Feature Article

Genetically modified pigs as donors of cells, tissues, and organs for xenotransplantation

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Implications

- For many patients with chronic organ failure, transplantation is the only therapeutic option, but the number of donated human organs and tissues falls far short of the need.
- Porcine cells, tissues, and organs likely will be an alternative transplant source, since pigs can be genetically engineered to overcome rejection mechanisms and physiological incompatibilities, and to reduce the risk of transmitting zoonotic pathogens.
- Significant progress has been made in many areas of xenotransplantation, including pancreatic islets, neuronal cells, and corneas, but also vascularized organs, especially kidneys and hearts.
- In view of recent preclinical breakthroughs, such as consistent long-term survival of baboons after orthotopic transplantation of a genetically multimodified porcine heart, xenotransplantation can be considered as a realistic future therapeutic option.

Key words: heart, organ donor, pancreatic islet, pig, xenotransplantation

Introduction

The number of donated human organs and tissues for patients with terminal organ failure falls far short of the need. According to the United Network for Organ Sharing (www.unos.org), more than 113,000 candidates for transplant are currently on the U.S. national waiting list, but only 36,527 organ transplants could be performed in 2018. Alternative sources, such as organs and tissues from animals, are therefore urgently needed. For a number or reasons, including size, anatomical, and physiological similarities with humans, the pig is the preferred donor species (reviewed in Cooper et al., 2016). Importantly, pigs can be optimized by genetic engineering as a source of cells, tissues, and organs for xenotransplantation. Recent advances in gen(om)e editing are speeding up progress in this field. Numerous genetically (multi-)modified pig lines have been generated to prevent immune rejection of xenotransplants, to overcome physiological incompatibilities, and to reduce the risk of transmitting zoonotic pathogens (Table 1; reviewed in Kemter et al., 2018).

Genetic modifications to overcome hyperacute and acute vascular rejection of pig-to-primate xenotransplants

Hyperacute rejection of vascularized pig-to-primate xenotransplants is triggered by binding of preformed antibodies of the recipient to specific antigens on the xenogeneic tissue and subsequent activation of the complement system. The major xeno-antigen is galactose-α1,3-galactose (αGal) synthesized by α-1,3-galactosyltransferase (GGTA1). Humans and Old World monkeys lack GGTA1 and αGal epitopes, but are exposed to bacterial αGal epitopes eliciting a persistent anti-αGal antibody response in early life. Other prominent xeno-antigens are N-acetylneuraminic acid (Neu5Gc) synthesized by cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) and an Sd(a)-like glycan made by porcine β-1,4-N-acetyl-galactosaminyl transferase 2 (B4GALNT2) (reviewed in Byrne et al., 2015).

An important step toward long-term survival of vascularized porcine xenotransplants in nonhuman primates was the generation of pigs lacking functional *GGTA1* alleles (Phelps et al., 2003). Subsequently, multiple *GGTA1* knockout pig lines were generated, initially by gene targeting (reviewed in Klymiuk et al., 2010) and later by gene editing (e.g., Hauschild et al., 2011). In addition, pigs with knockout mutations of *CMAH*, *B4GALNT2* or combinations of these modifications were generated (Estrada

Table 1. Selection of genetic modifications of donor pigs for xenotransplantation

