Kidneys From $\alpha_{1,3}$-Galactosyltransferase Knockout/Human Heme Oxygenase-1/Human A20 Transgenic Pigs Are Protected From Rejection During Ex Vivo Perfusion With Human Blood

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Background. Multiple modifications of the porcine genome are required to prevent rejection after pig-to-primate xenotransplantation. Here, we produced pigs with a knockout of the $\alpha_{1,3}$-galactosyltransferase gene (GGTA1-KO) combined with transgenic expression of the human anti-apoptotic/anti-inflammatory molecules heme oxygenase-1 and A20, and investigated their xenoprotective properties. Methods. The GGTA1-KO/human heme oxygenase-1 (hHO-1)/human A20 (hA20) transgenic pigs were produced in a stepwise approach using zinc finger nuclease vectors targeting the GGTA1 gene and a Sleeping Beauty vector coding for hA20. Two piglets were analyzed by quantitative reverse-transcription polymerase chain reaction, flow cytometry, and sequencing. The biological function of the genetic modifications was tested in a $^{51}$Chromium release assay and by ex vivo kidney perfusions with human blood. Results. Disruption of the GGTA1 gene by deletion of few basepairs was demonstrated in GGTA1-KO/hHO-1/hA20 transgenic pigs. The hHO-1 and hA20 mRNA expression was confirmed by quantitative reverse-transcription polymerase chain reaction. Ex vivo perfusion of 2 transgenic kidneys was feasible for the maximum experimental time of 240 minutes without symptoms of rejection. Conclusions. Results indicate that GGTA1-KO/hHO-1/hA20 transgenic pigs are a promising model to alleviate rejection and ischemia-reperfusion damage in porcine xenografts and could serve as a background for further genetic modifications toward the production of a donor pig that is clinically relevant for xenotransplantation.

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the ischemic tissue is damaged after reperfusion by reactive oxygen species. The complement system, proinflammatory changes of the endothelium, and neutrophils are critically involved in this cascade of deteriorating events. Strategies to overcome AVR and IRI include the expression of human anti-coagulant, anti-apoptotic, and/or anti-inflammatory transgenes on porcine tissues and organs.

Previously, we had produced a human heme oxygenase-1 (hHO-1) transgenic pig line and demonstrated significant protection of hHO-1 transgenic porcine aortic endothelial cells against TNF-α-mediated apoptosis and prolonged survival of hHO-1 transgenic kidneys in ex vivo xenoperfusion experiments with human blood. The anti-apoptotic and anti-inflammatory properties of hHO-1 are mediated by catalyzing cytotoxic free heme to biliverdin, carbon monoxide (CO), and free iron. Biliverdin is reduced to bilirubin, which acts as a potent antioxidant. The CO provides anti-apoptotic and anti-inflammatory effects via activation of the p38 MAPK pathway. Free iron upregulates ferritin which protects cells from oxidative damage. This renders hHO-1 promising for attenuating the detrimental effects during xenograft rejection.

Human TNF-α-induced protein 3, also known as human A20 (hA20), is a cytoplasmic zinc-finger protein that prevents inflammation by blockade of NF-κB activation and inhibition of TNF-α–mediated apoptosis. We had shown that transgenic hA20 expression in pigs also provided immune modulatory effects in porcine aortic endothelial cells by rendering them less susceptible to CD95(Fas)L-mediated cell death. Because of previous experience, we decided to construct a new hA20 expression vector based on the Sleeping Beauty (SB) transposon system to ensure high ubiquitous expression of the transgene. The SB transposons are less susceptible to rearrangements and not affected by reverse transcriptase-induced mutations, usually leading to stable gene expression profiles.

