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Original Article

Transgenic expression of human heme oxygenase-1 in pigs confers resistance against xenograft rejection during ex vivo perfusion of porcine kidneys

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Abstract: Background: The major immunological hurdle to successful porcine-to-human xenotransplantation is the acute vascular rejection (AVR), characterized by endothelial cell (EC) activation and perturbation of coagulation. Heme oxygenase-1 (HO-1) and its derivatives have anti-apoptotic, anti-inflammatory effects and protect against reactive oxygen species, rendering HO-1 a promising molecule to control AVR. Here, we report the production and characterization of pigs transgenic for human heme oxygenase-1 (hHO-1) and demonstrate significant protection in porcine kidneys against xenograft rejection in ex vivo perfusion with human blood and transgenic porcine aortic endothelial cells (PAEC) in a TNF-α-mediated apoptosis assay.
Methods: Transgenic and non-transgenic PAEC were tested in a TNF-α-mediated apoptosis assay. Expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) was measured by real-time PCR. hHO-1 transgenic porcine kidneys were perfused with pooled and diluted human AB blood in an ex vivo perfusion circuit. MHC class-II up-regulation after induction with IFN-γ was compared between wild-type and hHO-1 transgenic PAEC.
Results: Cloned hHO-1 transgenic pigs expressed hHO-1 in heart, kidney, liver, and in cultured ECs and fibroblasts. hHO-1 transgenic PAEC were protected against TNF-α-mediated apoptosis. Real-time PCR revealed reduced expression of adhesion molecules like ICAM-1, VCAM-1, and E-selectin. These effects could be abrogated by the incubation of transgenic PAECs with the specific HO-1 inhibitor zinc protoporphorine IX (Zn(II)PPIX, 20 μM). IFN-γ induced up-regulation of MHC class-II molecules was significantly reduced in PAECs from hHO-1 transgenic pigs. hHO-1 transgenic porcine kidneys could successfully be perfused with diluted human AB-pooled blood for a maximum of 240 min (with and without C1 inh), while in wild-type kidneys, blood flow ceased after ~60 min. Elevated levels of d-Dimer and TAT were detected, but no significant consumption of fibrinogen and antithrombin was determined. Microthrombi could not be detected histologically.
Conclusions: These results are encouraging and warrant further studies on the biological function of heme oxygenase-I expression in hHO-1 transgenic pigs in the context of xenotransplantation.

Björn Petersen,[1]* Wolf Ramackers,[2]* Andrea Lucas-Hahn,[1] Erika Lemme,[1] Petra Hassel,[1] Anna-Lisa Queißer,[1] Doris Herrmann,[1] Brigitte Barg-Kues,[1] Joseph W. Carnwath,[1] Johannes Klose,[2] Andreas Tiede,[3] Lars Friedrich,[4] Wiebke Baars,[2] Reinhard Schwinzer,[2] Michael Winkler[2] and Heiner Niemann[1]

[1]Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt, Germany, [2]Transplant Laboratory, Clinic for Visceral- and Transplantation Surgery, [3]Haematology, Haemostasis and Oncology, [4]Clinic for Anaesthesiology and Intensive Care, Hanover Medical School, Hannover, Germany

*Both authors contributed equally to this work.

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Abbreviations: AVR, acute vascular rejection; CO, carbon monoxide; DAF, decay accelerating factor; DIC, disseminated intravascular coagulation; EC, endothelial cells; GFP, green fluorescent protein; MAP, mean arterial pressure; MPV, mean venous pressure; MVP, minimum mean venous pressure; PAEC, porcine aortic endothelial cells; RVR, renal vascular resistance.

Address reprint requests to Heiner Niemann, Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Hoeltystrasse 10, Mariensee, D-31535 Neustadt, Germany (E-mail: heiner.niemann@fli.bund.de)

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Introduction

The growing shortage of human donor organs has prompted significant efforts to establish porcine-to-human xenotransplantation in a clinically acceptable manner. The hyperacute rejection response represents the first immunological barrier to xenotransplantation. It can be overcome by the expression of human complement regulating factors in porcine organs [1] or removing the major porcine cell surface antigens (Gal-epitopes) by knocking out the gene for α1,3-galactosyltransferase [2–6]. The next barrier is the activation of the pig endothelium observed in models of pig-to-primate xenotransplantation [7–12]. The acute vascular rejection response (AVR) is characterized by anti-non-Gal anti-pig antibody induced endothelial cell (EC) activation, up-regulated expression of adhesion molecules, platelet adhesion, and aggregation, followed by fibrin and thrombin deposition, and ultimately thrombosis and disseminated intravascular coagulation (DIC).

Heme oxygenase-1 (HO-1), which is encoded by the HMOX1 gene, is a stress-responsive enzyme that degrades free heme to yield equimolar amounts of carbon monoxide (CO), free iron, and biliverdin. Free iron induces the expression of heavy chain (H-) ferritin (an iron-sequestering protein) [13,14], which constitutes an ATPase-dependent iron pump that decreases the level of intracellular Fe^{2+}. Biliverdin is further converted to bilirubin by biliverdin reductase [15,16]. HO-1 is found intracellularly in microsomes and is regulated at the transcriptional level. It is ubiquitously expressed in mammalian tissue at low levels under physiological conditions, with the exception of the spleen, where HO-1 is critical for recycling iron from senescent erythrocytes [15,16]. A wide variety of stimuli can induce HO-1 expression, including heme, heavy metals, hydrogen peroxide, oxidized low-density lipoprotein, hypoxia, endotoxin, nitric oxide and nitric oxide donors, cytokines (IL-1, IL-6 or TNF-α), growth factors, angiotensin II, bacterial lipopolysaccharides, shear stress, heat shock, and UV radiation [17,18].

Transplanted organs express a variety of "protective genes," including HO-1 that previously was shown to be critical for prolonged survival of mouse cardiac xenografts in rats [19,20]. Expression of HO-1 is up-regulated in the vascular endothelium of transplanted organs [19,21], likely via exposure of ECs to circulating free heme, a potent activator of HO-1 in most cell types [13].

The cytoprotective properties of HO-1 induction were first shown two decades ago when exposure of a rat kidney to small quantities of hemoglobin up-regulated HO-1 expression and conferred protection against tissue injury to larger quantities of hemoglobin or myoglobin. Conversely, the presence of an HO-1 inhibitor exacerbated renal injury [22]. The postulated mechanisms are the removal of the reaction substrate (free heme), biological effects of the reaction products (CO, biliverdin, bilirubin), and free iron inducing ferritin production and a suppressive effect on monocyte chemoattractant protein 1 (MCP-1). These findings render HO-1 a promising molecule for xenotransplantation. Here, we produced and characterized transgenic pigs expressing human HO-1 (hHO-1) and utilized an established ex vivo perfusion model using freshly pooled and diluted human blood [23] to investigate the effects of hHO-1 expression on porcine kidney survival. We also evaluated the effects of transgenic hHO-1 expression on porcine endothelial cell activation in an in vitro system.

