC class I molecules on the surface of pig cells, and blocking molecular events leading to NK recruitment. Porcine UL16-binding protein 1 (pULBP1) expressed on endothelial cells has been identified as the primary functional ligand for the human NKG2D receptor on NK cells. Two groups have generated *ULBP1* knockout pigs and found that the cytotoxicity of human NK cells was reduced [59,60]. This suggests that elimination of the pig *ULBP1* gene may be a promising strategy for mitigating NK cell-mediated rejection.

Strategies aimed at inhibiting interactions between NKp44 and NKG2D on human NK cells through the expression of human MHC I molecules may prevent direct NK responses against xenografts [61]. By presenting human MHC I molecules on the surface of pig cells, the activation and cytotoxicity of NK cells may be reduced. Genetically modified pigs with HLA-E or HLA-G expression have been produced, and *in vitro* studies have shown that both of these molecules can reduce NK cell mediated rejection [62,63]. However, *in vivo* studies from these pigs have not yet been reported.

T cell-regulateing protein

T-cell-mediated rejection poses a significant challenge to the long-term survival of xenografts. To reduce T-cell responses after pig-to-NHP xenotransplantation, several strategies have been proposed. Major

histocompatibility complex (MHC) molecules play a pivotal role in chronic rejection by inducing T-cell-mediated rejection. It has been reported that MHC class I induces CD8+ T-cell-mediated rejection, while MHC class II molecules induce CD4+ T-cell-mediated rejection [64]. Pig MHC class I and class II molecules, also known as swine leukocyte antigen class I and class II (SLA-I and SLA-II), can be eliminated from pig cells to evade the recipient's T-cell responses and prolong xenograft survival. Beta2-microglobulin (*B2M*) is indispensable for the assembly of SLA-I receptors, and disrupting the B2M gene leads to the elimination of SLA class I molecules in pig cells. The Wang group successfully generated an SLA I-deficient pig by knocking out the *B2M* gene with TALENs, and skin grafts from these *B2M*-KO pigs exhibited remarkably prolonged survival [65], indicating that the knockout of the pig *B2M* gene represents an effective means of alleviating T-cell rejection. However, a report from the Sake group [66] found that disrupting the pig *B2M* gene had a negative impact on the animal's viability, including increasing the risk of infection and susceptibility to NK cell response. Therefore, *B2M*-KO pigs should be raised in a specialized facility that is free of germs to minimize the risk of infection

The Class II transactivator (*CIITA*) serves as the master regulator of MHC class II. By knocking out *CIITA* in combination with *GGTA1* and *B2M* simultaneously in pig, researchers have produced GBC-3KO pigs that are reported to effectively alleviate T-cell-mediated immune responses [67,68]. In addition to the knockout of the pig *CIITA* gene, another approach to reduce CD4(+) T-cell response involves the expression of a human dominant-negative mutant of *CIITA* (CIITA-DN) in the pig endothelium [69]. An *in vivo* study performed by the same group confirmed that the CIITA-DN gene could reduce the adaptive immune response [70].

In addition to genetic modification of SLA-I and SLA-II, the expression of proteins that inhibit T-cell activity represents an effective approach to reducing adaptive immunity. CTLA4-Ig is a protein that prevents the activation of naive T cells by binding to B7-proteins and blocking engagement of CD28 [71]. The Bähr group [72] established a genetically modified pig with ubiquitous expression of LEA29Y, a human CTLA4-Ig derivative. However, the pig was found to display an immune-compromised phenotype, indicating that the approach of ubiquitous CTLA4-Ig expression is not suitable. Alternative strategies for mitigating T cell-mediated rejection involve expressing CTLA4-Ig under the control of a tissue-specific promoter, which could limit the expression of CTLA4-Ig to specific tissues and minimize potential side risks. Guided by this idea, genetically modified pigs were bred to specifically express CTLA4-Ig in neurons [73], cornea [74] skin [75,76], and islets [77], which could protect pig tissues and cells from T-cell-mediated rejection and reduce side effects on the pig immune system.