Aim/Genetic modification (GM)	Reference
Deletion of sugar moieties of pig cells with pre-formed recipients' antibodies	
α-1,3-galactosyltransferase knockout (GGTA1-KO)	(Phelps et al., 2003)
Cytidine monophosphate-N-acetylneuraminic acid hydroxylase knockout (CMAH-KO)	(Kwon et al., 2013; Lutz et al., 2013)
β-1,4-N-acetyl-galactosaminyl transferase 2 knockout (B4GALNT2-KO)	(Estrada et al., 2015)
Complement regulation by human complement-regulatory gene expression	
Human membrane cofactor protein transgenic (hCD46-tg)	(Diamond et al., 2001)
Human decay-accelerating factor transgenic (hCD55-tg)	(Cozzi and White, 1995)
Human protectin or membrane inhibitor of reactive lysis transgenic (hCD59-tg)	(Fodor et al., 1994)
Human complement-regulatory protein C1 inhibitor transgenic (hC1-INH-tg)	(Kwon et al., 2017)
Coagulation regulation by human coagulation-regulatory gene expression	
Human thrombomodulin transgenic (hTBM-tg)	(Wuensch et al., 2014)
Human endothelial protein C receptor transgenic (hEPCR-tg)	(Iwase et al., 2014)
Human tissue factor pathway inhibitor transgenic (hTFPI-tg)	(Lin et al., 2010)
Human ectonucleoside triphosphate diphosphohydrolase-1 transgenic (hCD39-tg)	(Wheeler et al., 2012)
Human ecto-5′-nucleotidase transgenic (hCD73-tg)	(Lee et al., 2017)
Prevention of cell-mediated rejection - T cells	
Human LEA29Y transgenic (LEA29Y-tg)	(Klymiuk et al., 2012; Bähr et al., 2016)
Human CTLA4-Ig transgenic (hCTLA4-Ig-tg)	(Martin et al., 2005)
Porcine CTLA4-Ig transgenic (pCTLA4-Ig-tg)	(Phelps et al., 2009)
SLA class I knockout	(Reyes et al., 2014)
Human dominant-negative mutant class II transactivator transgenic (CIITA-DN-tg)	(Hara et al., 2013)
Human TNF-related apoptosis-inducing ligand transgenic (hTRAIL-tg)	(Klose et al., 2005)
Human-programmed cell death 1 ligand 1 transgenic (PD-L1-tg)	(Buermann et al., 2018)
Prevention of cell-mediated rejection - natural killer cells and macrophages	
HLA-E/human b2-microglobulin transgenic (HLA-E/b2M-tg)	(Weiss et al., 2009)
Human CD47 transgenic (hCD47-tg)	(Tena et al., 2014)
Expression of anti-inflammatory proteins or knockout of pro-inflammatory proteins	
Human tumor necrosis factor α–induced protein 3 (TNFAIP3) transgenic (A20-tg)	(Oropeza et al., 2009)
Human heme oxygenase 1 transgenic (hHO-1-tg)	(Petersen et al., 2011)
Soluble human TNFRI-Fc transgenic (shTNFRI-Fc-tg)	(Yan et al., 2016)
Reduction/elimination of the risk of PERV transmission	
Knockdown of PERV expression	(Miyagawa et al., 2005; Dieckhoff et al., 2008; Ramsoondar et al., 2009)
Genome-wide inactivation of PERV pol gene	(Niu et al., 2017)
Genetically multimodified pigs	
GGTA1-KO/hCD46-tg/hCD39-tg	(Bottino et al., 2014)
GGTA1-KO/hCD46-tg/hTFPI-tg/pCTLA4-Ig-tg	(Bottino et al., 2014)
GGTA1-KO/hCD46-tg/hTFPI-tg/pCTL4-Ig-tg/hCD39-tg	(Bottino et al., 2014)
GGTA1-KO/hCD55-tg/hCD59-tg/human fucosyltransferase (HT)-tg	(Le Bas-Bernardet et al., 2011)
GGTA1-KO/hCD55-tg/hCD59-tg	(Hawthorne et al., 2014)
GGTA1-KO/hCD55-tg/hCD39-tg/TFPI-tg/hC1-INH-tg/hTNFAIP3-tg	(Kwon et al., 2017)
GGTA1-KO/CMAH-KO/hCD46-tg/hCD55-tg/hCD59-tg/hA20-tg/hHO1-tg	(Fischer et al., 2016)

et al., 2015). The authors showed that cells from GGTA1/CMAH/B4GALNT2–deficient pigs exhibited reduced human IgM and IgG binding compared with cells lacking only GGTA1 and CMAH.