Here, we report the production and characterisation of pigs that express cytoprotective hHO-1 and hA20 transgenes on a α1,3-galactosyltransferase knockout (GGTA1-KO) background. The GGTA1 gene was disrupted by targeting exon 9 of the GGTA1 gene coding for the catalytic domain of the α1,3 galactosyltransferase with zinc-finger nucleases (ZFN) as recently described. These genetic modifications were combined in 1 porcine genotype to evaluate if the triple genetic modifications would result in a better control of xenograft rejection. We studied the biological function of the transgenes in vitro in a Chromium (51Cr) release assay and performed ex vivo kidney xenoperfusions with human AB-pooled blood to investigate its protective effects on IRI.

**MATERIALS AND METHODS**

**Animals**

The animals used in this study were German Landrace pigs from the experimental herd of the Institute of Farm Animal Genetics, Mariensee. Animal experiments were conducted in accordance with the German Animal Welfare Act and had been approved by the local authority (Lower Saxony State Office for Consumer Protection and Food Safety, LADES, TVA: 33.14-42302-04-12/0891).

**Design of hA20 Expression Vector**

The cDNA of the hA20 gene (kindly provided by Rudi Beyaert, LMBP 3778) was expressed by the 9.7-kb vector pSB-CAG-hA20-IRES-neo, based on the hyperactive SB transposon system (kindly provided by Dr. Zoltán Ivics, Paul-Ehrlich-Institut, Langen, Germany). The transposable element consists of the cytomegalovirus early enhancer / chicken β actin promoter (CAGGS) followed by an E-tag coupled upstream to the hA20 cDNA. A neomycin resistance cassette was linked via IRES to the hA20 sequence to allow antibiotic selection. Inverted terminal repeat sequences flank the integration unit which was cloned from the pCAGGS/hA20 vector into the SB vector via Spel and Xhol restriction sites.

**Production of GGTA1-KO/hHO-1/hA20 Donor Pigs**

Porcine ear fibroblasts from a well-characterized hHO-1 transgenic pig were transfected with a pair of ZFN-based plasmids targeting exon 9 of the porcine GGTA1 gene by electroporation (Gene PulserXcell unit, Bio-Rad). Gal-negative cells were enriched by counterselection with streptavidin-conjugated magnetic beads (Dynabeads, Life Technologies) in a magnetic field and served as donor cells for somatic cell nuclear transfer (SCNT). Fibroblasts from a GGTA1-KO/hHO-1 fetus were electroporated with the pSB-CAG-hA20-IRES-neo vector and the SB transposase 100× plasmid (molar ratio, 10:1). After antibiotic selection with 800 μg G418/mL medium for 14 days, cell colonies were screened for genomic integration of hA20 by polymerase chain reaction (PCR) and used for SCNT. One fetus from day 25 post conception was used for cloning.

**Transgenic Expression Analysis by Real-Time PCR**

The mRNA expression of hHO-1 and hA20 was analyzed in GGTA1-KO/hHO-1/hA20 transgenic fetuses and pigs by quantitative reverse-transcription (RT-q)PCR as previously described. In brief, 0.6 μg of total RNA was treated with DNase I (RNase-free, 1 U/μL, Biozym Scientific GmbH, Germany) before incubation with 0.8 μL 2.5 mM EDTA (Roth, Germany) solution. Reverse transcription (RT) was run for 10 minutes at 25°C, 60 minutes at 42°C, and 5 minutes at 99°C. Two microliters of 1:5 diluted RT mix were added to the real-time PCR mix. The real-time PCR was performed in the ABI 7500 Fast Real-Time System (Life Technologies) for 10 minutes at 95°C, 40 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute (for primers used see SDC, Materials and Methods). For quantification, threshold cycle values were fitted to the standard curves using the Sequence Detection Software 1.4. Normalization factors were calculated with the Excel based software geNorm (http://medgen.ugent.be/~jdevsomp/genorm/). The specificity of the PCR product size was confirmed by electrophoresis on a 3.5% agarose gel.