The programmed cell death-1 (PD-1, CD279)/PD-ligand1 (PD-L1, CD274) receptor system plays crucial role in controlling the balance between immune activation and induction of tolerance via the generation of inhibitory signals [78]. The Ding group [79] confirmed that hPD-L1 expression in pig PIEC can recruit CD4 +CD25hiFoxp3+ Tregs, which have higher suppressive potency. Buermann group produced hPD-L1 transgenic pigs, and peripheral blood mononuclear cells (PBMCs) from these pigs exhibited a significantly reduced capacity to stimulate the proliferation of human CD4+ T cells. Furthermore, ubiquitous expression of human PD-L1 does not appear to affect health of sourse pig, as human PD-L1 does not functionally interact with porcine PD-1 [80].

Another potential approach to evade host T cell rejection is to express monoclonal antibodies in pig tissues. In an early stage of xenotransplantation study, an anti-human CD2 antibody was transduced into pig neonatal islet cell clusters (pNICC) mediated by adenovirus (Adv). These results confirmed that local secretion of a

antibody against T cells could effectively reduce rejection [81]. In a subsequent study, the coding sequence for the anti-CD2 monoclonal antibody was knocked into the pig *GGTA1* locus, which eliminated the αGal epitope and allowed for secretion of the antibody. However, the anti-rejection performance of this approach has not been evaluated *in vivo* [82].

Humanizing functionally incompatible proteins

Although long-term survival has been achieved in xenotransplantation, additional attention to the issues of physical incompatibility of organs between pigs and humans should be paid. The sequences of functional gene-encoded proteins in a specific organ may be different between the two species, thus diminishing the functions of the donor organ over time.

The preclinical trials have suggested that pig kidneys are compatible with human physiology, while a few additional barriers beyond immune rejections were observed in pig-to-NHP kidney xenotransplant studies. Studies have reported that baboons with life-supporting pig kidney grafts suffered from hypovolemia, low blood pressure, and increased serum creatinine, and these symptoms were confirmed by the absence of rejection on biopsy [83,84]. These results imply that porcine renin is unable to cleave human angiotensinogen, impairing the renin-angiotensin-aldosterone system (RAAS). The prospect of humanizing renin in pigs could possibly address compatibility issues during the transplantation of porcine kidneys into primates. However, this modification might inhibit the cleavage of porcine angiotensinogen, leading to impairments in the renin-angiotensin-aldosterone system (RAAS). Subsequently, completely substituting porcine renin with a human equivalent might adversely affect the health of the pigs. A potential solution to this issue could be the utilization of humanized kidneys from heterozygous pigs that have undergone knock-in modification of the human renin gene. This measure would result in the pig carrying both human and porcine renin, maintaining the health of the organ donor pigs, while also ensuring effective cleavage when the kidneys are transplanted into human patients. Another incomparable protein between pigs and humans is erythropoietin. Several groups have reported occurrence of anemia after pig-to-baboon kidney xenotransplantation [85,86], and treatment with exogenous EPO could mitigate it. Pig EPO is unable to stimulate the bone marrow to produce red blood cells, which might be one explanation for anemia. If pig EPO cannot directly activate human hematopoietic stem cells (HSCs), human EPO gene expression in pigs should be seriously considered for kidney xenotransplantation.

Insulin extracted from pig pancreas was applied to treat T1D in the early part of the last century. The effectiveness decreases because one amino acid at the carboxy-terminal of the B chain of pigs is different from humans (alanine in pigs and threonine in humans). This difference may diminish pig islet function in T1D patients in the long term after islet xenotransplantation. Yang *et al*. [87] substituted alanine with threonine in the pig insulin gene and produced a humanized pig that exclusively secretes human insulin, providing a better resource for islet xenotransplantation. However, C-peptide produced by the cleavage of proinsulin also plays an important physiological role [88], and pig C-peptide may not be compatible with the human receptor. A recent study has produced a human insulin transgenic pig based on primary pig insulin KO fibroblasts [89]. Human insulin and C-peptide could be detected in piglets, but the expression level of insulin was low. Completely replacing the pig insulin locus with human insulin would be a better choice.