Material and methods

Production of cloned hHO-1 transgenic pigs

Generation of a human HO-1-DNA construct

A bicistronic expression cassette encoding green fluorescent protein (GFP), followed by IRES and neomycin resistance sequences was ligated in the multiple cloning site of the pSI plasmid (Promega, Madison, WI, USA) downstream of a SV40 promoter/enhancer and a chimeric intron, resulting in the plasmid pTSG1. The HMOX1 cDNA (gift from Dr. T. Ritter, Charité, Berlin) was fused with a 24-bp FLAG-tag, the GFP was removed, finally resulting in the plasmid pTSG1hmox1. The cloning sites and the human HMOX1 sequence were verified by DNA sequencing. The pTSG1hmox1 was amplified in XL10 bacteria, purified by anion exchange column method, and digested with *Bgl*II and *Bam*HI to release a 3706 bp fragment containing the SV40 promoter, intron, HMOX1, IRES, neo, and poly(A) sequence.

Cell culture and transfection of adult porcine fibroblasts

Primary cultures were prepared from connective explants from adult wild-type pigs as described [24]. A total of 3×10^6 fibroblasts were trypsinized and subsequently electroporated at 450 V, 350 μF in 0.8 ml PBS containing 10 μg of the linearized PTSG1hmox1-vector. Transfected cells were transferred to 50 ml of pre-warmed DMEM + 30% FCS and allowed to recover for 5 min before plating them in 96-well plates at a density of 8×10^3 cells per well.

Forty-eight hours after transfection, cells were selected for resistance against G418 (800 μg/ml; Gibco-BRL, Darmstadt, Germany) for 14 days. Cell clones were analyzed for the integration of the HMOX-1 construct by PCR (HMOX1: 5′-CAG-TCTTCGCCCCTGTCTAC-3′, HMOX2: 5′-TGT-TGGGGAAGGTGAAGAAG-3′) at 94 °C for 2 min, then 35 cycles at 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 45 s. Final elongation was 72 °C for 5 min. Positive cell clones were grown to confluency in a four-well dish and serum starved (0.5% FCS + DMEM) prior to use in somatic cell nuclear transfer.

Somatic cell nuclear transfer and transfer of cloned embryos

Somatic nuclear transfer was performed as recently described [25] using in vitro matured abattoir oocytes. Briefly, oocytes were enucleated by removing the first polar body along with the adjacent cytoplasm containing the metaphase plate. A small fibroblast from one of the positive cell clones or from an already generated pig (recloning approach) was placed in the perivitelline space in close contact with the oocyte membrane to form a couplet. After cell transfer, fusion was induced in Ca^{2+}-free SOR2 medium (0.25 M Sorbitol, 0.5 mM Mg-acetate, 0.1% BSA) with a single electrical pulse of 1.1 kV/cm for 100 μs (Eppendorf Multiporator®, Eppendorf, Germany). Reconstructed embryos were activated in an electrical field of 1.0 kV/cm for 45 μs in SOR2 activation medium (0.25 M Sorbitol, 0.1 mM Ca-acetate, 0.5 mM Mg-acetate, 0.1% BSA) followed by incubation with 2 mM 6-dimethylaminopurine (DMAP; Sigma, Hannover, Germany) in NCSU23 medium for 3 h prior to transfer to recipients. Peripubertal hormonally synchronized German Landrace gilts (100–130 kg bodyweight) served as recipients [26].

Expression analysis and assessment of the biological function of hHO-1 in vitro

Culture of porcine aortic endothelial cells (PAEC)

Endothelial cells were isolated by incubating aortal tissue from transgenic pigs with 0.1% type II collagenase (Biochrom, Berlin, Germany) for 30 min at 37 °C. Detached cells were washed twice in cold Dulbecco's medium and subsequently cultured in 2 ml Dulbecco's medium supplemented with 10% FCS, penicillin/streptomycin, glutamine, and 0.6 mg/ml EC growth factor (Boehringer, Mannheim, Germany) in 8 cm^2 tissue culture flasks. Cells were harvested by incubation with 0.5 ml Accutase (PAA Laboratories, Cölbe, Germany) for 5 to 10 min at 37 °C. Purity of cultured PAEC was assessed by the analysis of CD31 expression by FACS.

Caspase 3/7 Glo® assay

The resistance to hHO-1 transgenic PAEC against apoptotic damage after challenge with human TNF-α (BioMol GmbH, Hamburg, Germany) was determined by assaying caspase 3/7 activity (Promega, Mannheim, Germany), following the manufacturers' instructions. PAEC originating from three hHO-1 transgenic pigs and wild-type PAECs (passages 2–6) were used. PAEC were incubated for 4 h with increasing concentrations of TNF-α of 0.1, 1.0, 10, 30, and 50 ng/ml, respectively. PAEC were trypsinized, and following two washing steps in PBS, a total of 10 000 cells were added to a total volume of 100 μl PBS. The cells were mixed with 100 μl of Caspase-Glo® 3/7 reagent and incubated for 10 min at room temperature. Apoptosis was measured in a Sirius-e05/06 single tube luminometer (Berthold detection systems GmbH, Pforzheim, Germany). To prove that anti-apoptotic effects were hHO-1-mediated, transgenic PAEC were pre-incubated with the specific HO-1 inhibitor zinc protoporphorine IX (Zn(II)P-PIX, 20 μM, #sc-200329; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously described [27]. Aliquots of the cells were also used to determine the expression of adhesion molecules (VCAM-1, ICAM-1, E-selectin) by real-time PCR.

Immunofluorescence staining and flow cytometry

The mouse anti-rat CD31 monoclonal antibody TLD-3A12 (IgG1; BD Biosciences, Heidelberg, Germany), which cross-reacts with porcine CD31, was used to analyze cultured porcine endothelial cells. Binding of unlabeled primary reagents was visualized using phycoerythrin (PE)-conjugated rat anti-mouse IgG (BD Biosciences). Cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences) and data processed by WinMDI software (Purdue University Cytometry Laboratories, West Lafayette, IN, USA). Biotinylated mAb MSA3 (kindly provided by Prof. Dr. A. Saalmüller, Wien, Austria) was used for staining of porcine MHC class-II molecules. Binding of MSA3 was visualized by using Streptavidin-PE (BD Biosciences).

Reverse transcriptase-PCR (RT-PCR)

Transcripts of hHO-1 in transgenic PAEC and tissue samples were analyzed by RT–PCR. Total RNA was extracted using TRIZOL Reagent (Invitrogen, Karlsruhe, Germany) following the manufacturers' instructions. Two micrograms RNA were applied to DNAse treatment before

reverse transcription (RT). For RT, 500 ng RNA was added 10× PCR buffer (1×; Invitrogen), $MgCl_2$ (50 mM; Invitrogen), dNTP (10 mM, Amersham Biosciences, Freiburg, Germany), random hexamers (50 μm; Applied Biosystems, Foster City, CA, USA), RNase inhibitor (20 U/μl, Applied Biosystems), and reverse transcriptase (50 U/μl, Applied Biosystems) to a final volume of 20 μl. Reverse transcription was performed in a hot lid PTC-200 thermocycler (25 °C for 10 min, 42 °C for 60 min, 99 °C for 5 min). Two hundred nanogram of cDNA was applied for gene-specific PCR amplification. Human HO-1 amplification (95 °C for 15 s, 60 °C for 20 s, 72 °C for 15 s, 34 cycles) was carried out using the gene-specific primers HMOX1 and HMOX2 that were also used for normal PCR. The endogenous control gene was PolyA and PolyA-up (5′-GTTTCCTCGGTGGTG TTTCCTGGGCTATGC-3′) and PolyA-low (5′-TGGAGTTCTGTTGTGGGTATGCTGGTG TAA-3′) primers were applied, resulting in a fragment of 256 bp. A negative control for each sample, containing mRNA and sterile water instead of reverse transcriptase was included to exclude genomic DNA contamination. Water controls were added to assure absence of reagent contamination.

