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Will Genetic Engineering Carry Xenotransplantation of Pig Islets to the Clinic?

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Abstract

Purpose of Review Porcine islets represent a potentially attractive beta-cell source for xenotransplantation into patients with type 1 diabetes, who are not eligible to islet allo-transplantation due to a lack of suitable human donor organs. Recent progress in genetic engineering/gene editing of donor pigs provides new opportunities to overcome rejection of xeno-islets, to improve their engraftment and insulin secretion capacity, and to reduce the risk for transmission of porcine endogenous retroviruses. This review summarizes the current issues and progress in islet xenotransplantation with special emphasis on genetically modified/gene edited donor pigs.

Recent Findings Attempts to overcome acute rejection of xeno-islets, especially after intraportal transplantation into the liver, include the genetic elimination of specific carbohydrate antigens such as αGal, Neu5Gc, and Sd(a) for which humans and—in part—non-human primates have natural antibodies that bind to these targets leading to activation of complement and coagulation. A complementary approach is the expression of one or more human complement regulatory proteins (hCD46, hCD55, hCD59). Transgenic attempts to overcome cellular rejection of islet xenotransplants include the expression of proteins that inhibit co-stimulation of T cells. Expression of glucagon-like peptide-1 and M3 muscarinic receptors has been shown to increase the insulin secretion of virally transduced porcine islets in vitro and it will be interesting to see the effects of these modifications in transgenic pigs and islet products derived from them. Genome-wide inactivation of porcine endogenous retrovirus (PERV) integrants by mutating their *pol* genes using CRISPR/Cas9 is a recent approach to reduce the risk for PERV transmission by xeno-islets.

Summary Genetic engineering/gene editing of xeno-islet donor pigs facilitated major progress towards clinical islet xenotransplantation. The required set of genetic modifications will depend on the source of islets (fetal/neonatal vs. adult), the mode of delivery (encapsulated vs. free), and the transplantation site.

Keywords Pig · Islet transplantation · Xenotransplantation · Gene editing

Introduction

Since publication of the Edmonton islet transplantation and immunosuppression protocol [1], human islet transplantation entered successfully the clinic as beta-cell replacement therapy for insulin-dependent patients with beta-cell failure accompanied by problematic glycemic instability [2–4]. While progress in human pancreas procurement, islet isolation and quality, transplantation techniques, and immunosuppressive regimens led to a marked improvement in transplant outcomes, the number of procedures remains small, mainly due to the shortage of human donor pancreata [2].

Currently, different strategies are followed to develop alternative sources for beta-cell procurement. These include the targeted differentiation of human stem cells into endocrine

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progenitors or mature beta cells (reviewed in [5]), stimulation of endogenous beta-cell proliferation or reprogramming non-beta cells to beta-like cells (reviewed in [6]), the generation of human pancreas in animal hosts by chimeric embryo or organ complementation strategies (reviewed in [7]), and the use of xenogeneic pancreatic islets from animals. For a number of reasons, the pig is the favorite donor species for xeno-islets:

- Porcine insulin is active in humans.
- The fecundity of pigs is high and the generation time short (1 year).
- Pigs can be maintained under designated pathogen-free conditions.
- Genetic engineering and gene editing tools have been adapted to pigs to overcome rejection mechanisms, improve islet function, and reduce the risk for zoonoses.

Fetal, neonatal, and adult pig islets have been tested in preclinical transplantation experiments and each of these sources has advantages and disadvantages (reviewed in [8, 9]). Adult pig islets (APIs) are fully functional, but their isolation is technically demanding and expensive. Conditions need to be optimized to achieve reasonable quality and yields of API isolates [10]. The isolation of neonatal pig islets (NPIs) is straightforward and can be scaled to therapeutic quantities [11]. However, their insulin content is only 10–20% compared with APIs (reviewed in [12]) and NPIs require maturation in vitro or in vivo before they become fully functional [13–15]. Furthermore, NPIs—in contrast to APIs—have high levels of αGal epitopes which trigger an instant blood-mediated inflammatory response (IBMIR) after intraportal transplantation into the liver (reviewed in [16]). Fetal pig islets (FPIs) are usually derived from fetuses at 66 to 86 days of gestation and require long-term maturation (2–3 months) to achieve in vivo functionality after transplantation (reviewed in [17]).

