REVIEW

Xenotransplantation: back to the future?

Raphael P. H. Meier[1,*], Yannick D. Muller[2,3,*], Alexandre Balaphas[1], Philippe Morel[1], Manuel Pascual[3], Jörg D. Seebach[2] & Leo H. Buhler[1]

1 Visceral and Transplant Surgery, University Hospitals of Geneva, Geneva, Switzerland
2 Division of Clinical Immunology and Allergy, Department of Medical Specialties, University Hospitals and Medical Faculty, Geneva, Switzerland
3 Transplantation Center, Lausanne University Hospital, Lausanne, Switzerland

Correspondence
Dr. Raphael P. H. Meier and Dr. Yannick D. Muller, Geneva University Hospitals, Rue Gabrielle-Perret-Gentil 4, 1211 Geneva, Switzerland.
Tel.: +41 22 372 33 11;
fax: +41 22 372 77 81;
e-mails: raphael.meier@hcuge.ch (RPHM); Yannick.muller@hcuge.ch (YDM)

*These authors contributed equally.

SUMMARY

The field of xenotransplantation has fluctuated between great optimism and doubts over the last 50 years. The initial clinical attempts were extremely ambitious but faced technical and ethical issues that prompted the research community to go back to preclinical studies. Important players left the field due to perceived xenozoonotic risks and the lack of progress in pig-to-nonhuman-primate transplant models. Initial apparently unsurmountable issues appear now to be possible to overcome due to progress of genetic engineering, allowing the generation of multiple-xenoantigen knockout pigs that express human transgenes and the genomewide inactivation of porcine endogenous retroviruses. These important steps forward were made possible by new genome editing technologies, such as CRISPR/Cas9, allowing researchers to precisely remove or insert genes anywhere in the genome. An additional emerging perspective is the possibility of growing humanized organs in pigs using blastocyst complementation. This article summarizes the current advances in xenotransplantation research in nonhuman primates, and it describes the newly developed genome editing technology tools and interspecific organ generation.

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Key words
blastocyst complementation, cell transplantation, CRISPR Cas/9, genome editing technologies, interspecific organ generation, nonhuman primates, nucleases, safety, TALEN, transplantation, xenotransplantation, xenozoonosis

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Introduction

Xenotransplantation has made tremendous progresses over the last 10 years [1–3]. Notably, pig kidneys can be functional for more than 1 year in rhesus macaques [4–6], pig islets reverse diabetes for more than 2.5 years in nonhuman primates (NHP) [7], and heterotopic pig heart survival in baboons also exceed 2.5 years [8]. These achievements largely benefited from new technologies, including genome editing tools, such as zinc finger nucleases, TALEN, and CRISPR/Cas9 technologies. Now, the genome of large animals can be more easily manipulated resulting in multiple gene knockouts (KO), human transgene insertions, and, more recently, specific animal organ KO and replacement with a humanized organ. For the recipients, the use of costimulation blockade with anti-CD154 has progressively been replaced by CD40-specific blockade [8], which should be compatible with clinical use. Other recent improvements made in immunosuppressive/immunomodulation therapy include the use of IL-6 receptor antagonist [9,10] such as tocilizumab and the perspective of using anti-C5a drugs such as eculizumab [11]. Improvements are still needed for liver xenotransplantation as severe coagulation and xenoprotein compatibility issues have to be resolved. Lung

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transplantation is mostly limited to ex vivo experiments so far. Finally, regarding safety issues, the recent genomewide inactivation of porcine endogenous retroviruses represents a very interesting advance [12].

In this article, we aim to (i) report the current survival of xenotransplanted organs and cells in preclinical models; (ii) describe the new genome editing technologies; (iii) summarize the available KO/transgenic pigs; (iv) describe the most recent advances in stem cell technologies and their utilization for chimera generation; and (v) discuss the latest advances in terms of safety.