Real-time gene expression analysis

A real-time gene expression analysis was performed to determine expression levels of adhesion molecules like VCAM-1, ICAM-1 and E-selectin after treatment for PAEC with human TNF-α (Table 1). RNA from PAEC was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. An On-Column-based DNase Digestion was performed; the RNA was eluted, and aliquots were frozen at −80 °C. Reverse transcription (RT) was performed in a 20 μl volume consisting of 2 μl of 10× RT buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.3; Invitrogen), 2 μl of 50 mM $MgCl_2$ (Invitrogen), 2 μl of 10 mM dNTP solution (Amersham Biosciences), 1 μl (20 Units) of RNAsin® (Applied Biosystems), 1 μl (50 Units) of MULV reverse transcriptase (Applied Biosystems), and 1 μl random hexamers (50 μM) (Applied Biosystems) and 0.1 μg total RNA of each treatment. The samples were incubated at 25 °C for 10 min for primer annealing and then incubated at 42 °C for 1 h. Finally, the samples were heated to 99 °C for 5 min. A negative control for each sample, containing mRNA and sterile water instead of reverse transcriptase was included to exclude genomic DNA contamination. Water controls were added to assure absence of reagent contamination. The cDNA was diluted 1/10, and 2 μl of the diluted sample was used for real-time PCR. Real-time PCR was performed in 96-Well Optical Reaction Plates (Applied Biosystems). The PCR mix in each well included 10 μl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 6.4 μl H_2O, 0.8 μl each of the forward and reverse primers (5 μM), 2 μl of diluted cDNA in a final reaction volume of 20 μl.

The PCR reaction was carried out in an ABI 7500 Fast Real-Time System (Applied Biosystems) using the following program, activation of the Taq

Table 1. Primer sequences used for real-time PCR

Genes	Primer sequences 5′–3′	Number of cycles	Annealing temperature (°C)	Fragment sizebp	Accession number
E-Selectin	5′primer: 1257-1278 TTGGTGCTGTGTGTACATTTGC 3′primer: 1382-1363 TGGACTCAGCAGGAGCTTCA	38	60	126	U08350.1
Icam1	5′primer: 303-323 GGCACTCTACTCTGCCATGCA 3′primer: 392-372 TTTCACATACTCCGGGAACCA	38	60	90	NM_213816
Vcam1	5′primer: 144-163 GGATGGTGTTTGCCGTTTCT 3′primer: 214-192 AATCTGCGCAATCATTTTGTCTT	38	60	71	U08351.1
EEF1A1	5′primer: 1056-1075 CAAAAATGACCCACCTATGG 3′primer: 1104-1123 GGCCAGGATGGTTCAGGATA	38	60	67	NM_001097418.1
Human HMOX1	5′primer: 842-860 AGGGAAGCCCCCACTCAAC 3′primer: 922-903 ACTGTCGCCACCAGAAAGCT	38	60	81	NM_002133

Polymerase for 10 min at 95 °C followed by 38 cycles of denaturation at 95 °C for 15 s, and extension at 60 °C for 1 min. Fluorescent data were acquired during each extension phase. Reaction specificity was controlled by post-amplification melting curve analyses as well as by gel electrophoresis of the obtained products.

Data generated by the Sequence Detection Software 1.4 were transferred to Microsoft Excel for analysis. Differential mRNA expression of each gene was performed using a relative expression software tool (REST©) [28,29], which is based on an efficiency-corrected mathematical model for data analysis using the $2^{-\Delta\Delta C_T}$ method. Using the $2^{-\Delta\Delta C_T}$ method point method, the data are presented as the fold change gene expression normalized to an endogenous reference gene (*EEF1A1*) and relative to the untreated control (PAEC cultured in D10-FCS).

Ex vivo perfusion of porcine kidneys

Survival of hHO-1 transgenic porcine kidneys

Perfusion circuit All perfusion experiments were performed as described previously [22]. A total of 30 kidneys were used from 12 transgenic and 18 non-transgenic German Landrace pigs. Ex vivo perfusion experiments were performed for a maximum of 240 min (Table 2).

For the xenogenic perfusion experiments, freshly drawn and organ-size-matched volumes (350–750 ml) of heparinized (1 U/ml) pooled human whole blood from healthy AB donors was used to eliminate interference from anti-A and anti-B antibodies. Blood was diluted to a hematocrit of 24% with Tyrodes solution and Ringer's lactate and added to the system's filtered Hardshell Venous Reservoir (1316 Minimax Medtronic GmbH; Duesseldorf, Germany). Final perfusate volume was adapted to kidney weight (8 ml/g kidney), and 1 U/ml blood C1 inhibitor (Berinert P, Behring, Germany) was added to the human blood in all groups except for HAR and HH01 without C1 INH to inhibit the hyperacute rejection.

A porcine kidney was perfused via a vascular cannula, and arterial perfusion was maintained by a doublehead roller pump equipped with pressure and flow monitoring devices and a pressure control unit (Stoeckert Instrumente GmbH, München, Germany) under physiological conditions (mean arterial pressure [MAP] 70–130 mmHg, flow 50–150 ml) (minimum mean venous pressure [MVP] was 2–9 mmHg). The perfusion was automatically interrupted by the pump's pressure control unit when MAP exceeded a systolic pressure of 150 mmHg. Oxygenation with constant blood gases and perfusate temperature (pH 7.4, $p(O_2) < 350$ mmHg, $p(CO_2)$ 13–34 mmHg, 37 °C) was achieved by high flow rate perfusion with 500 ml/min in a second parallel circuit using a heated neonatal diffusion membrane oxygenator (3381 Minimax Medtronic, Duesseldorf, Germany). The parameters mean arterial pressure (MAP), mean venous pressure (MPV), flow, temperature, urine volume, pH, pCO_2, and hematocrit were recorded before and during the perfusion continuously. Blood samples were collected before and after 5, 15, 30, 60, 120, 180, and 240 min of perfusion to determine fibrinogen, antithrombin activity, D-dimer, and thrombin antithrombin (TAT). Renal vascular resistance (RVR) was calculated from the following formula: RVR (mmHg/ml/min/g) = (MAP/flow)/kidney weight.

Rejection was defined as a 100% increase in RVR above baseline and was characterized by marked macroscopic pathological changes including hemorrhage and edema of the perfused kidney. Survival time was defined as the time from the start of perfusion to rejection or end of perfusion after 240 min.

After perfusions, tissue samples were obtained for immunohistological staining (H&E staining) to determine the presence of microthrombi. Histological sections were analyzed at 600-fold magnification.