Remarkable progress has been made in transplantation studies of free porcine islet into diabetic non-human primate (NHP) models with immunosuppression (reviewed in [18]), with one animal being insulin independent for more than 900 days [19]. However, large islet doses and intense immunosuppressive regimes including blockade of the CD40/CD154 co-stimulation pathway with anti-CD154mAb, that is thrombogenic in humans, were necessary. More recent studies focus on improving immunosuppressive protocols to regimes applicable in the clinic (e.g., [20]).

Encapsulation is one strategy to overcome the need for immunosuppression (reviewed in [9]). Microencapsulated NPIs have been tested in clinical studies and proved to be safe [21, 22] with limited clinical improvements in hemoglobin A1c levels and a reduction in the frequency of hypoglycemic events [23]. Porcine C-peptide levels were not reported. Macroencapsulated APIs were successfully tested in diabetic rhesus monkeys. Although additional insulin treatment was still required, the dose could be lowered and porcine C-peptide secretion consistently detected [24].

Genetic modification of the donor pigs is an option to reduce the need for immunosuppression after transplantation of free islets, which can be readily vascularized by the recipient and are thus expected to provide a more physiological glucose control than encapsulated islets. Of note, islets derived from younger donors exhibited superior graft revascularization compared to older donors [25, 26]. The type of genetic modifications required depends on the type of islets used and on the transplantation site. Currently, major efforts are undertaken in islet allo- and xenotransplantation research to define the most suitable islet transplantation site, as intraportal islet transplantation is hampered by severe adverse effects on islet survival [9, 27]. This overview summarizes genetic engineering/gene editing strategies of islet donor pigs to overcome humoral and cellular rejection of xeno-islets, to improve their engraftment and insulin secretion capacity, and to reduce the risk for porcine endogenous retrovirus (PERV) transmission.

Tailoring of Islet Donor Pigs by Genetic Engineering/Gene Editing

Most of the currently used genetically (multi-)modified islet donor pigs were generated by pronuclear DNA microinjection into zygotes or by somatic cell nuclear transfer (SCNT) from genetically modified donor cells (reviewed in [28]). The latter technique can be used for random insertion of transgenes, but allowed for the first time also targeted genetic modifications of pigs [29]. Gene editing opened a new era in tailoring donor pigs for xenotransplantation. Designer nucleases, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or the RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system, are used to introduce a site-directed DNA double strand break (DSB) in a cell. DSBs can be repaired by two cellular DNA repair pathways. Non-homologous end-joining often leads to frameshift mutations and a functional knockout of the gene. The homology-directed repair pathway facilitates targeted replacements or insertions in the pig genome (reviewed in [30]). For the generation of gene edited pigs, designer nucleases can be applied to cultured cells which are then used for SCNT, or they can be applied on fertilized oocytes with the risk of generating mosaics. Methods for targeted placement and assembly of multiple xenoprotective transgenes at a single genomic locus (reviewed in [31]) will speed up the generation of novel genetically multi-modified pig lines that can provide cells and tissues with superior properties for xenotransplantation.

Genetic Modifications to Overcome Rejection of Xeno-Islets

The mechanisms of islet xeno-graft rejection depend on the type of islet product (APIs vs. NPIs) and the transplantation site. Genetic modifications of donor pigs to overcome these rejection mechanisms are summarized in Table 1.