Mechanisms of rejection involved in xenotransplantation

Xenografted organs trigger both humoral and cellular immune responses against xenogenic endothelial cells. Four main types of rejection can occur in a successive manner: (i) hyperacute xenograft rejection, (ii) acute humoral xenograft rejection (also called acute vascular rejection or delayed xenograft rejection), (iii) acute cellular rejection, and (iv) chronic rejection. (i) In hyperacute rejection, preformed human natural antibodies recognize xenogenic endothelial antigens such as pig Gal within minutes to hours following the transplant procedure [13]. This is followed by antibody deposition and complement activation resulting in membrane attack complex formation and endothelial activation. Subsequently, intravascular coagulation, platelet aggregation, and thrombosis occur and are mainly due to species incompatibilities of membrane-bound coagulation-regulatory proteins such as thrombomodulin and tissue factor pathway inhibitor, and interactions with xenogeneic von Willebrand factor [13]. In the context of xenogeneic cell infusion, instant blood-mediated inflammatory reaction (IBMIR) can occur. IBMIR is an innate immune response attacking allogeneic and xenogeneic cells following their contact with blood. It is characterized by complement and coagulation activation, and platelet aggregation, and leads to thrombosis and endothelial damage [14]. IBMIR results in the loss of approximately 50% of the cells [15]. When hyperacute rejection is prevented by avoiding preformed antibodies to exert their function and/or complement to activate, a delayed form of antibody-mediated rejection known as (ii) acute vascular rejection occurs within hours to days and is mediated by humoral and cellular mechanisms, together with activated endothelia and inflammation [16]. During this process, neutrophils release inflammatory cytokines and oxygen-reactive species. Concurrently, xenoantibodies bound to endothelia and trigger an antibody-dependent cell-mediated cytotoxicity by natural killer (NK) cells and macrophages [13]. Key molecules include NKG2D/UL16 binding protein 1, NKp44, CD28/CD86, and MHC class I [13]. CD4 + T cells can also exert direct cytotoxic effects through the Fas-Fas ligand lytic pathway [17] and produce interferon gamma that activate macrophages and NK cells [18]. Macrophage phagocytosis is mainly triggered by species incompatibility involving signal regulatory protein alpha and CD47 binding [13]. Interestingly, NK cells directly participate in xenorejection as their depletion leads to a prolongation of graft survival [19,20]. (iii) Acute cellular rejection includes T-cell and B-cell infiltration of the xenograft. This type of rejection is typically not observed in xenotransplantation experiments as intense immunosuppressive agent regimens are used to prevent preceding acute vascular rejection [16]. Costimulation blockade agents, such as an anti-human CD154 monoclonal antibody, have been found to be particularly effective in preventing T-cell activation in the xenotransplantation setting [21]. Delayed xenograft rejection is predominantly cellular in nature, and it occurs within weeks to months. This response includes not only the cytotoxic CD8 T- and CD4 T-cell responses, but also the formation of induced antixenograft antibodies (e.g., to pig Annexin A2, CD9, CD46, CD59, MHC) by B cells [13].

Survival of pig organs and cells xenotransplanted in nonhuman primates

Solid organs

Recently, vascularized life-sustaining solid xenotransplants reached an important milestone: an over-400-day survival (Fig. 1). Tector *et al.* reported GalT-KO and CD55 transgenic pig kidney xenografts sustaining life for up to 499 days in NHP recipients treated with T-cell depletion (anti-CD4 +/− anti-CD8), costimulation blockade using either anti-CD154 mAb or belatacept and daily mycophenolate mofetil/glucocorticoids [4–6]. These results already surpassed historical NHP-to-human kidney transplant that sustained life up to 9 months [23]. Heterotopic pig cardiac xenografts, galactose-α1,3-galactose (Gal)-free (by α1,3-galactosyltransferase gene KO (GalT-KO)), transgenic for human complement regulatory protein CD46 and human thrombomodulin transgenic (CD46 and TBM) survived up to 945 days in baboons [8]. The immunosuppressive regimen consisted of anti-thymocyte globulin and anti-CD20 antibodies, followed by maintenance with

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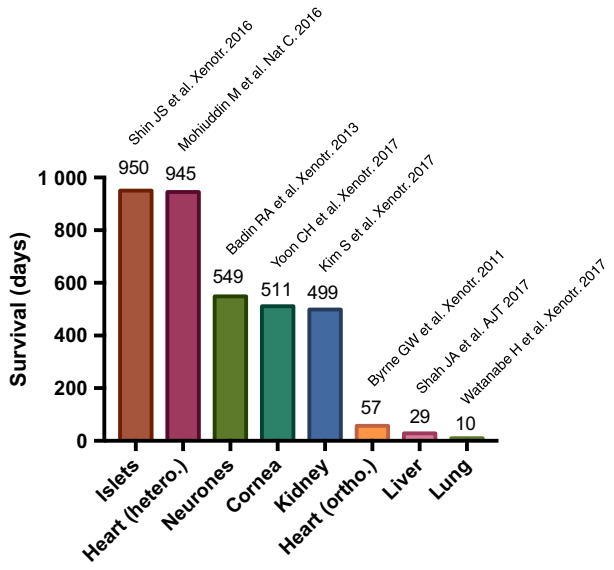

Figure 1 Survival of xenogeneic organs/tissue transplanted in nonhuman primates.

mycophenolate mofetil and anti-CD40 mAb. As the heterotopic cardiac xenotransplantation model is not life-sustaining, heart function remains generally poorly defined and thus, this model is not clinically applicable. The longest survival of orthotopic pig-to-NHP heart xenotransplantation was achieved using Gal-positive and human CD46 transgenic hearts treated with a polyethylene glycol alpha-Gal polymer which lasted for up to 57 days in baboon recipients [24] which is largely exceeding historical survival of NHP to human xenografts [25–27].