A complementary strategy is the generation of transgenic pigs that express human complement-regulatory proteins, such as CD46 (membrane cofactor protein, MCP), CD55 (complement decay-accelerating factor, DAF), or CD59 (membrane inhibitor of reactive lysis, MIRL), singly or in combination. These complement-regulatory proteins attenuate complement activation and significantly prolong survival of pig-to-nonhuman primate xenotransplants (reviewed in Cooper et al., 2016).

By combination of *GGTA1* knock-out with the expression of one or more human complement-regulatory proteins, the problem of hyperacute rejection of porcine xenotransplants in nonhuman primates has been solved. For clinical trials, additional knock-outs of *CMAH* and *B4GALNT2* may be required (reviewed in Kemter et al., 2018).

Besides preformed antibody binding to carbohydrate antigens, xenotransplantation of porcine cells, tissues, or

organs elicits a humoral immune response (reviewed in Vadori and Cozzi, 2015). The risk is likely increased in presensitized patients with antibodies against major histocompatibility complex (MHC) class I molecules/human leukocyte antigens, since these antibodies may cross-react with conserved epitopes of swine MHC subclasses/swine leukocyte antigens (Mulder et al., 2010). To overcome this problem, pigs lacking MHC class I have been generated. These pigs showed reduced levels of CD4$^-$ CD8$^+$ T cells in the peripheral blood, but appeared healthy and developed normally (Reyes et al., 2014).

Genetic modifications to overcome cellular rejection of pig-to-primate xenotransplants

Cellular rejection of pig-to-primate xenotransplants involves both innate and adaptive components of the cellular immune system. Immune cell infiltration of tissue and solid organ xenotransplants starts with neutrophils, followed by macrophages and T cells (reviewed in Vadori and Cozzi, 2015). In addition, natural killer cells may induce endothelial cell activation in the xenotransplant and lyse porcine cells directly and via antibody-dependent cytotoxicity (reviewed in Weiss et al., 2009).

Cellular xenotransplants such as porcine islets in nonhuman primates are mainly rejected by CD4$^+$ T cells. Their activation can be induced by direct binding of primate T-cell receptors to swine leukocyte antigen class 1 and class 2 molecules of porcine cells, or indirectly by antigen-presenting cells of the recipient expressing MHCs with processed xeno-antigens (reviewed in Vadori and Cozzi, 2015). In addition, co-stimulatory signals, which may induce and amplify an effective immune response, or exhibit an inhibitory function, are involved in the regulation of T-cell function. The most prominent T-cell co-stimulatory signaling complexes are CD40 (on APCs)-CD154 (on T cells) and CD80/CD86 (on antigen-presenting cells)-CD28 (on T cells). The CD80/CD86-CD28 co-stimulation pathway can be blocked by systemic treatment with CTLA4-Ig (abatacept) or its affinity-optimized version LEA29Y (belatacept) (reviewed in Bartlett et al., 2016). These molecules can also be expressed in genetically modified donor pigs, opening the prospect of inhibiting T-cell activation locally at the graft site, thus avoiding systemic immunosuppression of the recipient and the consequent risk of infection. Protective effects of human CTLA4-Ig expression on porcine cells and tissues were shown in xenogeneic neuronal cell (Aron Badin et al., 2016) and skin transplantation experiments (Wang et al., 2015).