After intraportal transplantation of islets, a large proportion is lost due to the IBMIR, which is associated with activation of complement and coagulation, endothelial activation, cytokine and chemokine release, inflammatory cell activation, infiltration of the graft, platelet aggregation on the islet surface, and thrombus formation (reviewed in [83]). Due to preformed antibodies in humans against specific carbohydrate antigens on pig islets, IBMIR is likely exacerbated after islet xeno- vs. allo-transplantation. These specific oligosaccharide antigens are galactosyl-α1,3-galactose (αGal) synthesized by α-1,3-galactosyltransferase (GGTA1), N-acetylneuraminic acid (Neu5Gc, also called Hanganutziu-Deicher antigen) synthesized by cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) and an Sd(a)-like glycan made by β-1,4-N-acetyl-galactosaminyl transferase 2 (B4GALNT2) (reviewed in [84]). αGal epitopes are present at high levels on NPIs, but almost absent on APIs [85]. To eliminate these carbohydrate antigens from porcine islets, pigs with knockout (KO) mutants of *GGTA1*, *CMAH*, *B4GALNT2*, or combinations of these were generated (Table 1). *GGTA1*-KO/*CMAH*-KO pigs did not show alterations in islet architecture, insulin secretion, and glucose homeostasis. After transplantation of islets from these pigs into CMAH-deficient mice, no antibodies against Neu5Gc were observed [35]. Deletion of all three oligosaccharide antigens led to greatly reduced human antibody binding to pig cells in vitro [36, 86].

Intraportal transplantation of αGal-deficient NPIs of *GGTA1*-KO piglets into immunosuppressed STZ-induced diabetic rhesus monkeys resulted in improved rates of insulin independence after transplantation likely due to decreased IBMIR compared to transplanted αGal-containing WT NPIs [32]. This beneficial effect of αGal deficiency was previously not observed after intraportal transplantation of adult pig islets [39], presumably due to the naturally low level of αGal on adult islets [85]. In the absence of immunosuppression, a robust inflammatory response may precede IBMIR, masking the beneficial effect of αGal deficiency [87]. *GGTA1*-KO pigs are now used as background for further genetic modifications to improve free islet xenograft survival [88].

Binding of preformed or de novo synthesized antibodies to their xeno-antigens leads to activation of the complement system. Furthermore, there are molecular incompatibilities of the human/NHP and porcine coagulation homeostasis systems. For example, tissue factor, which is expressed and secreted by porcine pancreatic alpha and beta cells, activates the coagulation cascade [89]. It is a matter of debate if porcine tissue factor pathway inhibitor (TFPI) can prevent activation of the human coagulation cascade (reviewed in [90]). Attempts to protect cellular xenotransplants against complement-mediated injury and coagulation dysfunction include expression of one or more human complement-regulatory proteins (e.g., hCD46, hCD55, hCD59) and human coagulation-regulatory proteins (e.g., thrombomodulin, endothelial protein C receptor, TFPI, CD39, CD73). Expression of hCD46 had no effect to mitigate IBMIR and adult islet loss in the early post-transplant period, but was beneficial for long-term survival due to limiting antibody-mediated rejection [39]. However, hCD55 and hCD59 expressing NPIs from αGal-deficient pigs led to significantly reduced activation of coagulation and complement in vitro and efficiently attenuated IBMIR after intraportal transplantation into immunosuppressed non-diabetic baboons in vivo [41]. Long-term graft survival was limited due to cell-mediated rejection requiring more effective immunosuppression or further genetic modifications.

Beside the above-mentioned preformed antibodies, de novo synthesis of antibodies by B cells after recognition of xeno-epitopes can occur, triggered by T cells and natural killer cells (reviewed in [91]). Antibody-mediated graft rejection is exacerbated if patients are pre-sensitized and their serum contains antibodies against donor major histocompatibility complex class I molecules/human leukocyte antigens (HLAs). Human T-cell receptors can bind swine leukocyte antigen (SLA) complexes, triggering human T-cell activation [56], with different SLA polymorphisms eliciting strong or weak stimulatory effects. Xenograft survival can therefore be supported by avoiding donor pigs with strongly stimulating SLA alleles [92].