The initial studies testing pig liver xenografts in NHP recipients showed survival of only 8 days. This short survival was due to a rapid onset of a lethal coagulopathy characterized by bleeding, severe thrombocytopenia, and thrombotic microangiopathy caused by the destruction of NHP platelets by the pig liver endothelial cells and resulting in diffuse bleeding [28]. Recently, a Massachusetts General Hospital team increased liver xenograft survival to 29 days [29,30] using GalT-KO donors, continuous post-transplant infusion of human prothrombin concentrate complex, and intensive immunosuppression, including costimulation blockade with belatacept or anti-CD40 mAb. These survivals are approaching historical survival of 70 days in the setting of NHP-to-human liver xenotransplantation [31]. Most recent strategies include the infusion of human coagulation factors II, VII, factor VIIa, IX, X, protein C, and protein S that renders post-transplant thrombocytopenia transient and manageable without platelet transfusions [32]. Lung pig-to-NHP xenografts still hold short graft survival rates [33]; the most recent progress consists of a 10-day survival when transplanting lungs from GalT-KO, CD47, CD55 transgenic pigs to NHPs treated with anti-thymocyte globulin, rituximab, anti-CD154 mAb, and mycophenolate mofetil [34]. Eight-day survival was obtained transplanting GalT-KO, CD46, CD55, endothelial protein C receptor, CD47, TFPI transgenic pig lungs to NHPs treated with methylprednisolone, C1 inhibitor, heparin, antiplatelet GPIb antigen-binding fragments, thromboxane synthase inhibitor, histamine receptor blockers, and vWF depleting agents [35,36].

Cells

Islet xenotransplantation currently holds the record for the longest xenograft survival time with nearly 1 000 days (Fig. 1). In 2016, a team from South Korea reported long-term survival for 512 and 950 days, respectively, of two pig islet graft NHP recipients [7]. Islets were isolated from pathogen-free wild-type miniature pigs and transplanted into streptozotocin-induced diabetic NHP at a dose of 100 000 IEQ/kg [37]. The immunosuppressive regimen consisted of anti-thymoglobulin, anti-TNF, cobra venom factor to deplete complement, anti-human CD154 monoclonal antibodies (mAb), and sirolimus. The previous use of anti-CD154 mAb, which was thrombogenic, was successfully replaced by anti-CD40 mAb, which should be compatible for clinical use [7]. Overall, these results demonstrated a proof-of-principle concept; that is, pig islet xenografts respond to glucose, control glycemia, and show long-term survival in NHP. It is likely that genetically modified pigs will be further developed to decrease the need for immunosuppression [38]. Indeed, the use of transgenic pigs led to comparable results in vascularized heterotopic heart xenografts [8]. Another potential strategy, namely encapsulation of pig islets [39–42], should also benefit from further development of genetically modified pigs. Of note, islet xenotransplantation from nongenetically modified pig-to-human achieved islet survival but no significant clinical improvement [43–45]. Explanations for these mitigate results may be the inability to prevent xenorejection, insufficient IEQ numbers, or inability of neonatal pig islet to respond sufficiently to glucose challenge. Regarding the replacement of liver function for acute and chronic liver failure, another bridge strategy is the use of encapsulated pig hepatocytes and mesenchymal stem cells [22,46–49]. In a wild-type pig-to-NHP model, encapsulated pig hepatocytes allowed a higher survival rate (60% vs. 40%

at one month) in NHPs subjected to a 75% hepatectomy and a 60 minutes of liver ischemia [50].

Pig neurons were used as cell therapy to cure Parkinson's disease in a NHP model [51]. In these experiments, the donor pigs expressed high levels of neuronal CTLA4-Ig and the recipient NHPs were subjected to standard immunosuppression (cyclosporin A, mycophenolate mofetil, and prednisone). This strategy to prevent rejection by combining immune privilege, local and systemic immunosuppression allowed pig neuron xenografts recipients to survive and to provide full recovery of spontaneous locomotion for up to 6 months.

Finally, wild-type pig cornea xenografts which are immune-privileged and nonvascularized survived up to 511 days in a pig-to-NHP model using an anti-CD40-based immunosuppression regimen [52,53].

Overall, selected pig-to-NHP organ or tissue xenotransplants can now achieve survival rates that appear to be sufficient to be considered for clinical trials. Islet and kidney xenografts are close to meeting the requirements to start such trials.

Genome editing technologies for generation of knockout or transgenic animals

The first transgenic pigs were generated using DNA microinjection into the pronuclei or nuclei of eggs from superovulated pigs [54]. The lackluster efficiency of such technique is the random transgene integration associated with mosaicism during embryonic development. The next generations of transgenic pigs were produced using somatic cell nuclear transfer (SCNT) technologies on transfected or genetically modified cells lines or embryonic stem cells, either by egg electroactivation or intracytoplasmic injection [55,56]. Reliable DNA integration at the target site remained an important limiting step to efficiently generate KO or transgenic animals. The discovery of homologous recombination was a key development in the field [57]. Homologous recombination consists of breaking the DNA and inducing the cellular DNA repair mechanisms to insert a linearized plasmid DNA construct with homologous arms into the native DNA [58]. Nonhomologous end-joining repair, an important cell repair system, is responsible for short insertions or deletions in the target sequence (the indel) resulting in loss of gene function (Fig. 2). More recently, synthetic nucleases were engineered. These enzymes cleave the genome at specific sites, which are repaired in either a homologous or nonhomologous fashion [59]. Three major synthetic nucleases are currently available: (i) zinc finger nucleases, (ii) transcription activator-like effector nucleases (TALEN), and more recently (iii) clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) nucleases (Fig. 2).