LEA29Y expressing transgenic porcine neonatal islet-like cell clusters transplanted into immunodeficient diabetic mice normalized blood glucose levels and, in contrast to wild-type neonatal islet-like cell clusters, were not rejected after the recipient mice were reconstituted with human immune cells (Figure 1) (Klymiuk et al., 2012). A subsequent study using diabetic mice with a long-term "humanized" immune system as recipients showed that LEA29Y expressing porcine neonatal islet-like cell clusters survived for several months and normalized the recipients' blood glucose levels, whereas wild-type islets did not engraft in this model (Wolf-van Buerck et al., 2017). Neonatal islet-like cell clusters have a number of advantages over adult porcine islets, 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 (reviewed in Kemter and Wolf, 2018). However, neonatal islet-like cell clusters are immature and not fully functional after isolation. To visualize the maturation and proliferation of neonatal islet-like cell clusters, we generated transgenic pigs expressing enhanced green fluorescent protein (eGFP) under the control of the porcine insulin gene (INS) promoter. The reporter gene is expressed specifically in beta cells and the level of expression increases upon beta-cell maturation (Kemter et al., 2017). 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 molecular profiling studies, such as single-cell RNA sequencing (Kemter and Wolf, 2018).

To prevent lysis of xenogeneic cells by natural killer cells, transgenic pigs expressing HLA-E/beta2-microglobulin were generated. Their cells were effectively protected against human natural killer-cell mediated cytotoxicity, depending on the level of CD94/NKG2A expression on the natural killer cells (Weiss et al., 2009). To control macrophage activity, human CD47 has been expressed on porcine cells to activate the "don't eat me signal" receptor SIRPα on (human) monocytes/macrophages and to suppress phagocytic activity (reviewed in Cooper et al., 2016).

Genetic modifications to overcome dysregulation of coagulation and inflammation

Dysregulation of coagulation and disordered hemostasis are frequent complications in preclinical pig-to-nonhuman primate xenotransplantation studies. Inflammation, vascular injury, innate, humoral and cellular immune responses, and, in particular, molecular incompatibilities between porcine and primate regulators of coagulation are discussed as potential causes (reviewed in Cowan and Robson, 2015).