Both innate (neutrophils, natural killer cells, and monocytes/macrophages) and adaptive (B and T lymphocytes) components of the cellular immune system contribute to allo- and xenograft rejection (reviewed in [93]), in which, irrespective of transplant site, T-cell-mediated rejection is seen as major barrier for long-term islet graft survival. T-cell activation requires—in addition to T-cell receptor signaling—a co-stimulatory signal, which may—depending on its nature—induce and amplify an effective immune response, or have an inhibitory tolerogenic function. In xenotransplantation, the best studied T-cell co-stimulatory signaling complexes are CD80/CD86-CD28 and CD40-CD154, with CD28 and CD154 (= CD40L) being localized on T cells and CD80/CD86 and CD40 on APCs. Klymiuk et al. [51] generated transgenic pigs expressing the T-cell co-stimulation blocking molecule LEA29Y (binding CD80/CD86 with high affinity) specifically in beta cells. LEA29Y expressing NPIs transplanted under the kidney capsule of diabetic immune deficient mice (NOD-SCID Il2rg$^{-/-}$; NSG) were able to normalize glucose homeostasis and were not rejected by transplanted human peripheral blood mononuclear cells (PBMCs) [51]. Importantly, the concept of local immune modulation by LEA29Y was supported, as only marginal levels of LEA29Y were detectable in the circulation of mice grafted with LEA29Y

Table 1 Selection of genetic strategies to bring free islet xenotransplantation to the clinic

Aim/genetic modification (GM)	GM pigs	Islet XTx in rodents	Islet XTx in NHPs
Deletion of sugar moieties of pig cells with pre-formed recipients' antibodies			
α-1,3-galactosyltransferase knockout (GGTA1-KO)	[29]		[32]
cytidine monophosphate-N-acetylneuraminic acid hydroxylase knockout (CMAH-KO)	[33, 34]	[35]	
β-1,4-N-acetyl-galactosaminyl transferase 2 knockout (B4GALNT2-KO)	[36]		
Complement regulation by human complement-regulatory gene expression			
human membrane cofactor protein transgenic (hCD46-tg)	[37]	[38]	[39]
human decay-accelerating factor transgenic (hCD55-tg)	[40]		[41, 42]
human protectin or membrane inhibitor of reactive lysis transgenic (hCD59-tg)	[43]		[41]
human complement-regulatory protein C1 inhibitor transgenic (hC1-INH-tg)	[44]		
Coagulation regulation by human coagulation-regulatory gene expression			
human thrombomodulin transgenic (hTM-tg)	[45]		
human endothelial protein C receptor transgenic (hEPCR-tg)	[46]		
human tissue factor pathway inhibitor transgenic (hTFPI-tg)	[47]		[48]
human ectonucleoside triphosphate diphosphohydrolase-1 transgenic (hCD39-tg)	[49]		[48]
human ecto-5′-nucleotidase transgenic (hCD73-tg)	[50]		
Prevention of cell-mediated rejection-T cells			
human LEA29Y transgenic (LEA29Y-tg)	[51]	[51, 52•]	
human CTLA4-Ig transgenic (hCTLA4-Ig-tg)	[53]		
porcine CTLA4-Ig transgenic (pCTLA4-Ig-tg)	[54]		[48]
SLA class I knockout	[55]		
human dominant-negative mutant class II transactivator transgenic (CIITA-DN-tg)	[56]		
human TNF-related apoptosis-inducing ligand transgenic (hTRAIL-tg)	[57, 58]		
human programmed cell death 1 ligand 1 transgenic (PD-L1-tg)	[59]		
Prevention of cell-mediated rejection-natural killer cells and macrophages			
HLA-E/human b2-microglobulin transgenic (HLA-E/b2M-tg)	[60]		
human signal regulatory protein alpha transgenic (hCD47-tg)	[61]		
Expression of anti-inflammatory proteins or knockout of pro-inflammatory proteins			
human tumor necrosis factor α–induced protein 3 (TNFAIP3) transgenic (A20-tg)	[62]		
human heme oxygenase 1 transgenic (hHO-1-tg)	[63, 64]	[65, 66]	
soluble human TNFRI-Fc transgenic (shTNFRI-Fc-tg)	[64]	[65, 66]	
Reduction/elimination of the risk of PERV transmission			
Knockdown of PERV expression	[67–70]		
Genome-wide inactivation of PERV pol gene	[71••]		
Genetically multi-modified pigs			
GGTA1-KO/hCD46-tg/hCD39-tg	[48, 72]		[48]
GGTA1-KO/hCD46-tg/hTFPI-tg/pCTLA4-Ig-tg	[48, 72]		[48]
GGTA1-KO/hCD46-tg/hTFPI-tg/pCTLA4-Ig-tg/hCD39-tg	[48, 72]		[48]
GGTA1-KO/hCD55-tg/hCD59-tg/human fucosyltransferase (HT)-tg	[73]		[74]
GGTA1-KO/hCD55-tg/hCD59-tg	[41, 75]		[41]
GGTA1-KO/hCD55-tg/hCD39-tg/TFPI-tg/hC1-INH-tg/hTNFAIP3-tg	[44]		
GGTA1-KO/CMAH-KO	[34, 35, 76]	[35]	
GGTA1-KO/CMAH-KO/hCD46-tg/hCD55-tg/hCD59-tg/hA20-tg/hHO1-tg	[77]		
GGTA1-KO/CMAH-KO/shTNFRI-Fc-tg/hHO-1-tg	[78]		
GGTA1-KO/CMAH-KO/B4GALNT2-KO	[36, 79, 80]		
GGTA1-KO/CMAH-KO/isoglobotrihexosylceramide synthase (iGb3S)-KO	[81]		
GGTA1-KO/hCD39-tg	[82]		
GGTA1-KO/pCTLA4-Ig-tg	[54]		