Coagulation analysis

Fibrinogen concentrations (in g/l) were determined according to the Clauss method (reagents provided by Dade Behring, Marburg, Germany). Antithrombin activity (in % of normal) was determined using

Table 2. Experimental groups employed in the ex vivo perfusion experiment

Group	*N*	Kidney	Origin of the blood	Additional complement inhibition
Autologous	5	Wild-type porcine	Porcine	C1 inhibitor
Xenogenic	10	Wild-type porcine	Human	C1 inhibitor
HAR-Control	3	Wild-type porcine	Human	None
hHO-1	6	hHO-1 tg porcine	Human	C1 inhibitor
hHO-1	6	hHO-1 tg porcine	Human	None

a chromogenic anti-Xa-based assay (Dade Behring). D-dimer concentrations were assessed using a latex particle-based immunoassay (Roche, Mannheim, Germany). TAT concentrations were determined by ELISA (Haemochrom Diagnostica, Essen, Germany). C3a levels were determined by ELISA (Quidel, San Diego, CA, USA).

Statistical analysis

Different statistical tests were run on the results of this study. Statistically significant differences between the hHO-1 and the xenogenic group were calculated with the aid of Mann–Whitney *U*-test for perfusion time, resistance index (RI), C3a levels, and the coagulation parameters D-Dimer and TAT. Differences between wild-type and hHO-1 transgenic cells for fibrinogen and antithrombin were analyzed with the unpaired *t*-test.

Differences in MHC class-II expression between wt and hHO-1-tg cells were statistically analyzed using Student's *t*-test. In all experiments, P-values < 0.05 were considered to be significant.

Results

Production of hHO-1 transgenic pigs

In total, 205 reconstructed embryos were transferred to two synchronized peripubertal German Landrace gilts, which gave birth to nine live piglets, all with normal birth weights. Seven of them survived the perinatal period. Clone 57 (hHO-1) served as cell donor for recloning to produce larger groups of genetically identical animals (Table 3). In total, 13 clones from clone 57 donor cells were produced and used for the experiments. PCR (Fig. 1A) and Southern blot analysis (data not shown) revealed that all offspring had integrated the hHO-1 construct.

Expression of hHO-1 in transgenic pigs

Expression of porcine HO-1 was detected in cells from wild-type and hHO-1 transgenic pigs (Fig. 1B). As expected, hHO-1 was only found in EC from hHO-1 transgenic pigs (data not shown). The cloned transgenic animals showed hHO-1 expression at low levels in various organs including the heart and kidney (Fig. 1B,C). The highest hHO-1 expression level was found in kidneys. Although hHO-1 transgenic pigs were cloned from the same donor cell line, differences in the expression levels of hHO-1 between clones could be detected. No hHO-1 expression was determined in wild-type pigs. A direct detection of the hHO-1 protein was not possible due to the cross-reactivity of the five commercially available antibodies with the porcine analog. Even the use of a FLAG-specific antibody (# 600-401-383; Rockland Immunochemicals, Gilbertsville, PA, USA) showed strong background staining in porcine tissue. Nevertheless, the capability of the construct to produce an intact hHO-1/FLAG fusion protein was demonstrated in transfected immortalized porcine endothelial cells (PED-SV15) using the FLAG-specific Ab (Fig. S1). PED-SV15 cells were kindly provided by Prof. J. Seebach, Geneva and have been described previously [30].

Transgenic hHO-1 expression reduces the activation of endothelial cells and protects against apoptosis in vitro

Activation of ECs by various stimuli can result in up-regulation of adhesion molecules. To study whether over-expression of hHO-1 affects the responsiveness of EC to activating stimuli, we established EC cultures (PAEC) from hHO-1 transgenic and wild-type control animals. Cell cultures were characterized morphologically and by flow cytometry. The cells expressed CD31 confirming that they were indeed EC. Staining of PAEC by CD31 mAb resulted in a homogeneous shift in fluorescence intensity compared to cells stained by an isotype-matched control antibody, suggesting CD31 expression on the vast majority of the cells (data not shown). A quantitative comparison of control and CD31 histograms revealed that usually more than 85% of cells exhibited fluorescence intensity above background (wt: $86.3 \pm 8.1\%$, $n = 4$; hHO-1 transgenic: $89 \pm 2.6\%$, $n = 3$).

Porcine aortic endothelial cells were treated with TNF-α and expression of ICAM-1, VCAM-1, and E-selectin was determined by real-time PCR. Expression of all three adhesion molecules was markedly reduced after TNF-α treatment in hHO-1 transgenic PAEC compared to wild-type

Table 3. Somatic cell nuclear transfer results using hHO-1 transgenic donor cells

Donor cells	Transfers (n)	Embryos transferred (n)	Pregnancies, n (%)	To term, n (%)	Born piglets (live born)	Cloning efficiency (%)
Primary transfected cell clone	2	205	2 (100)	2 (100)	9 (7)	4.4
Clone 57 (hHO-1)	8	715	4 (50)	4 (100)	15 (13)	2.1
Total	21	1932	14 (66.7)	13 (92.9)	70 (53)	3.6

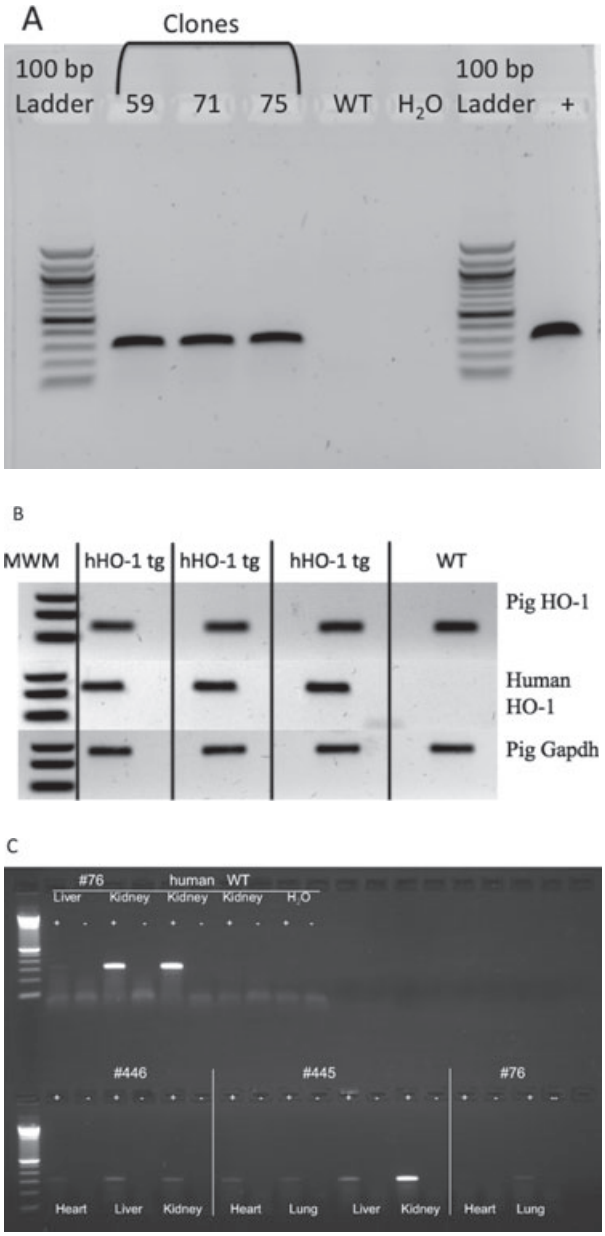

Fig. 1. (A) Human heme oxygenase-1 (hHO-1)-specific PCR of transgenic pigs showing integration of the hHO-1 vector in cloned pigs. (B) Products from real-time PCR on ethidium bromide gel. Data from three human HO-1 transgenic porcine kidneys and a wild-type control animal to differentiate porcine and human HO-1 expression. Human heme oxygenase-1 mRNA expression was only detected in hHO-1 transgenic animals. (C) Human heme oxygenase-1 expression was detected in various organs by RT–PCR. Expression levels were very low and differed among the pigs although they were cloned from the same cell line. Highest expression levels were found in kidneys of transgenic pigs. Clones 76 and 445 showed expression levels similar to human kidneys.