Key endothelial anticoagulant/antithrombotic proteins that have been modified/supplemented by genetic engineering of donor pigs include human thrombomodulin (TBM), endothelial protein C receptor, tissue factor pathway inhibitor, and ectonucleoside triphosphate diphosphohydrolase 1 (CD39) (reviewed in Cowan and Robson, 2015). Porcine thrombomodulin binds human thrombin, but is a poor co-factor for activation of human protein C. Therefore, we generated transgenic pigs expressing human thrombomodulin under the control of the porcine thrombomodulin gene (THBD) promoter (Figure 2) (Wuensch et al., 2014). A GGTA1 knockout, hCD46/hTBM transgenic pig heart survived for 945 d after heterotopic abdominal transplantation into a baboon with appropriate immunosuppression (Mohiuddin et al., 2016).
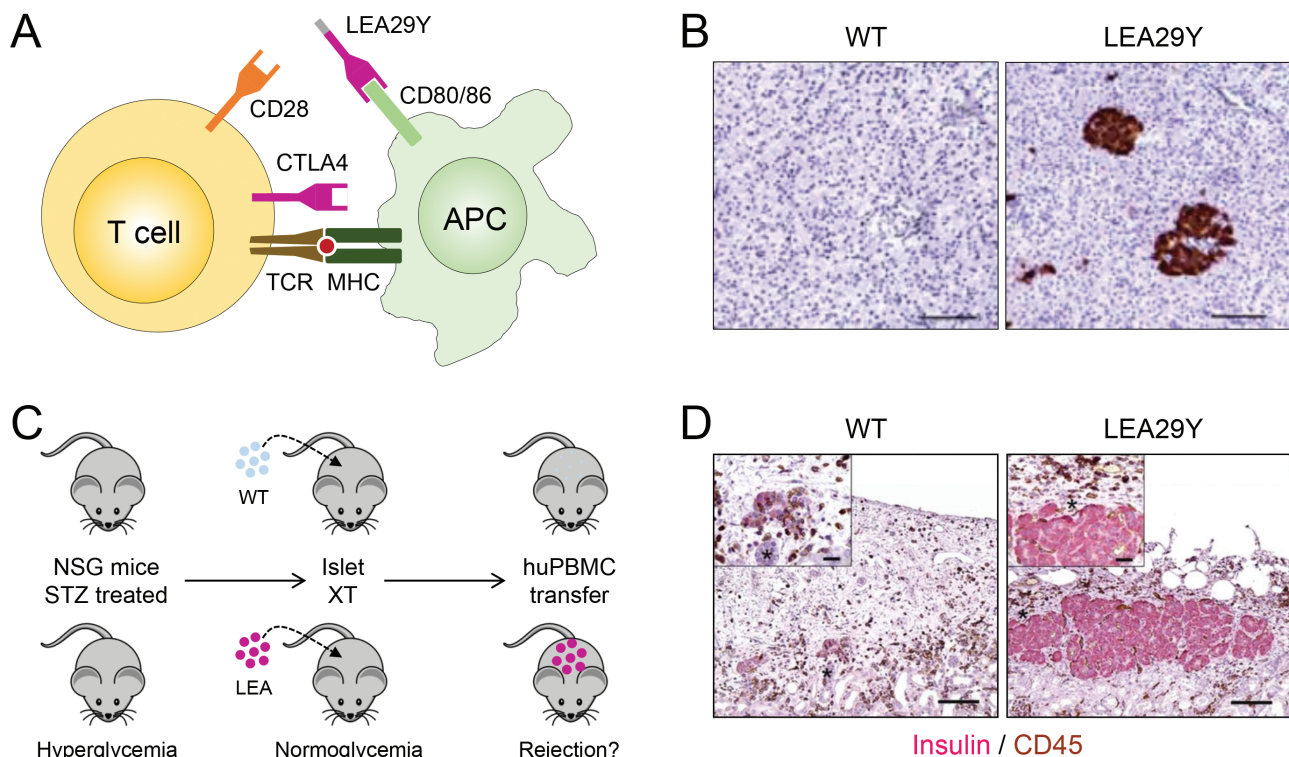
Figure 1. Protection of xenotransplanted porcine pancreatic islets against T-cell mediated rejection by local expression of LEA29Y. (A) Principle of co-stimulation blockade of T cells. Activation of T cells requires interaction between the T-cell receptor and a peptide-loaded MHC on an antigen-presenting cell (APC). In addition, a second signal such as the interaction between CD28 und CD80/CD86 is required. The interaction of CTLA4 and CD80/CD86 blocks T-cell activation. The latter can also be achieved by the soluble molecule CTLA4-Ig or its affinity-optimized version LEA29Y. (B) Immunohistochemical staining of LEA29Y in pancreas sections from INS-LEA29Y transgenic pigs. (C) Transplantation of neonatal islet-like cell clusters (NICCs) from wild-type (WT) or INS-LEA29Y transgenic pigs (LEA29Y) under the kidney capsule of immune deficient streptozotocin (STZ)-diabetic NSG mice results in an insulin-positive cell mass that is able to normalize their blood glucose level. If the mice are subsequently reconstituted with human peripheral blood mononuclear cells (hPBMCs), the WT islets are rejected while the LEA29Y transgenic islets are protected (Klymiuk et al., 2012). (D) Histology of the transplantation site. In recipients of WT NICCs, very few insulin-positive cells were found, but a massive T-cell infiltration (shown by CD45 staining) was evident. In contrast, LEA29Y expressing NICCs survived and formed large clusters of insulin-positive cells. T-cell infiltration was observed in the periphery, but not within the insulin-positive cell clusters.

In addition to modifications targeting coagulation disorders in xenotransplantation, transgenic pigs expressing antiapoptotic and antiinflammatory proteins, such as human tumor necrosis factor-alpha-induced protein 3 (A20) (Oropeza et al., 2009) and human heme oxygenase-1 (HO-1) (Petersen et al., 2011), have been produced.