Multiple genetic modification of pigs for xenotransplantation

Dozens of genes related to immune rejection and/or functional genes encoding incompatible proteins in a specific organ are crucial for the long-term survival and maintenance of the normal function of organs transplanted into humans (Table 1). Although various kinds of genetically engineered pigs are currently available (Table 2), none of them contains modifications that effectively address all hurdles associated with xenotransplantation. To achieve this goal, multiple gene modifications for eliminating antigen genes, overexpressing foreign genes, or replacing porcine functional genes with corresponding human genes should be implemented in pigs (Table 1).

The advent of artificial endonuclease-mediated gene editing tools, such as ZFN, TALEN, and CRISPR/Cas9 [109], particularly CRISPR-based toolkits, including base editors and prime editors [110,111], has led to significant advancements in precise genome editing.

Table 1 Genes related to long-term survival and normal function maintenance of transplated organs

Gene and protein		The types of modification required and protein expression
Xenoreactive genes	*Pig GGTA1*	Knockout
	Pig CMAH	Knockout
	Pig β4GalNT2	Knockout
Complement regulate proteins	Human CD46	Expression
	Human CD55	Expression
	Human CD59	Expression
Coagulation regulates proteins	Human TBM	Expression
	Human EPCR	Expression
	Human TFPI	Expression
	Human vWF	Partial replacement
	Pig *vWF*	Knockout
Anti-inflammatory proteins	Human CD39	Expression
	Human HO-1	Expression
	Human A20	Expression
	shTNFRI-Fc	Expression
Macrophage regulation proteins	Human CD47	Expression
	Human CD24	Expression
	Human IL-10	Expression
T cell regulation proteins	Pig *B2M*	Knockout
	Pig *CIITA*	Knockout
	Human B2M	Expression
	Human CIITA-DN	Expression
	Human CTLA4-Ig	Expression
	Human PD-L1	Expression
NK cell regulation proteins	Pig *ULBP1*	Knockout
	Human HLA-E	Expression
	Human HLA-G	Expression
Functional proteins	Human Renin	Replacement
	Human EPO	Replacement
	Human insulin	Replacement
	Human serum albumin	Replacement
	Growth hormone receptor	Knockout for reduction of porcine organ size

Table 2 Genetically modified pigs reported for xenotransplantation

Gene knockout	Expression of exogenous gene	Ref.
GGTA1		[16]
GGTA1, *CMAH*, *iGb3S*		[90]
	hHLA-E, hB2M	[91]
	hA20	[52]
	hHO-1	[92]
	hCIITA-DN	[69]
	hHO-1, hTNFRI-Fc	[93]
vWF		[45]
SLA I		[94]
GGTA1, *CMAH*, *B4GalNT2*		[31,95]
GGTA1	hHO-1, hA20	[53]
ASGR1		[96]
GGTA1, *CMAH*	hCD46, hCD55, hCD59, hHO1, hA20	[97]
GGTA1	hCD39	[98]
	CTLA4-Ig	[72,75]
B2M		[65,66]
GGTA1	hCD55, hCD39, hTFPI, C1-INH, hA20	[99]
GGTA1	Anti-CD2 antibody	[82]
GGTA1	shTNFRI-Fc, hHO-1	[100]
GGTA1, *CMAH*	shTNFRI-Fc, hHO-1	[101]
GGTA1	hCD55, hCD59	[102]
	hPD-L1	[80]
GGTA1, *CMAH*, *B4GalNT2*	hCD46, hCD55, hEPCR, hTBM, hHO-1,hCD47	[103]
GGTA1, *CMAH*, *B4GalNT2*, *SLA-1*	hCD46, hCD55, hCD59, hHO1, hA20	[104]
GGTA1, *CMAH*, *B4GalNT2*, *PERV*	hCD46, hCD55, hCD59, hB2M, hHLA-E, hCD47, hTHBD, hTFPI, and hCD39	[105]
GGTA1, *GHR*	hCD46, hTBM	[106]
GGTA1, *CMAH*, *B4GalNT2*	hCD46, hCD55, hCD59, hTM, hTFPI, hCD39, hCD47, hHLA-E.	[107]
GGTA1, *B2M*, *CIITA*		[67,68]
GGTA1	hCD46, hTBM	[108]
GGTA1	h*pvWF, hCD46	[46]

For eliminating antigen genes, out-of-frames by deleting or inserting fragments with different lengths [112], or the introduction of premature stop codons through base editing in exons [113] can be simultaneously created in multiple genes using artificial endonuclease-mediated gene editing tools to efficiently silence xenoreactive antigen genes in pigs.