**Flow Cytometry Analysis**

Absence of Gal epitopes on ear fibroblasts from the GGTA1-KO/hHO-1/hA20 transgenic pigs was investigated by flow cytometry. Approximately 1 x 10⁹ fibroblasts were incubated with Fluorescin isothiocyanate-conjugated Isolectin-B4 (3 μg/mL cell suspension; isolated from Griffonia simplicifolia; Enzo Life Sciences) for 5 minutes at 37°C. After centrifugation, the cell pellet was resuspended in 1 mL phosphate-buffered saline. Fluorescence was measured in a Gallios Flow Cytometer (Beckman Coulter). The emitted signal was collected at wavelength range of 505 to 545 nm, converted by a logarithmic amplifier, and processed using company-owned software. Fibroblasts from a GGTA1-KO pig, and wild-type (WT) fibroblasts incubated with or without lectin served as controls.
TABLE 1.
Experimental setup for kidney perfusion

| Experimental group | Blood          | Kidney donor pig | n   | C1 inhibitor |
|--------------------|----------------|------------------|-----|--------------|
| Autologous         | Porcine        | Wild-type        | 5   | Yes          |
| Xenogeneic         | Human          | Wild-type        | 10  | Yes          |
| Transgenic         | GGTA1-KO/hHO-1/hA20 transgenic | 2   | No            |

Sequencing of GGTA1 Gene Locus in Cloned Pigs

The success of genetic modification of the targeted region within the GGTA1 gene was confirmed by allele-specific sequencing of the GGTA1 gene. The PCR products of the ZFN target site within the GGTA1 gene were purified with the Invirob Fragment CleanUp Kit (STRATEC Molecular, Germany) and ligated with the commercial pGEM-T Easy Vector (Promega). The ligation product was transformed into E. coli XL10-Gold ultracompetent cells (Stratagene). Colonies were picked the following day for control PCR and were sent for sequencing with T7/SP6 primers.

Assay for Complement-Mediated Lysis of Porcine Fibroblasts

The $^{51}$Cr release assays were performed to study the susceptibility of GGTA1-KO/hHO-1/hA20 cells and controls to lysis by human antibody/complement. Porcine fibroblasts ($3 \times 10^6$) were labelled with $100 \mu$Ci of sodium $^{51}$Cr-chromate (GE Healthcare, Buckinghamshire, Great Britain; $1 \text{Ci} = 37 \text{GBq}$) and plated at $1 \times 10^4$ cells/well in microtiter plates. After 18 hours, dead fibroblasts were removed by “dumping.” Cells were incubated with increasing concentrations of pooled complement preserved normal human serum (Dunn Laborteknik, Asbach, Germany). After 4 hours, $25 \mu$L of the cell supernatant were removed, and the amount of radioactivity was measured in a Microbeta scintillation counter (Wallac, Turku, Finland). The mean counts per minute obtained in triplicate cultures was used for all calculations. The spontaneous release of $^{51}$Cr was determined by incubating the target cells with medium alone, whereas the maximum release was determined by incubating in $2\%$ Triton X-100. Specific lysis was calculated as follows: % specific lysis = (experimental $^{51}$Cr release – spontaneous $^{51}$Cr release) / (maximum $^{51}$Cr release – spontaneous $^{51}$Cr release) $\times 100$.

Ex Vivo Kidney Perfusion

Kidneys from WT or GGTA1-KO/hHO-1/hA20 transgenic pigs were perfused with human (xenogeneic) or porcine blood (autologous, WT) in a well-established ex vivo perfusion circuit.$^6,16$ The perfusion experiments with WT kidneys were not run in parallel with the triple transgenic organs, but taken from previous studies.$^6,16$ Perfusion of WT organs consistently results in symptoms of a hyperacute rejection.