(i) Zinc finger nucleases are composed of linked zinc fingers, each one being specific for a triplet DNA sequence and a type II restriction endonuclease named FokI [60]. Zinc finger nucleases are designed to bind and cleave a region of interest (e.g., a functional domain of a specific gene) (Fig. 2); dimerization is needed to achieve double-strand DNA cleavage. The development of zinc finger nucleases has revolutionized the generation of KO pigs. Using this technology, the team of Tector *et al.* sequentially disrupted the GalT and cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) genes in cultured cells and performed somatic cell nuclear transfer to yield viable double-KO pigs in 7 months [61]. Of note, this technology is also currently used in clinical trials for gene editing of C-C chemokine receptor type 5 (CCR5) in autologous CD4 T cells of persons infected with human immunodeficiency virus [62]. Nevertheless, zinc finger nucleases bear a risk of oncogenic translocation [63,64].

(ii) TALENs consist of repetitive conserved motifs of 33-35 amino acids with a fixed two-amino acid variation at position 12-13. This variable two-residue repeat confers nucleotide specificity. Like other artificial restriction endonucleases, TALENs can recognize a specific sequence and cleave it, allowing *in situ* genome modification. As for zinc finger nucleases, in TALEN, dimerization is required for double-strand DNA cleavage. TALENs are much easier to assemble, and a library of them has been generated [65]. Furthermore, TALEN toxicity seems to be lower than zinc finger toxicity and generates fewer off-target sites [66]; however, TALENs are sensitive to cytosine methylation, a well-known mechanism for DNA silencing [67]. TALEN was used to efficiently generate GalT biallelic KO inbred mini pigs [68].

(iii) More recently, a novel synthetic endonuclease system, CRISPR/Cas9, was developed consisting of a RNA-guided DNA endonuclease associated with CRISPR [59,69]. This complex activates Cas9 endonuclease activity and provokes double-strand DNA cleavage [70]. Cas9 checks the DNA for the complementary 20-bp spacer region of its guide RNA. If the DNA sequence is complementary to the guide RNA, Cas9 cleaves the DNA site at the specific site determined by the guide RNA. The target DNA must contain a protospacer adjacent motif (PAM) consisting of the 3-nucleotide

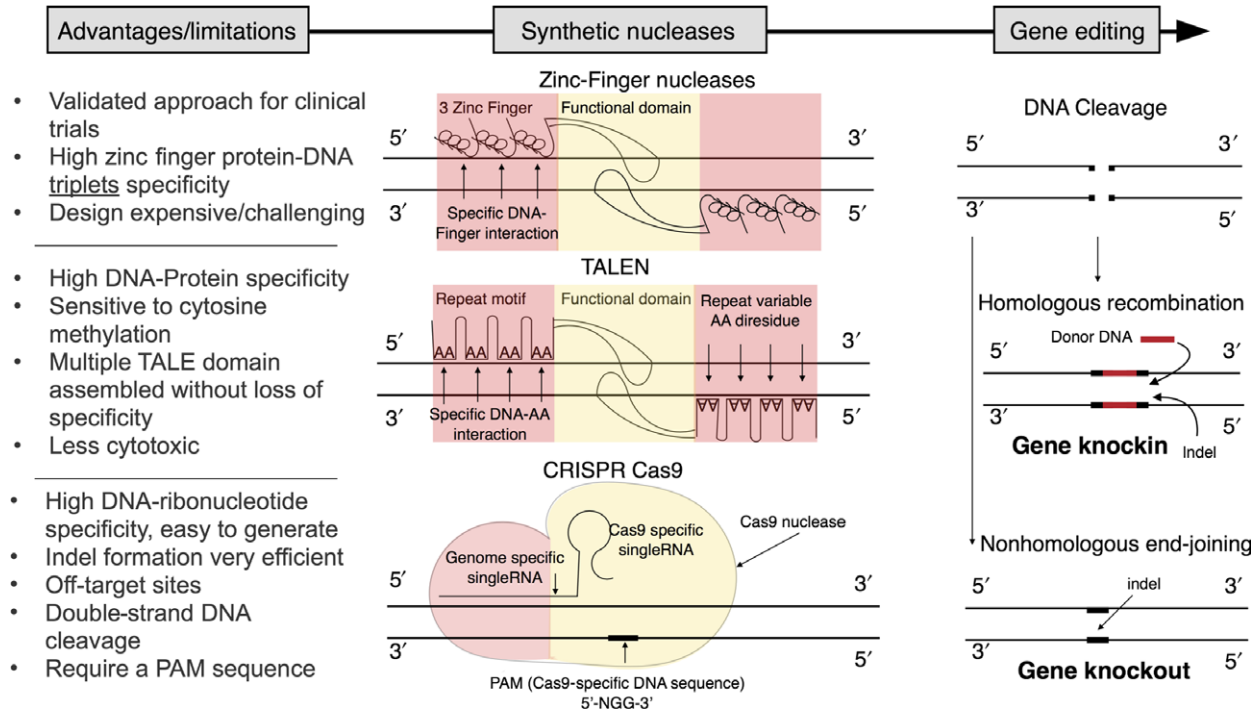

Figure 2 Genome editing technologies: advantages, limitations, and mechanisms of action of zinc finger, TALEN, and CRISPR Cas9 synthetic nucleases.

sequence NGG; the PAM is recognized by the PAM-interacting domain of Cas9 before cleavage. The DNA break can lead to gene inactivation or the introduction of heterologous genes through nonhomologous or homologous recombination. Several double-strand breaks can be introduced at once using multiple guide RNAs [71]. For example, Cas9 allowed to target multiple genes in a single reaction and generated pigs of one or multiple genetic strains in a single pregnancy [72]. Recently, different team corrected a pathogenic gene mutation in human embryos using CRISPR/Cas9 [73,74].