controls. A significantly reduced expression of VCAM-1 and ICAM-1 was observed at higher TNF-α concentrations (30 and 50 ng), with E-selectin expression showing the strongest reduction (Fig. 2A). This effect was abrogated by use of the HO-1-specific inhibitor ZnPPIX (Fig. 2B). Furthermore, IFN-γ induced up-regulation of MHC class-II molecules was markedly reduced in PAEC from hHO-1-tg pigs (Fig. 2C).

We also investigated the susceptibility of PAEC from wt and hHO-1-tg pigs to TNF-α-mediated apoptosis. PAEC from hHO-1 transgenic pigs were significantly better protected against TNF-α-mediated apoptosis compared to wild-type PAEC (Fig. 3A). The protection was abrogated when hHO-1 transgenic PAEC were pre-incubated with the specific HO-1 inhibitor zinc protoporphorine IX (Zn(II)PPIX, 20 μM) prior to TNF-α exposure. After Zn(II)PPIX treatment, hHO-1 transgenic PAEC showed similar or even higher levels of apoptosis than wild-type controls (Fig. 3B).

Prolonged survival of hHO-1 transgenic porcine kidneys in ex vivo perfusion

Ex vivo perfusion of hHO-1 transgenic and non-transgenic porcine kidneys

A drastic increase in the renal intravascular resistance index was observed during perfusion of wild-type porcine kidneys with human blood (Fig. 4A). In contrast, kidneys from hHO-1 transgenic animals perfused with human blood, and autologous perfusions were not associated with an increase in the renal intravascular resistance index (Fig. 4A).

D-Dimer and TAT complexes were not elevated, and fibrinogen was not consumed in autologous perfusions (Fig. 4B). In contrast, elevated concentrations of D-Dimers and thrombin antithrombin complex (TAT) together with the consumption of fibrinogen and antithrombin were found after perfusions of wild-type porcine kidneys (Fig. 4B). D-Dimer and TAT showed a similar increase in hHO-1 transgenic and wild-type (xenogenic) control kidneys. In contrast, antithrombin activity (at 120, 180, and 240 min) and fibrinogen concentration (at 180 min) were significantly higher in hHO-1 transgenic kidneys as compared to wild-type (xenogenic) kidneys indicating reduced consumption of these coagulation factors (Fig. 4B).

Autologous perfusions were terminated after 4 h (Fig. 5A). The average survival of wild-type porcine kidneys during organ perfusion with human blood under the addition of heparin without soluble complement inhibition (C1 inhibitor) was 60 min with no variation between the three replicates. Application of soluble complement inhibition (C1 inhibitor) prolonged wild-type organ survival to 126 ± 72 min (Fig. 5A). In contrast to wild-type kidneys, perfusion of hHO-1 transgenic porcine kidneys was significantly prolonged to 230 ± 25 min with supplementation of C1