RESULTS

Generation of GGTA1-KO/hHO-1/hA20 Transgenic Pigs

Eleven fetuses were obtained from 1 recipient after transfer of 88 GGTA1-KO/hHO-1 embryos. Fibroblasts isolated from 1 GGTA1-KO/hHO-1 transgenic fetus were cotransfected with the pSB-CAG-hA20-IRES-neo vector and the SB transposase.
fetuses were obtained, of which 1 fetus (682/2) was used for recloning. A total of 580 triple transgenic reconstructed embryos were transferred to 6 recipients. Three recipients gave birth to 6 stillborn and 5 liveborn piglets with low birth weights between 0.5 and 0.9 kg. Three piglets died within 24 hours after birth, 2 pigs (K746 and K747) survived and developed normally (Table 2). All piglets were confirmed as GGTA1-KO/hHO-1/hA20-transgenic by PCR (data not shown).

Transgene Expression in Offspring

The RT-qPCR analysis revealed that both transgenes were expressed in all fibroblast samples from GGTA1-KO/hHO-1/hA20 transgenic offspring. The mean hHO-1 mRNA expression level varied among piglets and was increased approximately 1.7-fold in 8 of 11 live and stillborn GGTA1-KO/hHO-1/hA20 transgenic piglets compared to 3 fetuses (Figure 1A). Expression of hA20 was compared to a pig (K271) originating from our previous hA20 line.12 The hA20 mRNA levels were increased 37-fold in GGTA1-KO/hHO-1/hA20 transgenic fetuses and 27-fold in pigs recloned from these fetuses (Figure 1B). The WT-controls showed neither hHO-1 nor hA20 expression.

Analysis of ZFN-Mediated GGTA1-KO

Flow cytometry analysis revealed functional disruption of the GGTA1 gene as demonstrated by absence of Gal epitopes on fibroblasts from pigs K746 and K747. The negative WT control (without GSIB4-lectin) showed no fluorescence, whereas the positive WT control (with GSIB4-lectin) showed massive Gal expression (Figure 2A). Sequencing of genomic DNA from K746 and K747 revealed identical mutations of the GGTA1 gene: a single nucleotide deletion on 1 allele and a dinucleotide deletion on the other allele (Figure 2B).

Decreased Susceptibility of GGTA1-KO/hHO-1/hA20 Fibroblasts to Antibody/Complement-Mediated Lysis

Fibroblasts from WT animals were lysed by increasing concentrations of human antibody/complement. Although %

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FIGURE 1. A, HHO-1. B, HA20 transgene expression detected by real-time PCR. A, Expression of hHO-1 mRNA in GGTA1-KO/hHO-1/hA20 transgenic fetuses (n = 3, dotted, set = 1) and live or stillborn recloned pigs (n = 8, striped). Wild-type controls are negative (n = 2). Values from 2 technical replicates per sample. Results are given as fold change ± standard deviation. B, Expression of hA20 mRNA in clone K271 (bred from previous hA20 pig line, black bar, set = 1) compared to GGTA1-KO/hHO-1/hA20 transgenic fetuses (n = 3; dotted) and live- or stillborn recloned pigs (n = 8, striped). Wild-type controls are negative (n = 2). Values from 2 technical replicates per sample. Results are given as fold change ± standard deviation.

FIGURE 2. Analysis of ZFN-mediated GGTA1-KO. A, Flow cytometric analysis of fibroblasts from WT controls and both clones (K746 and K747) after incubation with FITC-conjugated isoelectin B4 or without (WT negative control). B, Sequencing analysis of the ZFN target region within the GGTA1 gene. The original wild-type sequence is given in the upper line. Both cloned pigs (K746 and K747) carry identical mononucleotide deletion on one and dinucleotide deletion on the other allele (boxed region). Bold letters represent ZFN target sequences.
specific lysis was low, a clear-cut dose-response effect could be demonstrated. At the highest antibody/complement concentration (1:5 dilution) with an average of 18.1% (±1.1%) specific lysis was found in fibroblasts from WT pigs. In contrast, specific lysis remained low (5% ± 1.1%) in transgenic cells (Fig. 3).