Xenotransplantation has directly benefitted from these developments, and various transgenic pigs with up to 7 genetic modifications have been generated [10,75–81].

Genetically modified pigs

Several different molecular species incompatibilities in pig-to-human xenotransplantation demanded the generation of animals with multiple genetic modifications to minimize xenorejection and IBMIR. Table 1 summarizes the pigs with genetic modifications currently available for xenotransplantation, based on previously published reviews [12,82,83]. The first transgenic pig was generated in the early 1990s with the membrane-associated complement regulator CD55 (human decay-accelerating factor, DAF) inserted randomly by DNA microinjection [84,85]. As the presence of Gal on porcine endothelial cells results in hyperacute or early humoral rejection in Gal-negative recipients [86], a second important step was achieved in the early 2000s when GalT-KO pigs were generated by homologous recombination and somatic cell nuclear transfer [87–89]. Indeed, GalT-KO pigs allowed to remove the main xenoantigen that caused hyperacute rejection through preformed antibodies. Additional xenoantigens present on pig endothelial cells include N-glycolylneuraminic acid (Neu5Gc), encoded by the cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH) gene, and glycans, produced by β1,4 N-acetylgalactosaminyl transferase (β4GalNT2) enzymes. Pigs lacking all three major carbohydrate xenoantigens, that is, GalT/CMAH/β4GalNT2 triple gene KO, were recently produced using the CRISPR/Cas9 technology [90]. As complement activation with formation of the membrane attack complex (MAC) is part of the effector humoral response leading to organ dysfunction after xenotransplantation, human complement regulatory proteins, such as CD46 (membrane cofactor protein), CD55 (critical for C3 activation), and CD59 (MAC-inhibitory protein), have also been inserted by homologous recombination and pronuclear microinjection of CD46 and CD59

Table 1. Genetically modified pigs available for xenotransplantation.

Category	Abbreviation	Name/Alternate name	Function
Gal or non-Gal deletion	GalT-KO	α1,3-galactosyl-transferase KO (GGTA1 KO)	Deletion of αGal xenoantigen
	EndoGalC	Endo-B-Galactosidase transgene	Reduction in αGal xenoantigen
	GLA	α-galactosidase transgene	Reduction in αGal xenoantigen
	NeuGc/CMAH KO	N-glycolylneuraminic acid/Cytidine monophosphate-N-acetylneuraminic acid hydroxylase	Deletion in xenoantigen Neu5Gc
	α2FucT	Human H-transferase transgene	Masking of xenoantigens by adding H blood group antigen
	β4GalNT2 KO	α2FucT	Synthesize xenoantigens
	GnT-III	N-acetylglucosaminyltransferase III	Masking of xenoantigens αGal and NeuGc
Complement regulation	CD46	Human complement regulatory protein transgene	Inactivation complement factors C3b and C4b
	CD55	Human complement decay-accelerating factor (DAF) precursor transgene	Acceleration of complement decay
	CD59	Human MAC-inhibitory orotein transgene	Inhibition of the complement membrane attack complex C5b-9
Cellular immune response	CIITA-DN	MHC class II transactivator knockdown, major histocompatibility complex class II, swine leukocyte Ag II, SLA-II	Transcription factor essential for porcine histocompatibility antigens II (SLA-II) expression
	MHC Class I KO	Major histocompatibility complex class I, swine leukocyte Ag I, SLA-1, SLA-2, and SLA-3	Antigen presentation
	HLA-E/human β2-microglobulin	Human leukocyte antigen class I histocompatibility antigen transgene, α chain E/human β2-microglobulin	Inhibition of NK cells cytotoxicity
	CD178	FAS ligand transgene, CD95L	Inhibition of NK cells cytotoxicity
	CTLA4-Ig	Cytotoxic T lymphocyte antigen 4 transgene, CD152, LEA29Y	Inhibition of T-cell costimulation via CD86/CD80
	CD253/TRAIL	TNF-α-related apoptosis-inducing ligand transgene	Induction of apoptosis of activated T cells
	CD47	Human integrin-associated protein transgene	Regulation of macrophage activation and phagocytosis
	SIRPα	Human signal regulatory protein-α transgene	Regulation of macrophage activation and phagocytosis
	ASGR1-KO	Porcine asialoglycoprotein receptor 1	Decreases human platelet phagocytosis by pig sinusoidal endothelial cells
	iGb3S KO	Isoglobotrihexosylceramide, isogloboside 3 synthase	Critical for NK cell development and self-recognition
Anticoagulation and other	vWF-deficient	Von Willebrand factor	Platelet adhesion
	TFPI	Tissue factor pathway inhibitor	Human protein C activation
	CD141	Human thrombomodulin transgene	Human protein C activation
	CD73	5′-nucleotidase	Platelet aggregation
	CD201	Human endothelial protein C receptor, EPCR	Human protein C activation
	CD39	Human ectonucleoside triphosphate diphosphohydrolase-1 transgene	Platelet aggregation
Anti-inflammatory/ Anti-apoptotic	A20	Human tumor necrosis factor-α-induced protein 3 transgene	Inhibition of NF-κB activation and TNF-mediated apoptosis
	HO-1	Human heme oxygenase-1 transgene	Degradation of heme
	sTNFRI-Fc	Human soluble TNF receptor inhibitor/Fc chimera	Inhibition of TNF/receptor binding
Other	PERV inactivation	Porcine endogenous retroviral viruses	Retroviruses