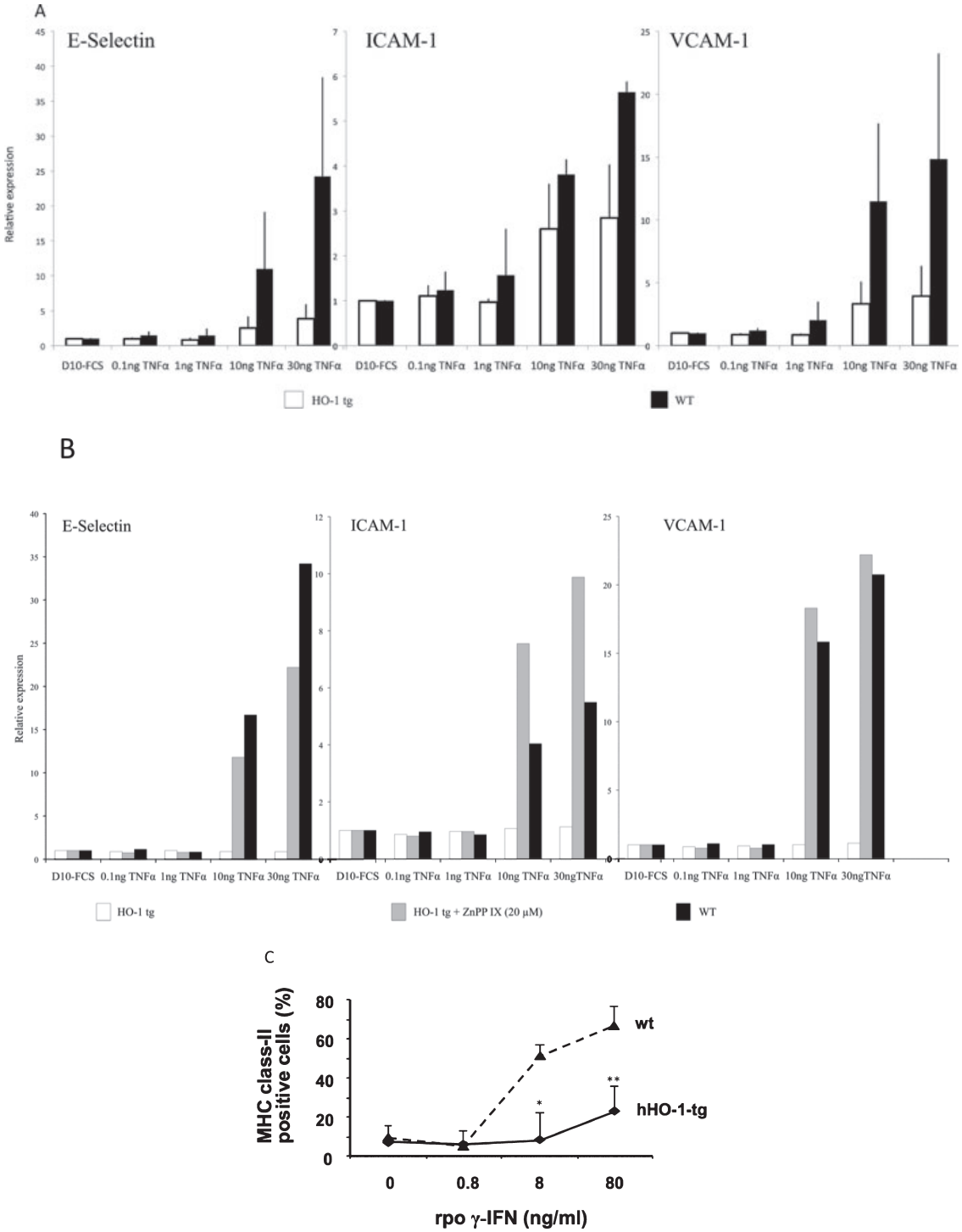

Fig. 2. (A) Real-time PCR expression analysis of adhesion molecules VCAM.1, ICAM-1, E-selectin in porcine aortic endothelial cells (PAECs) treated with increasing amounts of TNF-α (0.1–30 ng). hHO-1 transgenic PAECs showed lower expression of adhesion molecules at higher concentrations compared to wild-type controls (mean ± SD). (B) Representative data showing the protective effects of hHO-1 were abolished when cells were pre-incubated with Zn(II)PPIX. Expression levels of adhesion molecules were similar to wild-type controls or even higher due to the fact that also the endogenous porcine HO-1 is inhibited by Zn(II)PPIX. (C) Effect of hHO-1 on γ-IFN-mediated up-regulation of MHC class-II molecules in porcine endothelial cells. Wild-type cells and cells from hHO-1 transgenic pigs were incubated in the presence of increasing concentrations of porcine γ-IFN. MHC class-II expression was studied after 3 days by flow cytometry analysis. Data represent the mean percentage of MHC class-II-positive cells ± SD observed in three independent experiments. The differences in MHC class-II expression between wt and hHO-1-tg cells were statistically significant when 8 and 80 ng/ml γ-IFN were used for treatment (*P = 0.003 and **P = 0.01, respectively; Student's *t*-test).

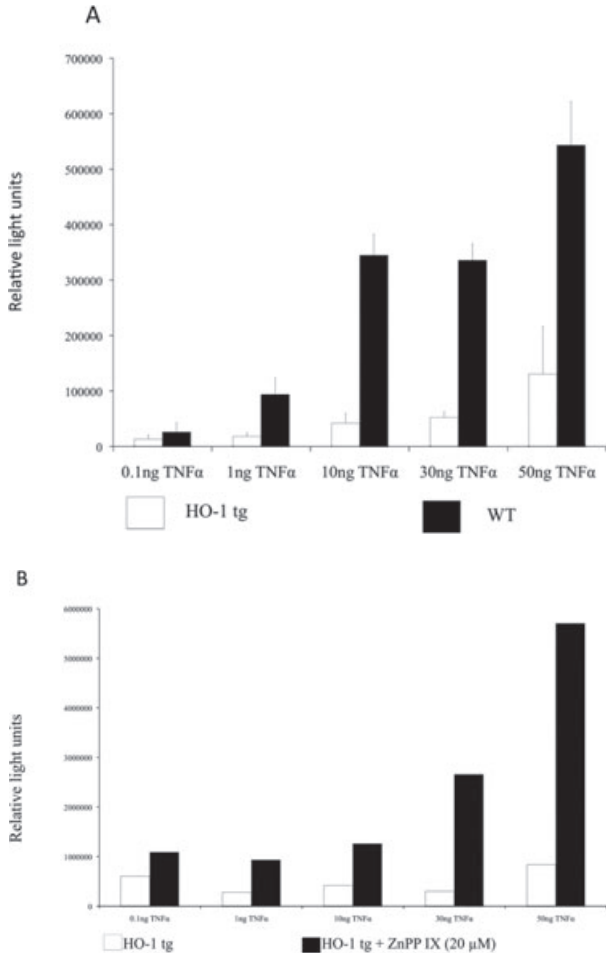

Fig. 3 (A) Human heme oxygenase-1 transgenic porcine aortic endothelial cells are protected against TNF-α-mediated apoptosis measured by a caspase 3/7 assay. Relative light units (RLU) corresponded to the amount of released caspase, which is an early indicator for apoptosis (mean ± SD). (B) The protective effect of hHO-1 expression can be abrogated by pre-incubation of cells with the specific HO-1 inhibitor Zn(II)PPIX. This indicates that the protective effects are hHO-1-specific (mean ± SD).

inhibitor (P-value < 0.05) and to 190 ± 80 min without C1 inhibitor, respectively (Fig. 5A).

Activation of complement as indicated by C3a levels was detected in all groups with the exception of the autologous perfusion (Fig. 5B). When no C1 inhibitor was used (wild-type kidneys; HAR group), C3a levels increased until termination of perfusion, whereas the presence of the C1 inhibitor resulted in decreased levels of C3a over time. There was no statistical difference between hHO-1 and wild-type kidney perfusions in the presence of the C1 inhibitor regarding C3a levels over time. Histological analysis revealed that glomeruli of wild-type porcine kidneys showed multiple microthromboses (Fig. 6). In contrast, in autologously perfused wild-type porcine kidneys as well as in hHO-1 transgenic porcine kidneys after perfusion with or without the addition of C1 inhibitor, we did not detect multiple microthrombi (Fig. 6). However, the occurrence of microthrombi cannot be totally excluded by this analysis.

Discussion

Here, we have shown that transgenic expression of hHO-1 confers resistance against xenograft rejection in the ex vivo perfusion system. Expression of hHO-1 could be an important factor when designing multi-transgenic pigs with improved survival of xenografts. The heme oxygenase-1 gene is highly conserved in different species underlining its critical role for maintaining an anti-inflammatory and anti-apoptotic status in mammalian organisms. Products from several genes can combat deleterious effects of inflammation and inflammatory diseases [31–34]. HO-1 expression is crucial in the regulation of inflammation and induction of HO-1 expression down-regulates inflammatory responses in a variety of experimental systems (reviewed in [35]). The most likely explanation for the broad protective effects of HO-1 is its enzymatic activity that modulates both inflammation and cytotoxicity, which in turn confines disease progression and thus enforces a rapid return to homeostasis.

The main biological function of HO-1 is to avoid the deleterious accumulation of free heme [36]. In addition, HO-1 mediates beneficial effects by degrading heme into three different biologically active molecules, CO, free iron, biliverdin, which synergize in preventing disease progression, confine the production of "endogenous" pro-inflammatory agonists, which in turn inhibit inflammation, and ultimately reduce cytotoxicity [36]. Growing evidence demonstrates that CO alone exerts anti-inflammatory and cytoprotective effects [21,37,38]. The cytoprotective effects of HO-1 involve protection against tumor necrosis factor-α (TNF-α), lymphotoxin, and Fas [39,40]. These cytotoxic agonists trigger several signal transduction pathways leading to activation of caspases [41] including the effector caspases-3 and -7 [39,40]. In the present study, hHO-1 expression protected PAEC from undergoing TNF-mediated apoptosis in vitro. Similar effects have been described in a mouse–rat transplantation model [19].