XTx xenotransplantation, *NHPs* non-human primates

transgenic islets. In diabetic NSG mice reconstituted with human $CD34^+$ hematopoietic stem cells, LEA29Y transgenic islets survived and maintained glucose control for more than 6 months without additional immunosuppressive treatment [52•]. Transgenic expression of human PD-L1, a co-inhibitory immunomodulating agent suppressing T-cell activation and thereby inducing tolerogenic T-cell responses [94, 95], represents a complementary strategy [59]. Transient expression of PD-L1 on human islet allo-transplants was recently reported to promote their indefinite survival (https://confman.tts2018.org/mobis/lecture/828).

Besides T cells, macrophages entered into the focus of xenograft rejection. In a dual transplant model where diabetic NHP with robust co-stimulation blockade-based regimen, using CTLA4-Ig, anti-CD154, and anti-LFA1 therapy, were transplanted with adult NHP islets into one liver lobe and with *GGTA1*-KO NPI into the other lobe, NPI xenotransplants showed augmented macrophage infiltration and antibody deposition compared with allografts [96•]. Therefore, engineering of transgenic pigs expressing the 'macrophage don't eat me' signal hCD47 (SIRPα) might be the next step to prevent macrophage infiltration. CD47 expressed on rodent islets allotransplanted intraportally in mice reduced IBMIR-associated early islet mass loss (https://confman.tts2018.org/mobis/lecture/419).

Inhibition of inflammatory and apoptotic stimuli of islets might enable a further step towards improved engraftment and prolonged graft survival. Inactivation of the *CCL2* gene [encoding the monocyte chemotactic protein 1 (MCP1)/chemokine (C-C motif) ligand 2] and transgenic overexpression of TFPI were recently proposed as a means of reducing IBMIR by diminishing pro-inflammatory and pro-coagulant signals from islet xenotransplants (reviewed in [16, 97]). Human TFPI transgenic pig islets (on a *GGTA1*-KO/hCD46-tg genetic background) were shown to mitigate IBMIR and reduce early cell losses, but no beneficial effect on long-term graft survival in NHPs was observed [48]. Adult porcine islets expressing human soluble TNF-α receptor-Fc (sTNF-αR-Fc) or heme oxygenase-1 (HO-1), transplanted under the kidney capsule into diabetic BALB/c nude or humanized NSG mice with no immunosuppressive regime, had significantly prolonged graft survival, decreased intragraft MCP1, TNF-α and IL-6 expression and decreased perigraft infiltration of macrophages and T cells [65, 66]. Additionally, HO-1 expressing islet xenotransplants exhibited decreased apoptosis during early engraftment.