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constructs into porcine fertilized oocytes of the CD55 transgenic background [91]. Overall, these achievements have largely resolved the hurdle of hyperacute rejection.

Physiological incompatibilities between pig and NHP activate IBMIR which ultimately results in the loss of the majority of the infused cells. To limit this hurdle, several genes have been inserted into pigs, including (i) CD39 and CD73 to avoid platelet aggregation, (ii) CD141 (thrombomodulin) or CD201 (endothelial protein C receptor) to enhance human C protein activation and inhibition of the clotting factors Va and VIIIa, and (iii) tissue factor pathway inhibitor (TFPI), a regulator of the clotting factors VIIa and Xa [92,93]. Interestingly, Hawthorne *et al.* demonstrated that GalT-KO pigs also expressing human CD55/CD59 were protected from IBMIR following intraportal islet xenotransplantation in immunosuppressed baboons [94]. This is consistent with the important role of complement activation in IBMIR process. Alternatively, von Willebrand factor (vWF)-deficient pigs were produced to reduce the interaction between human platelets and the pig endothelium [33]. Recently, genetically modified pigs expressing humanized vWF rather than KO have been developed [95]. Anti-apoptotic and anti-inflammatory genes, such as the human protein A20 (inhibiting NF-κB activation and TNF-mediated apoptosis) and HO1 (heme oxygenase 1, which degrades free heme and protects against reactive oxygen species), were also inserted to prevent endothelial activation and IBMIR [96–98]. Finally, several strategies have been employed to control innate cellular response against the endothelium. For example, the expression of FasL (CD178) or TRAIL (CD253) was used to induce apoptosis, by overexpression of HLA-E/human β2 microglobulin) to inhibit NK cells through the inhibitory C-type lectin receptor (CD94/NKG2), or human CD47 to regulate monocyte activation via its ligand SIRPα [98–101].

In conclusion, various types of KO and transgenic pigs already exist and they are being tested in preclinical pig-to-NHP xenotransplantation models. Importantly, new xenoreactive antigens are progressively discovered as well [102,103], and they may require the generation of new KO pigs. Currently available genetically modified pigs might be used clinically in the near future. Waitlisted patients have minimal xenoreactive antibodies binding to GalT/CMAH/B4GalNT2 KO pig endothelia [104], and recently used immunosuppressive protocols are clinically applicable. Further research may interest in epigenetic aberrations of the genome in genetically modified pigs that could result in an early animal death [105]. The fast development of novel and efficient genome editing technologies, such as CRISPR/Cas9, will facilitate the generation of multiple transgenic pigs, bringing xenotransplantation even closer to clinical application.

Xenogeneic chimera generated by blastocyst complementation

Pioneer work by Gurdon *et al.* in 1962 demonstrated that the nuclei of mature intestine-derived cells contain all the information necessary to generate a frog [106]. In 2010, Nakauchi *et al.* injected xenogeneic pluripotent cells into blastocytes and could generate interspecific chimeras, demonstrating for the first time that xenogenic cells can interfere with embryonic development [107].

Intraspecies (autologous) or interspecies (xenogeneic) organ-specific chimeric embryos can be generated only in the presence of a "developmental niche" [108]. Therefore, organ-specific developmental genes need to be removed or repressed for the pluripotent cells to restore the defect and normal organ development, a process named blastocyst complementation [109]. This can be achieved using either embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) [69,110]. As previously described, recent advances in the field of genome editing accelerated the success of these approaches (Fig. 2). Chen *et al.* first demonstrated that injecting autologous ESCs into RAG (−/−) blastocytes rescued T- and B-cell development [111]. In 2007, Stanger *et al.* demonstrated that embryonic development of the pancreas after depletion of Pdx1 + pancreatic progenitor cells can be rescued by blastocyst complementation with ESCs [112]. These experiments were extended in a xenogeneic setting and rat pancreases were generated in Pdx1(−/−) mice using rat iPSCs [107] (Fig. 3). However, although this work proved the concept, the generation of these animals remained technically challenging. It was only in 2017, that chimeric islets isolated from apancreatic rats complemented with murine iPSCs were transplanted into streptozotocin-induced diabetic mice receiving a combination of cyclosporine and anti-inflammatory agents (anti-interferon-γ mAb, anti-mouse TNF-α mAb, and anti-IL-1β) for only 5 days. These grafts survived for over one year [113]. The same group could generate apancreatic pigs using somatic cell cloning technology and transgenic approaches [114] (Fig. 3). Belmonte *et al.* recently successfully complemented heart-, pancreas-, and eye-deficient mice with interspecies iPSCs using rodent models and CRISPR/Cas9 technology