Xenogenic activation of coagulation is mediated by the activation of the graft endothelium. Ischemia/reperfusion (I/R) damage of the graft endothelium leads to the release of TNF-α and free heme, which in turn causes activation of the

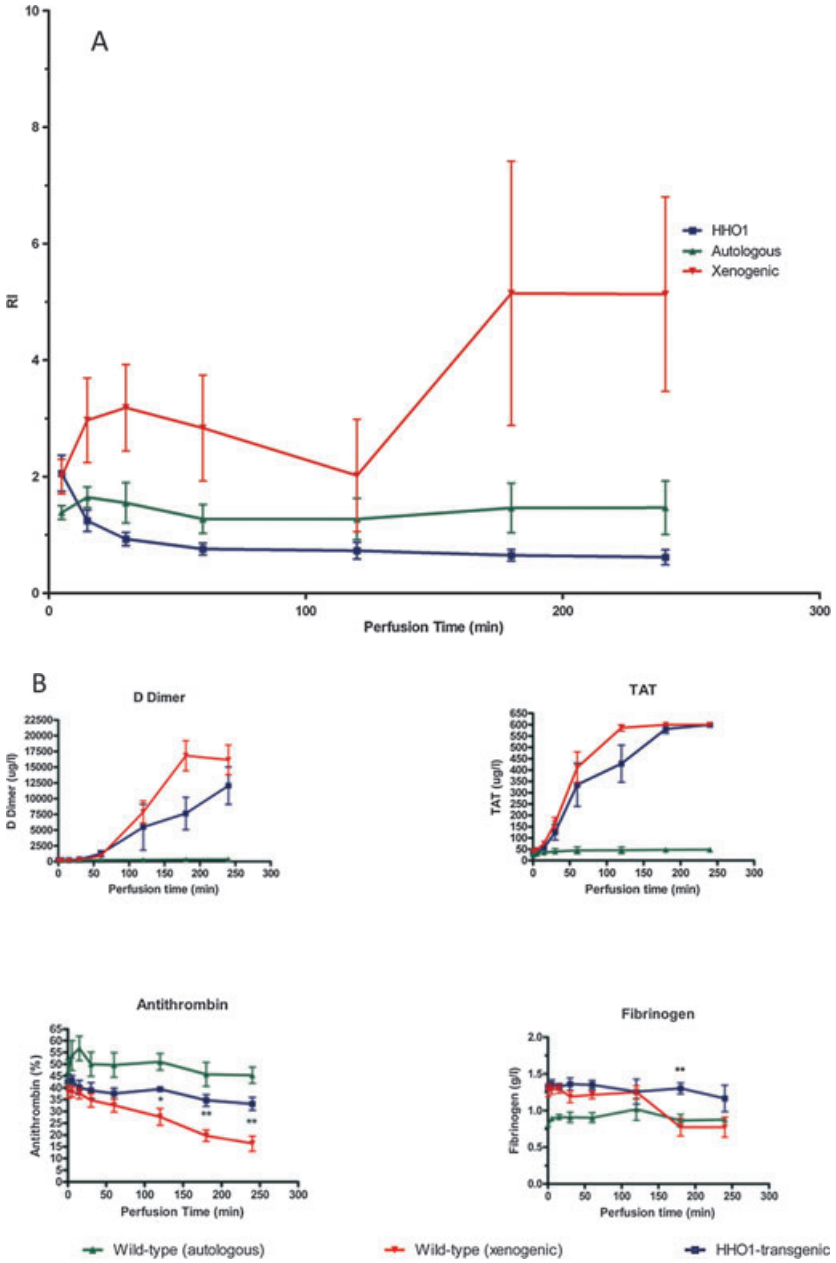

Fig. 4. (A) Resistance index (RI) during perfusion over time. The perfusions of hHO-1 transgenic porcine kidneys (blue) were compared to the autologous (green) and xenogenic (red) control perfusion experiments of wild-type porcine kidneys. Results show that the xenogenic perfusion led to significant elevation of the RI indicative of the failure of the organ to survive the perfusion. Mean values ± SEM are shown. P-value < 0.01 for hHO-1 with C1 INH vs. xenogenic with C1 INH. (B) Time course of the coagulation parameters D-Dimer, TAT, AT, and fibrinogen during perfusion of hHO-1 transgenic porcine kidneys (blue) compared to the autologous (green) and the xenogenic (red) control experiments of wild-type porcine kidneys. Mean values are shown ± SD. *P-Value < 0.05; **P-Value < 0.01 for hHO-1 vs. xenogenic.

endothelium. Adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, are the first factors known to be expressed by the endothelium upon a specific challenge. Here, we showed that the TNF-α challenge of PAEC reduced expression of adhesion molecules relative to wild-type controls and protected PAEC from becoming apoptotic by an hHO-1-specific mechanism. Heme oxygenase-1 and its product CO activate p38 MAPK, AKT, which in turn leads to over-expression of Bcl-xL, which shifts the bias of TNF-α-mediated apoptotic pathways to anti-apoptosis [42]. This may explain why hHO-1 transgenic PAEC were less susceptible to TNF-α-mediated apoptosis. However, one has to take into account that ZnPP IX inhibits endogenous porcine as well as human HO-1,

Results indicate that hHO-1 expression with its three end products is critically involved in preventing

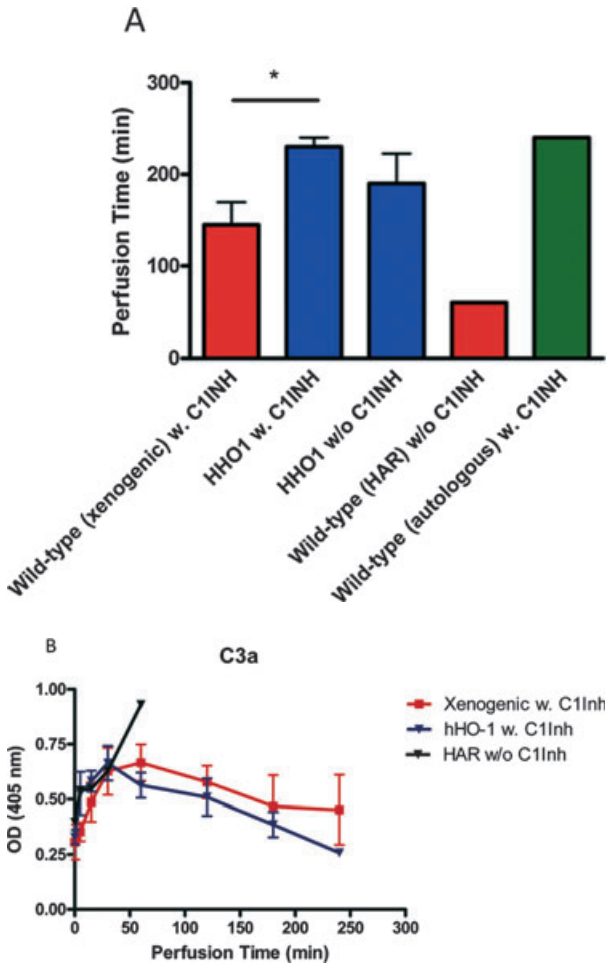

Fig. 5. (A) Perfusion times in the different experimental groups of wild-type kidneys and hHO-1 transgenic kidneys, with and without C1 inhibitor. The perfusion times of hHO-1 transgenic porcine kidneys (blue) are compared to the autologous (green) and xenogenic (red) control perfusion experiments of wild-type porcine kidneys. Mean values are shown ± SD; *P-Value < 0.05 for hHO-1 vs. xenogenic. (B) C3a levels are shown in the xenogenic (red), the HAR (black), and the hHO-1 group (blue) during perfusion. There was no statistical difference between the xenogenic and the hHO-1 group.

hyperacute rejection of the perfused transgenic kidneys. CO might have been the most important factor as it prevents platelet aggregation and has vasodilatory function [43–46]. Exogenous administration of biliverdin or bilirubin has been shown to exert beneficial effects, by modulating onset and severity of diseases, including I/R injury [47], graft rejection [48], endotoxic shock [49,50], neointima formation after balloon injury [51], and the development of autoimmune neuroinflammation [52]. Bilirubin derived from heme degradation suppresses MHC class-II expression in ECs [53].