Genetic Modifications for Optimizing Xeno-Islet Maturation and Function

NPIs have a number of advantages over APIs, most importantly their straightforward isolation, their proliferation capacity, their superior revascularization after transplantation, and the fact that donor animals do not need to be maintained for a long period under expensive designated pathogen-free conditions. However, NPIs are immature and not fully functional after isolation. It would therefore be important to gain a better understanding of factors affecting the maturation and proliferation of NPIs. Kemter et al. [26] generated transgenic pigs expressing enhanced green fluorescent protein (eGFP) under the control of the porcine *INS* promoter. The reporter gene is expressed specifically in beta cells, and the level of expression increases upon beta-cell maturation. This model is useful to study beta-cell maturation and expansion in vivo, e.g., after transplantation into the anterior eye chamber of mice. Moreover, eGFP-expressing beta cells can be recovered by fluorescence activated cell sorting and processed for omics analyses like single-cell RNA sequencing [98]. Systematic analyses of beta cells derived from different pre- and postnatal stages will improve our understanding of porcine beta-cell development and eventually reveal new markers and strategies to improve the maturation of NPIs and to assess the quality of islet products (Fig. 1).

Although pigs exhibit blood glucose concentrations comparable to those in humans, porcine beta cells contain less insulin and respond with lower insulin secretion to glucose stimulus than human beta cells (reviewed in [12]). Therefore, larger amounts of xeno-islets than allo-islets might be necessary to be transplanted in humans to produce physiologically relevant amounts of insulin and to achieve normoglycemia. By adenoviral transfer mediated transgene expression of glucagon-like peptide-1 (GLP1) and constitutively activated type 3 muscarinic receptor (M3R), porcine islets contained increased amounts of insulin, insulin granules, and improved islet secretory function in vitro [99•]. GLP1 activates a cAMP-dependent pathway and activation of M3R initiates the cholinergic pathway. Both pathways lead to 'amplification' of insulin production, thereby to an increased number of readily-releasable insulin granules in beta cells, resulting in greater secretory response to glucose stimulation. It will be interesting to see how glucose homeostasis of transgenic pigs expressing increased GLP1 and M3R levels in their islets is affected and if isolated and transplanted islets show improved insulin secretion.

Gene Editing to Prevent Zoonosis

Xenogeneic cell therapy products like porcine islet xenotransplants require regulatory approval before entering the clinic [100]. Beside graft functionality and avoidance strategies of graft rejection, zoonotic risk of xenografts is a critical issue.

Gene editing may play also a major role in preventing transmission of porcine microorganisms to the xenotransplant recipient. Xenotransplantation may be associated with the risk of transmission of porcine microorganisms including bacteria,