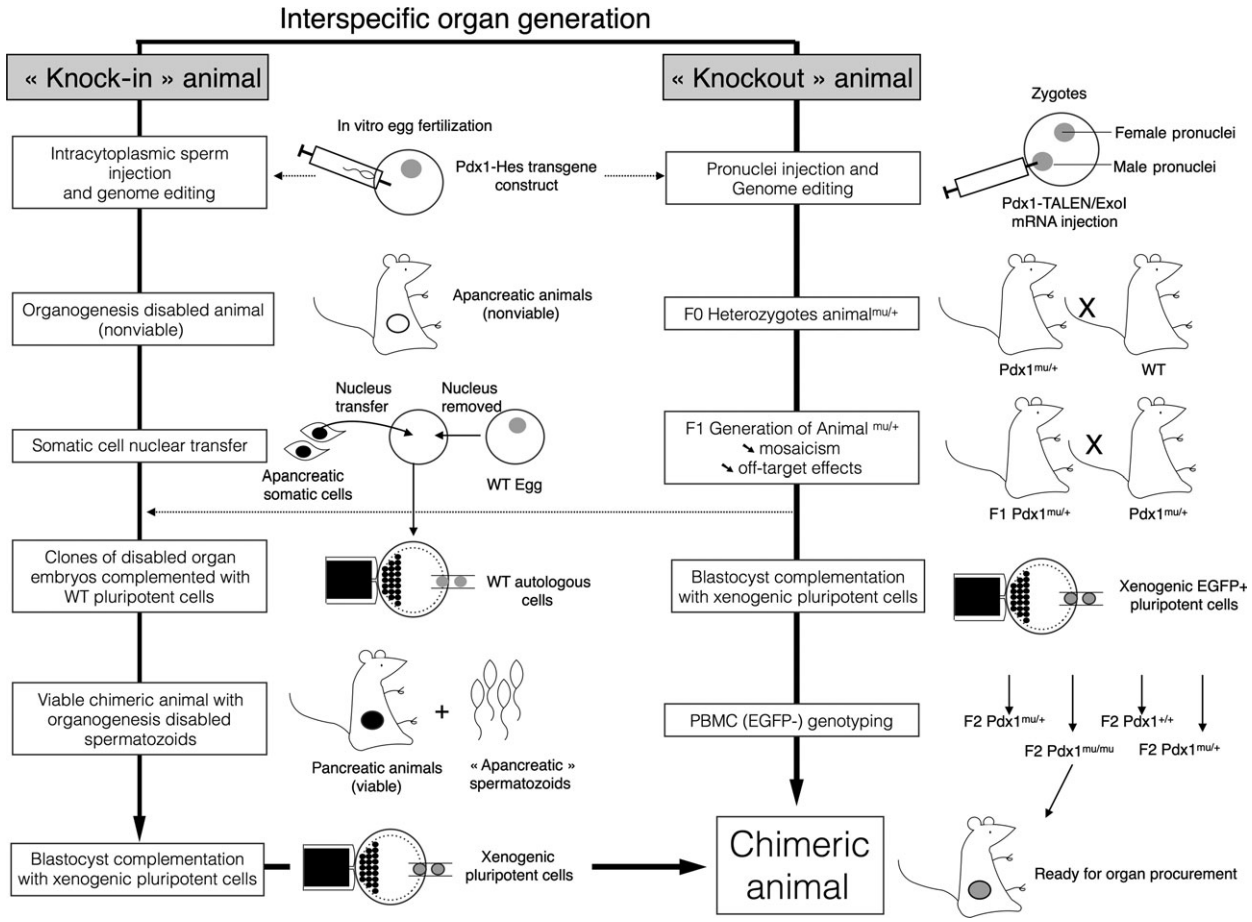

Figure 3 Interspecific organ generation steps for transgenic (knock-in) or knockout animals.

[115]; intraspecies kidneys were also generated using this approach [116]. Importantly, they also showed that concordant, but also discordant, interspecies chimeric embryos can be generated using iPSCs [115].

Overall, blastocyst complementation has the potential to solve both the organ shortage and the need for immunosuppression [108], but several issues need to be solved, such as the endothelium that develops from host cells in chimeric organs remains of host origin [117] and the full breeding of recently generated pig–human chimeras remains to be demonstrated [115]. Moreover, safety issues include the risk of uncontrolled high chimerism in pigs and the potential of carcinomatous degeneration. Interspecies iPSC injection into blastocytes results in low but detectable human–pig chimerism, with up to 10% of human cells in the pig heart [115]. The acceptable limits of human-pig chimerism percentages by organ must be defined. A solution would be to restrict iPSC differentiation to certain tissues, thus preventing unwanted integration in the brain or the ovaries, for example [118], and the use of inducible suicide genes [119].