Due to the lack of mAbs specific for hHO-1 that do not cross-react with pig HO-1, we could not directly demonstrate expression of hHO-1 protein. Nevertheless, we unequivocally demonstrate hHO-1 mRNA expression in these pigs albeit at low levels. The ability of the construct to generate HO-1/FLAG protein could be shown in transfected porcine endothelial cells using a FLAG-Ab (Fig. S1). Based on the substantial specific functional data, we conclude that the observed protective effects on ECs in vitro and ex vivo perfused kidneys are due to the transgenic expression of the HO-1, which is supported by the fact that all protective features in vitro were abrogated after addition of the HO-1-specific inhibitor Zn(II)P-PIX. Most likely, the enzymatic function of hHO-1 by catabolizing heme requires only a low expression level. Heme/heme oxygenase-1 is an enzyme/substrate system, whose functionality is mainly limited by the available substrate. Therefore, this system could compensate for the observed slight differences in the expression levels between individual cloned offspring. High expression of the transgene has been detrimental to development in mice (M. Soares, personal communication). Our results can be considered consistent with this observation because we never identified pigs with high hHO-1 expression. We show that low constitutive expression of HO-1 from the SV 40 promoter is sufficient to protect the porcine endothelium during ex vivo perfusion with human blood.

An important finding of our study with regard to xenotransplantation was that constitutive expression of human HO-1 without the application of soluble complement inhibition protected porcine kidneys from being hyperacutely rejected in the ex vivo perfusion model, In contrast, perfusion of porcine wild-type kidneys without complement inhibition (HAR group) led to hyperacute rejection and premature termination of perfusion. Interestingly, no significant difference in complement activation in the xenogenic and the hHO-1 group could be found, the C3a levels remained constant. In combination with prolonged perfusion times, this indicates the protective effect of hHO-1 expression in the perfused kidneys. In contrast to porcine wild-type kidneys, no consumption of fibrinogen and antithrombin was observed. D-dimer and TAT still increased during perfusion indicating that a moderate activation of the coagulation system occurred that was, however, not strong enough to result in consumption of coagulation proteins.

The data also show that hHO-1-mediated protection of the xenograft diminished the derangement of the coagulation process, normally leading to the consumption of coagulation factors and the formation of microthrombi. Transgenic expression of hHO-1 in porcine xenografts may also reduce thrombotic microangiopathy. Although our results are promising, the inherent limitations of our ex

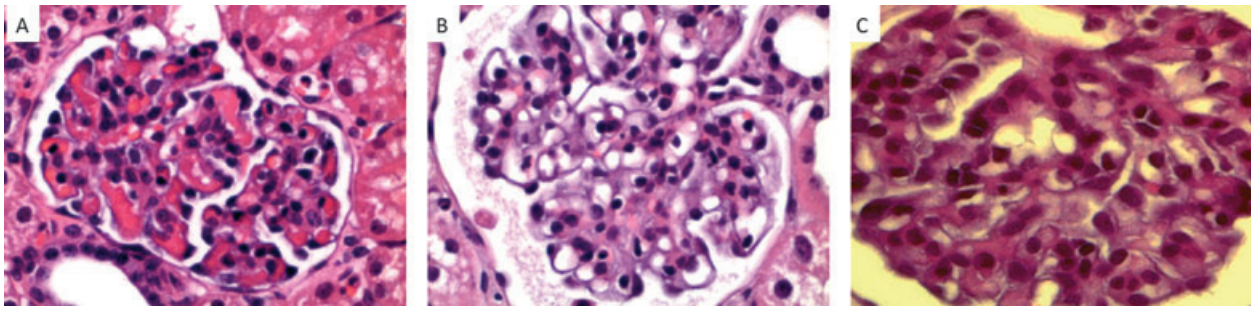

Fig. 6. Histology (H&E staining) of porcine kidneys after perfusion. (A) Upper left: H&E staining of a wild-type porcine kidney (xenogenic group, 110 min) with the addition of C1 inhibitor (magnification 600×). A single glomerulum with multiple microthromboses can be seen. (B) Upper right: H&E staining of a wild-type porcine kidney (autologous group, 240 min) (magnification 600×). A glomerulum without any microthrombi is shown. (C) Lower left: H&E staining of a hHO-1 transgenic porcine kidney (hHO-1 group, 240 min) with the addition of C1 inhibitor (magnification 600×). A glomerulum without any microthrombi is shown.

vivo model must be taken into account. First, the ex vivo perfusion system may not completely reflect the situation of a transplanted organ in vivo. Second, heparin is used in our system to prevent blood clotting in pumps and tubings. We have previously shown that the doses of heparin used here do not prevent coagulopathy of xenogenous blood. However, it may still affect EC function [23]. Activation of the coagulation process and the development of coagulopathy were not affected by adjusting the hematocrit by the small dilution [23]. Third, we could not study effects on platelets and other potentially relevant components of hemostasis, incl. von Willebrand factor. Fourth, the limited perfusion of up to 240 min was ideal to study short-term EC damage and coagulopathy, but may not be predictive for any long-term effects in vivo.

In contrast to a previous study [54], we did not detect up-regulation of porcine endogenous CD55 (decay accelerating factor) expression by constitutive hHO-1 expression. CD55 expression could further enhance protection of the endothelium from complement attack during ex vivo perfusion of the transgenic kidneys.

Results of this study suggest that transgenic expression of hHO-1 in pigs could provide significant protection against post-transplant vasculopathy and ischemia reperfusion injury in a xenotransplantation setting. Transgenic expression of hHO-1 in pigs is promising to prolong survival of porcine xenografts and should be part of multi-transgenic pigs for xenotransplantation.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PED-SV15 cells were transfected with the pTSG1 vector containing human HO-1 (PED-SV15, hHO-1 cells) or no insert (PED-SV15, vector control). Wild-type PED-SV15 cells and transfectants were lysed, the proteins were separated by 12.5% SDS–PAGE, transferred to PVDF membrane, and probed with an antibody detecting the FLAG-tag (polyclonal rabbit antibody; Rockland Immunochemicals, Gilbertsville, USA). As a protein-loading control, the same blots were stripped and reblotted with an anti-α-actin antibody (polyclonal mouse IgG2; Sigma, Deisenhofen, Germany). The anti-FLAG antibody cross-reacted with a protein of about 28 to 30 kDa in all samples (►). The hHO-1/FLAG fusion protein (←) corresponding to a relative molecular mass of 32 kDa was only detected in PED-SV15-hHO-1 cells.

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