Genetic modifications to decrease the risk for zoonoses

Xenotransplantation may be associated with the risk of transmission of porcine microorganisms including bacteria, fungi, and viruses able to adapt in the recipient and to induce a disease (zoonosis or xenosis) (reviewed in Fishman, 2018). Many microorganisms can be eliminated from the donor pigs by selection, treatment with antibiotics, antimycotics or antiviral drugs, by vaccination, by early weaning and colostrum deprivation, by caesarean delivery or embryo transfer, and by maintenance of the donor animals in designated pathogen-free housing facilities (reviewed in Kemter et al., 2018). An example is the elimination of porcine cytomegalovirus by early weaning of piglets, even if their mothers were infected (Egerer et al., 2018).

In contrast, porcine endogenous retroviruses (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. Until now, no transmission of PERV has been observed in preclinical and clinical trials (Denner, 2018). To prevent PERV transmission despite their integration in the pig genome, several strategies have been developed: 1) selection of pigs with a low copy number and a low expression of PERV-A and PERV-B proviruses; 2) selection of PERV-C free animals to avoid PERV-A/C recombinants with increased replication competence; 3) knockdown of PERV expression by RNA interference in transgenic pigs; and 4) vaccination against transmembrane and surface envelope proteins of PERV (reviewed in Kemter et al., 2018).

A breakthrough was achieved when the CRISPR/Cas9 technology was used to inactivate PERVs integrated in the pig genome. After proof of principle in immortalized PK-15 pig cells (Yang et al., 2015), all PERV copies (altogether 25) were inactivated in primary pig cells and these were used for somatic cell nuclear transfer to produce live healthy piglets (Niu et al., 2017). The technical feasibility of reducing the risk of PERV transmission to zero is exciting, but it is
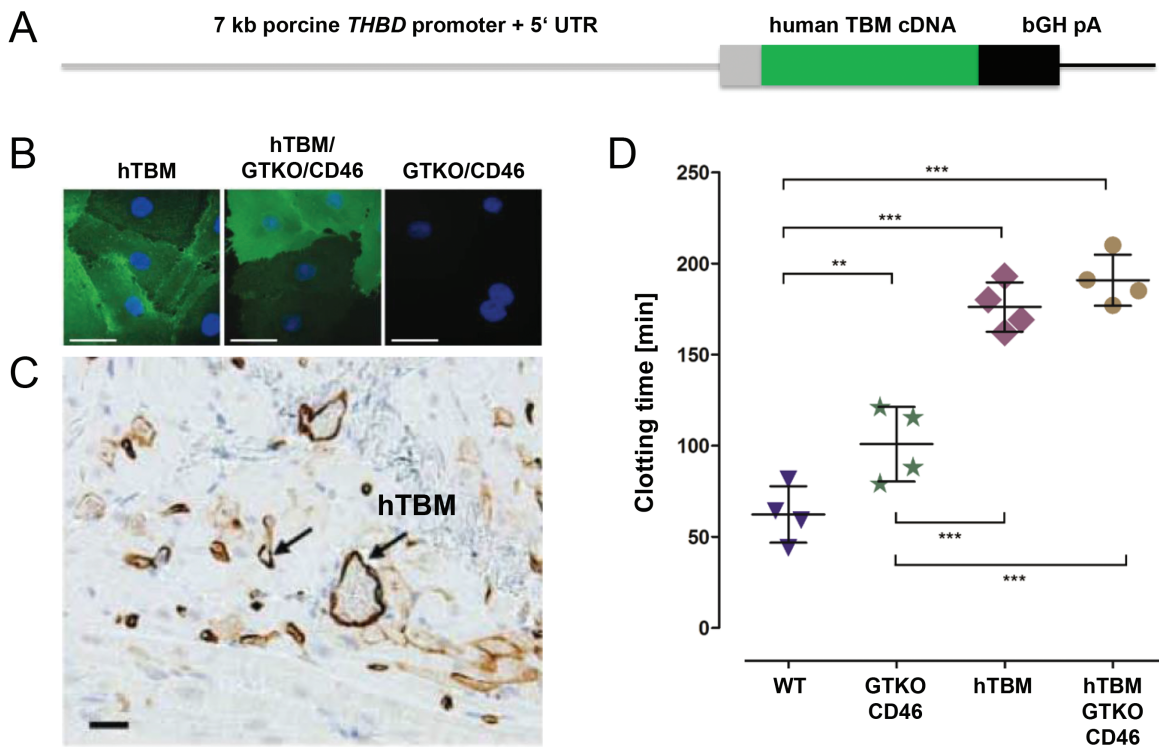
Figure 2. Expression of human thrombomodulin (hTBM) in genetically (multi-)modified pigs. (A) Expression vector with the porcine *THBD* gene promoter. (B) Immunofluorescence staining of hTBM in transgenic porcine endothelial cells. (C) Expression of hTBM in vascular endothelial cells of myocardium from transgenic pigs. (D) Beads covered with hTBM expressing endothelial cells from genetically (multi-)modified pigs delay clotting of human blood (Wuensch et al., 2014).