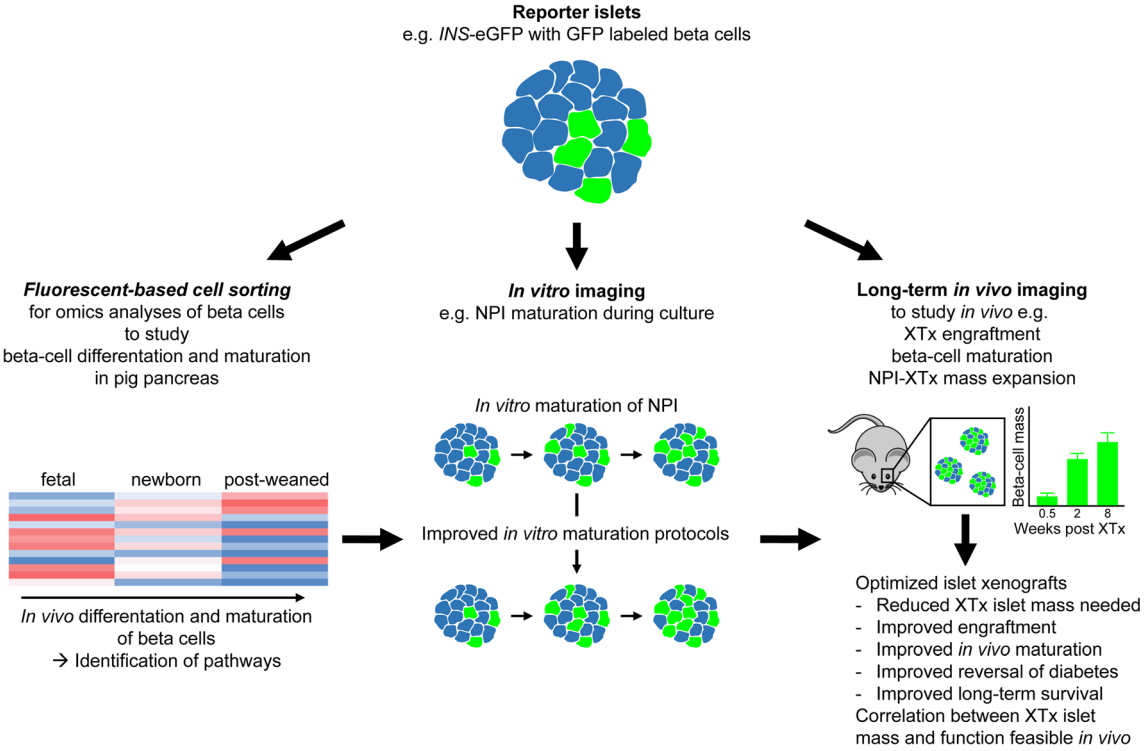

Fig. 1 Reporter pig islets to gain a better understanding of factors affecting the maturation and proliferation of NPIs. Reporter islet pigs like *INS*-eGFP pigs with GFP-labeled beta cells [26] are a useful tool (1) for omics analyses to identify markers and pathways of beta-cell differentiation and maturation in pig pancreas and to develop strategies to improve NPI maturation in vitro, (2) for monitoring and optimizing of the in vitro maturation process of NPIs before xenotransplantation, and (3) for non-invasive long-term in vivo imaging of xenotransplant engraftment, beta-cell maturation, and NPI mass expansion. By optimizing the NPI maturation process and obtaining high-quality, well-functioning and apoptosis-resistant NPIs for transplantation, reduced islet mass for xenotransplantation, improved engraftment and vascularization, and improved in vivo maturation of the xenograft might be feasible for obtaining a reliable transplant outcome with improved reversal of diabetes and improved short- and long-term survival

fungi, and viruses able to adapt in the recipient and to induce a disease (zoonosis or xenosis). Whereas many microorganisms may be eliminated from the donor pigs by selection, treatment with antibiotics, antimycotics, or antiviral drugs, by vaccination, by early weaning and colostrum deprivation, by Cesarean delivery or embryo transfer, and by maintenance of the donor animals in designated pathogen-free housing facilities, for others this is not so easy or not possible. Reasons for this are for example a wide distribution in all pigs, a high stability of the virus, an easy distribution by body fluids, or a transplacental transmission. This is true for the porcine circoviruses 1, 2, and 3 (PCV1, PCV2, PCV3) [101], the porcine cytomegalovirus (PCMV) [102], the porcine lymphotropic herpesviruses (PLHV-1, PLHV-2, PLHV-3) [103], and the hepatitis E virus (HEV) [104]. However, with great effort and excellent elimination programs, it can be achieved even in the case of these viruses (e.g., [105]). Even when donor pigs were positive for PCMV and PLHV in their PBMCs, their islet cells were negative demonstrating that the hygiene status of the product can be better than that of the herd [106].