Safety in xenotransplantation

A major risk in the field is represented by the possibility of xenozoonosis. Exogenous virus contamination of donor pigs, such as cytomegalovirus [120], gammalymphotropic herpes virus, and hepatitis E virus [121], can be easily avoided using specific breeding techniques in a clean environment. Porcine endogenous retroviral viruses (PERVs), however, cannot be eliminated by breading. Despite these concerns, several reassuring findings have emerged since the discovery of PERV transmission to human cells: (i) PERV transmission to patients exposed to porcine tissue has never been observed [122–125], (ii) *in vitro* pig-to-human PERV transmission occurred only in a human cell line that lacked the intracellular machinery that protects against retroviruses [126], and (iii) CRISPR/Cas9 technology allowed a genomewide inactivation of PERV copies from a pig cell line [12]. This latter crucial achievement was made by disrupting all copies of the PERV pol gene. The authors subsequently demonstrated a > 1000-fold reduction in PERV transmission

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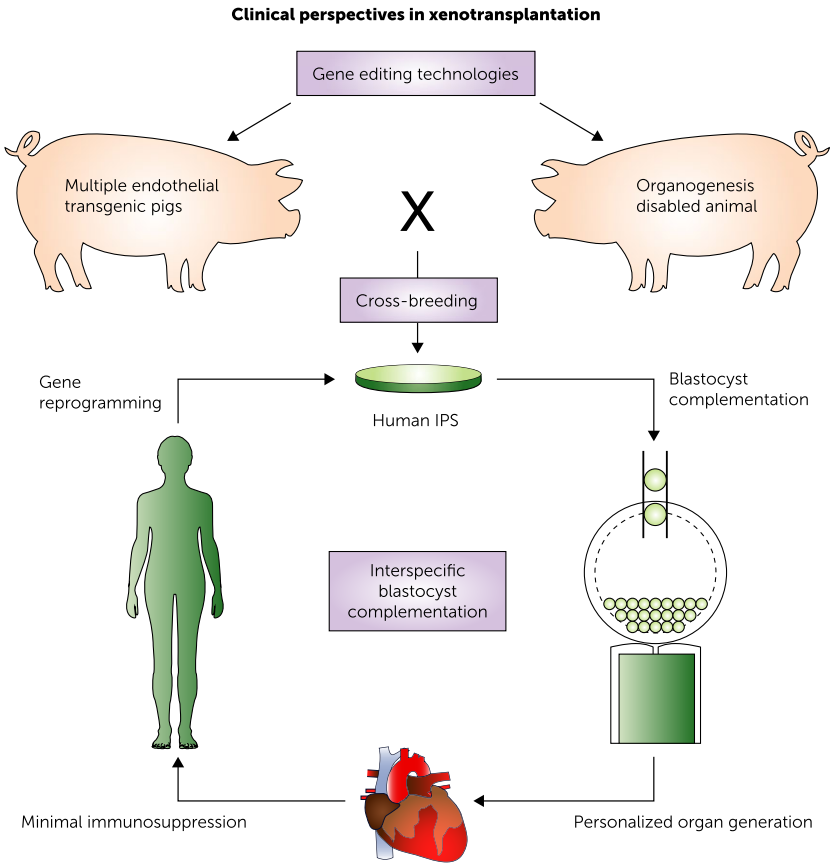

Figure 4 Clinical perspectives in xenotransplantation when crossing multiple transgenic pigs, targeting the immunogenicity of endothelial cells and organ-disabled animals with interspecific humanized organs.

to human cells. In their latest report, they further generated PERV-inactivated pigs via somatic cell nuclear transfer [127]. An additional safety issue includes the fact that xenoproteins produced by xenografts may cause diseases and/or medium/long-term compatibility issues similar to coagulopathies observed after liver xenotransplantation [128]. New hope for the field of xenotransplantation has evolved as these pigs are likely to be crossed with already available transgenic and KO pigs to generate the "perfect" donor pig for a first clinical trial. Altogether, these recent exciting advances move the field further toward a possible clinical application.

Conclusion

The field of xenotransplantation is entering a new era mainly based on advances in genetic engineering and stem cell research (Fig. 4). For several decades, research groups have worked on transgenic pigs with endothelial cells expressing inhibitory molecules controlling preformed antibody-induced humoral rejection, coagulation, and innate immune cells, such as monocytes or NK cells. The crossover of these animals with disabled-organ pigs complemented with patient-derived IPSCs may now allow for the generation of patient-specific solid organs to be transplanted in recipients receiving minimal or even no immunosuppression (Fig. 4). Novel genome editing technologies allow the generation of multiple transgenic, KO, and PERV-free animals in shorter periods of time and with greater efficiency. We however must acknowledge that only a few groups are active worldwide in the domain of pig-to-NHP xenotransplantation, highlighting the difficulty of mastering genome editing technologies together with the complexities and costs to generate and maintain large transgenic pigs in a clean environment. Furthermore, ethical issues remain at the forefront of this research, as it will imply the generation of human iPSCs and large organ-deficient chimeric animals. The fast and recent progress made in the last few years also urges regulatory authorities to re-examine their guidelines and regulations regarding xenotransplantation [129]. The adequate selection of recipients for initial clinical trials will be of crucial importance [130]. Moreover, as safety is paramount, any trial will be accompanied by rigorous and lifelong monitoring of patient recipients. We expect that in

the next decade, xenotransplantation will not anymore be "the future of transplantation" but a successful clinical reality [131].

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Conflicts of interest

The authors have declared no conflicts of interest.

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