not clear at this stage if genome-wide PERV inactivation by CRISPR/Cas9 is actually required for entering clinical xenotransplantation trials.

Recent breakthrough in orthotopic pig-to-baboon cardiac xenotransplantation

Heart transplantation is the only cure for patients with terminal cardiac failure, but the supply of human donor organs does not meet the clinical need. Xenotransplantation of genetically modified pig hearts is a potential alternative as demonstrated by long-term survival (up to 945 d) of genetically multimodified pig hearts (GGTA1 KO, hCD46/hTBM transgenic) after heterotopic abdominal transplantation in baboons (Mohiuddin et al., 2016). Although this model demonstrated long-term acceptance of discordant cardiac xenotransplants with safe immunosuppression, their life supporting function remained to be proven. Therefore, Längin et al. (2018) used the same genetic background of donor pigs and adapted the immunosuppressive regimen developed by Mohiuddin et al. (2016) to perform a series of orthotopic heart transplantation (= heart replacement) experiments in baboons, finally resulting in consistent long-term success with survival times up to 195 d (Figure 3). The most essential improvements were 1) specific perfusion preservation of the xeno-hearts after explantation and during implantation with 8 °C-oxygenated hyperoncotic cardioplegic solution containing nutrition, hormones, and erythrocytes; and 2) post-transplantation growth control of the xeno-hearts by early weaning of glucocorticoids, lowering the recipients' blood pressure, and inhibition of mTOR (mechanistic target of rapamycin) activation to counteract cardiomyocyte hypertrophy. Consistent life-supporting function of xeno-hearts for up to 195 d in the most relevant and stringent preclinical animal model is a milestone on the way to clinical cardiac xenotransplantation (Längin et al., 2018).