To overexpress immune-protective foreign genes, many alternative methods have been established for introducing foreign DNA fragments into predefined genomic loci to ensure a desirable expression level of foreign genes, such as the CRISPR-associated transposon [114–118], homology-independent targeted integration (HITI) [119–122], single-strand oligonucleotide (ssODN) mediated seamless knock-in [87,123], and prime editor-mediated insertion of small DNA fragments [111,124,125]. The overexpression of most exogenous genes can be regulated by ubiquitous promoters [88,95–97]. However, for genes whose universal expression may result in reduced survival ability of pigs, such as CTLA4-Ig [71,72], a tissue-specific promoter should be selected to permit expression in a specific organ without harming the pig's health [73–76]. For genes that may impact embryonic development and reduce resistance to pathogens, elements for

conditional regulation of gene expression controlled by doxycycline could be incorporated into the pig genome. This allows them to develop and grow normally without expressing the inserted embryo-lethal genes. Meanwhile, the immune-tolerant gene could be activated under chemical induction right before their use in organ transplantation. For genes that require stable and high expression level, they can be incorporated into safe harbor loci such as *Rosa26* [126] and *HIPP11* [127]. Additionally, for the foreign genes requiring an appropriate expression level or natural expression level, they could be introduced to downstream of an internal gene promoter. For instance, endothelial-specific promoters are potentially suitable for coagulation-related transgenes [128].

For the replacement of porcine functional genes with corresponding human genes, both double-strand DNA homology (dsDNA) [129] and single-stranded oligodeoxynucleotides (ssODNs) [87] for homology-directed repair (HDR) could be used as donor template to be inserted into the porcine genes to be replaced. As a result, the porcine genes is nullified, only inserted human gene will be expressed. Compared with dsDNA, ssODNs is shorter and easie to synthesize, and could achieve higher integration efficiency. In addition, when ssODNs are used as HDR donors, selection marker gene is not necessary, which enables to create seamless site-specific mutations, reducing safety conern.

Conventionally, genetically modified pigs were produced using genetically modified somatic cells, followed by somatic cell nuclear transfer (SCNT). However, somatic cells display limited proliferative capacity, necessitating multiple rounds of somatic cell modification and SCNT to produce a pig strain with comprehensive genetic modifications for xenotransplantation. This procedure is labor-intensive and time-consuming. Fortunately, the recent establishment of porcine pluripotent stem cells [130,131], with their limitless proliferative ability, presents ideal cell lines for gene editing. Successive rounds of gene editing could be directly performed on these stem cells. While the germline competency of these stem cells has not yet been validated in pigs, ruling out the generation of genetically modified pigs through a technique similar to the embryonic chimeric approach used in mice, the stem cells could serve as donor nuclei to produce pigs with multiple genetic modifications through just a single round of nuclear transfer, as suggested by Fan *et al*. [132]. This approach, undoubtedly, would speed up the generation of pigs with multiple genetic modifications, significantly accelerating progress in xenotransplantation from pigs to humans.

Notably, the first clinical trial in 2022 involving the transplantation of the heart from a pig genetically modified with 10 genes into a human patient has provided preliminary validation for the necessity of multiple modifications [18,133]. Nonetheless, the strategy of overexpressing an excessive number of protective genes may not always be ideal due to the risk of redundant genetic elements, each of which could potentially burden the physiology and affect the pig's health or the function of a specific donor organ. Moreover, the potential synergies and mutual limitations among the inserted multiple genes remain insufficiently understood, highlighting the need for further comprehensive research on the clinical implications of xenotransplantation using pig models with different combination of multiple genetic modifications.

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Conflict of interest

The authors declare no conflict of interest.

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