**Prolonged Organ Survival in Ex Vivo Kidney Perfusion**

Perfusion of kidneys from the 2 GGTA1-KO/hHO-1/hA20 transgenic pigs with human blood was feasible for the maximum duration (240 minutes) of the experiment without addition of the complement inhibitor C1-Inh. The RVR remained constantly low during the perfusion time. Perfusion was similarly successful after autologous perfusion of porcine WT kidneys (n = 5), whereas in xenogeneic perfused WT kidneys (n = 10; human blood) RVR increased dramatically and perfusion had to be terminated after 142 ± 26 minutes, despite addition of a soluble complement inhibitor (C1 inhibitor) (Fig. 4A and B). The maximal RI was 5.1 ± 1.7 in the xenogeneic group, 1.65 ± 0.1 in the autologous group, and 0.7 ± 0.1 in the transgenic group.

Platelet counts dropped over time in the xenogeneic group, but only slightly decreased in the autologous and the transgenic group, most probably due to shear stress in the roller pump (Fig. 5A). Coagulation parameters changed over time in plasma samples taken from the perfusion circuit at defined time points. Although fibrinogen was consumed in the xenogeneic group, the levels remained stable after an initial slight drop in the GGTA1-KO/hHO-1/hA20 transgenic group, similar to autologous perfusion. Likewise, antithrombin activity dropped in the xenogeneic group, but remained constant over time after an initial decrease in the transgenic group and stable during autologous perfusion (Fig. 5B and C). D-dimer and TAT complex concentrations remained low in the autologous group. In contrast, they were strongly increased in the xenogeneic WT group and also increased in the transgenic group, but to a lesser extent (Fig. 5D and E).

**DISCUSSION**

The production of multitransgenic donor pigs for xenotransplantation is considered critical for achieving long-term protection from the immunological rejection cascade associated with porcine-to-primate xenotransplantation, including the HAR, AVR, and cellular rejection. Previously, we reported the production of pigs with various single genetic modifications, including a homozygous ZFN-mediated GGTA1-KO,15 transgenic expression of hA20,12 and hHO-1.6 Moreover, we had demonstrated the efficacy of these genetic modifications in protecting porcine organs from rejection by in vitro assays and ex vivo perfusion experiments. Here, we report the successful production and characterization of pigs that combine all 3 genetic modifications to further improve their protective properties against ischemia-reperfusion damage and xenograft rejection.

The successful insertion of these genetic modifications was possible by 2 efficient new molecular technologies, that is, ZFN and the SB transposon system. ZFNs are known to have a ~10,000-fold higher efficiency than conventional homology-based gene targeting methods for gene disruption and can induce biallelic gene disruption within 1 step.15 Transposon systems, like the hyperactive SB transposon system, have emerged as efficient nonviral method for transgene delivery. The active genomic insertion by the transposase SB100X leads to integration of the transposon, preferably...
into permissive genomic loci, and is thus compatible with high and stable transgene expression. Transgenic porcine cells can easily be used to induce additional genetic changes by these 2 technologies.

The GGTA1-KO/hHO-1/hA20 pigs were produced in a stepwise manner, with a cloning step before adding the next genetic modification. This strategy is promising to arrive at pigs with multiple genetic modifications because it allows selection for the highest expression of each transgene and for normally developing fetuses to avoid problems putatively arising from the cloning process. However, the combined integration of several transgenes into a single permissive locus is preferred to avoid genetic segregation when producing the ultimate donor pig.

Sequencing and flow cytometry analysis revealed that both alleles of the GGTA1 gene were disrupted in the GGTA1-KO/hHO-1/hA20 pigs. Transgenic expression in the GGTA1-KO/hHO-1/hA20 offspring was analyzed by RT-qPCR. HHO-1 mRNA was detected in fibroblasts indicating that the transgene had not been silenced during the stepwise cloning process. Human A20 mRNA expression levels were dramatically increased in the GGTA1-KO/hHO-1/hA20 transgenic offspring compared to our previous hA20 line. The most likely explanation for this was the use of the SB-based hA20 transposon vector and the integration site; but also the copy number might have influenced expression levels, as transposons are known to integrate with multiple copy numbers.