Conclusions and Perspectives

Recent studies of life-supporting cardiac (Längin et al., 2018) and kidney xenotransplantation (survival > 400 d; Kim et al., 2019) in nonhuman primates have achieved survival times that the initiation of clinical xenotransplantation trials may be justified. This requires an internationally accepted regulatory framework covering safety and quality standards of donor pigs, requirements for preclinical data, selection and information of trial participants, post-transplant long-term patient follow-up, and storage of appropriate pre- and post-procedure specimens from donor pigs and patients. Pertinent recommendations from the Third WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials (Changsha, China, December 12–14, 2018) will be published as The 2018 Changsha Communiqué.
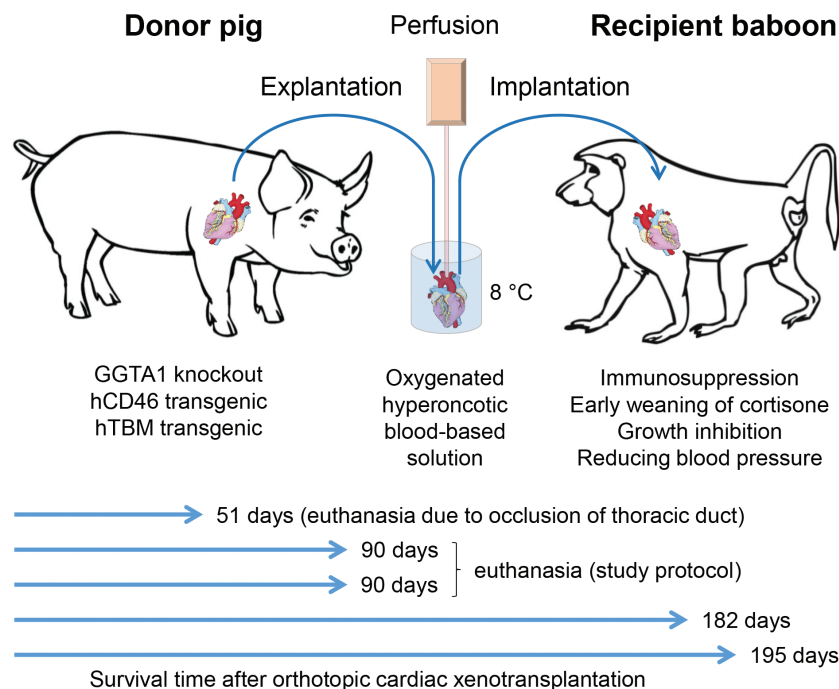
Figure 3. Factors enabling consistent success in life-supporting pig-to-baboon cardiac xenotransplantation. In addition to genetically multimodified porcine donor hearts (lacking αGal epitopes and expressing human CD46 as well as human thrombomodulin) and appropriate immunosuppression, two steps were key to success: 1) nonischemic preservation of the donor hearts by perfusion with oxygenated hyperoncotic blood-based solution; and 2) prevention of detrimental xeno-heart overgrowth by early weaning of cortisone, lowering of blood pressure and treatment with the mTOR inhibiting prodrug temsirolimus (Längin et al., 2018).

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Eckhard Wolf studied Veterinary Medicine at the LMU Munich, Germany. He is Head of the Institute for Molecular Animal Breeding and Biotechnology, Director of the Laboratory for Functional Genome Analysis, and Director of the Center for Innovative Medical Models at LMU Munich. His lab is specialized in the generation and characterization of genetically engineered pigs as models for human diseases (diabetes mellitus and rare monogenic diseases) and as organ donors for xenotransplantation. Wolf is Speaker of the DFG-funded Transregional Collaborative Research Center (TRR) 127 "Biology of xenogeneic cell, tissue and organ transplantation – from bench to bedside." **Corresponding author:** ewolf@lmu.de

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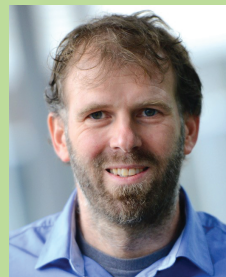
Nikolai Klymiuk studied Biochemistry at the University of Vienna, Austria. He has been a Senior Scientist at the Institute for Molecular Animal Breeding and Biotechnology and Center for Innovative Medical Models at LMU Munich since 2005 and focused his research on the design and generation of large animal models for biomedical research. Since May 2019, he is Associate Professor for Cardiovascular Translation in Large Animal Models at the Technical University Munich. He is Principal Investigator in the DFG-funded TRR 127 "Biology of xenogeneic cell, tissue and organ transplantation – from bench to bedside," where he is leading the porcine genetic engineering program.

Bruno Reichart is a German cardiothoracic surgeon who performed Germany's first successful combined heart-lung transplantation in 1983. In 1984, he succeeded Prof. Christiaan Barnard at Groote Schuur Hospital in Cape Town and was appointed President of the International Society for Heart and Lung Transplantation from 1988 to 1990. He returned to Munich in 1990 as Director of the Cardiac Surgery Clinic at the Klinikum Großhadern of LMU Munich, a position he held till 2011. He has been active in experimental research in discordant xenotransplantation from 1998, currently as Co-Speaker of the DFG-funded TRR 127 "Biology of xenogeneic cell, tissue and organ transplantation – from bench to bedside."

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