In contrast, PERVs cannot be eliminated this way, because they are integrated in the genome of all pigs and can be released from pig tissues as infectious virus particles [107]. Two of them, PERV-A and PERV-B, are able to infect human cells. When islets were macroencapsulated in an alginate patch, no release of PERVs was detected [108]. Until now, no transmission of PERVs has been observed in preclinical and clinical trials [109]. Unfortunately, NHPs are a very limited animal model to study efficacy and virus safety of pig islet cell transplantation [110•]. First, there are major differences in the glucose metabolism between humans and pigs on one side and NHPs on the other side, and, second, NHPs do not carry a functional receptor for PERVs (for details, see [110•]). With few exceptions, clinical trials have been performed with encapsulated pig islet cells without pharmaceutical immunosuppression, but still not with large vascularized organs and appropriate immunosuppression. At present, there are no additional experimental approaches available to evaluate whether PERVs pose a risk in clinical xenotransplantations (for details, see [111]).

To prevent PERV transmission despite their integration in the pig genome, several strategies have been developed. First of all, the selection of pigs with a low copy number and a low expression at the RNA or protein level of PERV-

A and PERV-B proviruses based on methods able to discriminate between high and low expression of PERVs in blood cells [112, 113]. Second, it has been shown that PERV-A, which is able to infect human cells, and PERV-C, which is able to infect only pig cells, can recombine. The resulting recombinant PERV-A/Cs are able to infect human cells and are characterized by an increased replication competence compared with the parental viruses [114–116]. Therefore, PERV-C-free animals should be selected to avoid recombination. For this, sensitive and specific methods to screen for PERV-C-positive animals have been developed [117, 118]. Third, RNA interference technology was successfully used to reduce the expression of PERVs in genetically modified animals expressing small interfering RNAs [67–70, 119, 120]. Fourth, a vaccine based on neutralizing antibodies against the transmembrane and surface envelope proteins of PERVs was developed, though it could not be tested in the absence of an appropriate animal model of infection [121–123].

A breakthrough was achieved when gene editing was used to inactivate PERVs integrated in the pig genome. This was a great challenge, because gene editing is usually applied to inactivate single genes in the genome. PERVs are present approximately in up to 130 copies in the genome [124, 125]. When gene editing was performed using a ZFN in a pig cell line and PERV-infected human cells, the expression of ZFN was very high inducing a toxic effect [126]. Obviously, the ZFN was cutting the genome at multiple sites and was destabilizing the genome [126]. The use of the CRISPR/Cas9 technology was another step forward. In a proof of principle experiment, 62 PERV proviruses were successfully inactivated in immortalized PK-15 pig cells [127•]. Meanwhile, all PERV copies (altogether 25) were inactivated in primary pig cells and these were used to produce live healthy piglets [71••]. The technical feasibility of reducing the risk of PERV transmission to zero is exciting, but it is not clear at this stage if genome-wide PERV inactivation by CRISPR/Cas9 is actually required for clinical islet xenotransplantation [111, 128].

To further increase the virus safety, it may be possible to inactivate receptors for porcine microorganisms if these molecules are known and without important functions in the animals.

Conclusions

Porcine islet xenografts have a high potential to pass the door towards the clinic as beta-cell replacement therapy due to following advantages: (1) “on demand” unlimited source of beta cells, (2) consistent and standardized, high quality beta-cell replacement therapy achievable, (3) likely potential to avoid recurrent autoimmunity, (4) potential to prevent allogeneic sensitization, and (5) avoid amyloid deposition [4]. Both immune destruction and the potential zoonotic risk of transmission of PERVs and other pig viruses so far hindered transition of free islet xenotransplantation towards the clinic. However, pig donors can be genetically modified, and enormous progress was achieved in recent years especially since the introduction of gene editing tools enabling efficient generation of every kind and combination of genetic modifications. To define which multiplex genetic modifications are necessary for an optimized xenograft with superior properties and enabling long-term graft survival without need of systemic immunosuppressive regime is a challenge but can be achieved.

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Compliance with Ethical Standards

Conflict of Interest Elisabeth Kemter, Joachim Denner, and Eckhard Wolf declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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Papers of particular interest, published recently, have been highlighted as:
- • Of importance
- •• Of major importance

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