FIGURE 5. Parameters of coagulation activation during kidney perfusion. A, Thrombocyte counts at defined time points of the perfusion are given for the autologous (circle), xenogeneic (square), and GGTA1-KO/hHO-1/hA20 transgenic perfusion group (triangle). The changes in parameters of coagulation activation in the experimental groups are shown for: (B) fibrinogen, (C) antithrombin activity, (D) D-dimer, and (E) TAT complex. Data for WT-perfusions are from Petersen et al and Ramackers et al.

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Detection of the transgenic proteins was complicated due to the highly conserved sequences between the human and porcine protein (protein sequence identity for heme oxygenase-1 [HO-1]: 83%; for A20: 88%), resulting in nonspecific protein bands. These difficulties were encountered already in the analysis of the previous hHO-1 transgenic pig line.6 Western blot analysis for hA20 expression in triple transgenic pigs revealed nonspecific protein bands caused by the cross-reactivity of the antibody (polyclonal rabbit antihuman-A20 antibody, A302-633A, Bethyl Laboratories) with porcine hA20 (Figure S1, SDC, http://links.lww.com/TXD/A6).

The xenoprotective potential of the GGTA1-KO/hHO-1/hA20 transgenic pigs was demonstrated in the 51Cr release assay which revealed significant protection against complement-mediated cytotoxicity similar to a previous GGTA1-KO pig line.15 Antibody-binding to Gal epitopes could be prevented by prolonged perfusion time compared to nontreated controls18 which in turn has shown to upregulate more TF on endothelial cells26 and monocytes.21 Thus, inflammation and coagulation are linked and interact with each other.

HHO-1 and A20 are well known “xenoprotective genes”19 and can protect tissue and organs from IRI.22,23 The hHO-1–derived CO suppresses endothelial cell apoptosis via activation of the p38 MAPK pathway,24 induces vasodilation, and inhibits platelet aggregation mediated by activation of guanylate cyclase.25 The A20 and HO-1 both block NF-κB activation, which in turn induces induction of apoptosis and reduced production of proinflammatory molecules. Thereby, less endothelium is damaged and less TF is expressed and exposed to the recipient’s blood.

Although results from this study are preliminary and require substantiation, these results provide important new insight into the combined effects of three genetic modifications on kidney perfusion in a xenoperfusion setting. The comparison with previously perfused hHO-1 kidneys showed that the GGTA1-/hHO-1/hA20 kidneys are advantageous over the hHO-1 single transgenic alternative.6 However, individual effects of each modification cannot be inferred from this study because their interaction with each other is not predictable. It is not clear whether the prolonged perfusion time of the transgenic kidneys in this study was related to the lack of Gal molecules, the additional hA20 expression, or both. So far, no perfusion with GGTA1-KO kidneys alone has been performed.

In conclusion, this is, to the best of our knowledge, the first report on the production of pigs with a combined expression of 2 functional anti-apoptotic and anti-inflammatory transgenes on a GGTA1–KO background. The triple transgenic approach provided significant protection against apoptosis in an in vitro cytotoxicity assay and ischemia-reperfusion damage during ex vivo kidney perfusion, holding great potential for future in vivo experiments. Additional anticoagulant strategies, such as transgenic expression of human thrombomodulin26 or knockdown of porcine TF,27 are required to overcome molecular incompatibilities of anticoagulant pathways to achieve complete inhibition of the coagulation activation. The next step is to test GGTA1-KO/hHO-1/hA20 transgenic pig organs in pig-to-non-human primate xenotransplantation to prove potential clinical relevance.
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