Cytokine-inducible promoters to drive dynamic transgene expression: The “Smart Graft” strategy

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Abstract

Background: Ubiquitous expression of T-cell regulatory transgenes such as the cytotoxic T lymphocyte-associated antigen 4 (CTLA4) or the high-affinity variant LEA29Y improves xenograft survival. Such donor pigs are however immunocompromised and susceptible to infection. Continuous high expression of CTLA4 or LEA29Y in the graft could also compromise the health status of recipients. The novel "Smart Graft" strategy is likely to avoid these problems by controlling the expression of T-cell regulatory transgenes as and when required.

Methods: Candidate promoters inducible by inflammatory cytokines were identified by in silico screening for potential NF-κB binding sites. Basal promoter levels and responsiveness to TNFα and IL1β were quantified by expression of secreted embryonic alkaline phosphatase in cultured cells. Promoters were modified to increase responsiveness by removing regulatory elements or adding SP-1 or NF-κB binding sites and again tested in vitro. The most promising promoters were then assessed in vivo. Porcine cells expressing inducible Renilla luciferase constructs were transplanted into immunodeficient NOD-Scid-IL2 receptor gamma null (NSG) mice. Following engraftment, the recipient’s immune system was reconstituted by splenocyte transfer raising an immune response to the porcine xenograft. The resulting induction of promoter activity was detected by in vivo bioimaging.

Results: Three human (hTNFAIP1, hVCAM1 and hCCL2), and one porcine promoter (pA20) were chosen for in vitro tests. In all experiments, the semi-synthetic and inducible ELAM promoter as well as the CAG promoter were used as references. In contrast to hTNFAIP1 and hVCAM1 the ELAM, hCCL2 and pA20 promoters showed significant induction after cytokine challenge. The hCCL2 and pA20 promoters were further optimized, resulting in increased responsiveness to TNFα and IL1β. Cytokine-dependent upregulation of promoter activity was tested in vivo, where the ELAM and the optimized hCCL2 promoters showed a 2-fold upregulation, while one of the improved A20 promoters showed almost 10-fold upregulation. Our results also revealed more than 4-fold cytokine inducibility of the CAG promoter.

Angelika Schnieke and Konrad Fischer are joint last authors.

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1 | INTRODUCTION

The worldwide shortage of donor organs for allotransplantation has encouraged research into xenotransplantation. However, several problems have to be overcome. Hyperacute rejection destroys the graft within several minutes due to preformed antibodies against the carbohydrate epitope galactose-α1,3-galactose (α-1,3Gal). This can be greatly reduced by ablating porcine α-1,3-galactosyltransferase expression by GGTA1 gene knockout and further improved by combining GGTA1 knockout with expression of human complement regulatory genes. The next hurdles, occurring within several days to weeks, are presented by delayed immune responses, notably acute vascular rejection. Binding of preformed antibodies induces activation of the graft endothelium, platelets and innate immune cells, leading to cytokine release, inflammation, complement activation, ischaemia, thrombotic microangiopathy and graft failure. Target antigens of the most important preformed antibodies have been identified and include N-glycolyneuraminic acid (Neu5Gc), synthesized by cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH) and the Sd(a) blood group antigen produced by β-1,4-N-acetyl-galactosaminyltransferase 2 (B4GALT2). Human leucocyte antigen (HLA) antibodies also cross-react with the porcine major histocompatibility complex class I antigens (swine leucocyte class I, SLA-I). Knockouts of all these antigens have been achieved.

Combinations of knockouts and expression of anti-apoptotic and anti-inflammatory transgenes can further diminish acute vascular rejection. Control of cellular rejection is the next main challenge. The cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is a potent immunosuppressive, costimulation-blocking molecule and negative regulator of T-cell activation. CTLA4 is a high-affinity CD28 homologue and binds to the CD80/CD86 (B7) receptors on antigen-presenting cells, directly preventing T-cell activation. It is already used for several clinical immunosuppression and transgenic expression of CTLA4 or its derivative LEA29Y has been suggested to mitigate xenograft rejection. However, pigs that ubiquitously over-express CTLA4-Ig or LEA29Y have developmental defects, diminished humoral immunity and are acutely susceptible to infection. One solution to this problem is tissue-specific transgene expression, with examples reported including a neuron-specific enolase promoter to drive expression in corneal keratocytes and various areas of the brain, and an insulin promoter to direct specific expression in pancreatic β cells. These animals are viable and healthy, but this approach would require separate donor pig lines for each xenograft or tissue.

Here, we demonstrate a new approach in which immune-suppressive genes are expressed in response to cytokines released upon imminent rejection such as tumour necrosis factor α (TNFα) and interleukin 18 (IL18). Both are potent pro-inflammatory cytokines produced by blood monocytes and tissue macrophages during the early response of the innate immune system. They are released after activation of innate immune cells, cell lysis, tissue damage or initial graft rejection mechanisms. Our aim was to identify cytokine-reactive promoters, assess whether inducibility could be improved through genetic modifications so that they direct high level transgene (eg LEA29Y) expression at the first sign of rejection.

2 | MATERIALS AND METHODS

2.1 | TNFα and IL18 inducible promoters

Genomatrix Genome Analyzer was used to identify promoter sequences with high similarity to the consensus sequence of the NF-κB binding site via Basic Local Alignment Search. Sequences of the promoter regions originated from Ensembl Genome Browser (Ensembl Release 95; https://www.ensembl.org/) using the genome assemblies GRCh38.p12 (Homo sapiens) and SSrofa11.1 (Sus scrofa). Detailed information is provided in Appendix S1. Functional elements of the porcine A20 promoter were identified by alignments with the human A20 promoter including an E-box element, two endogenous NF-κB and one SP-1 binding site. The proximal promoter of the endothelial cell-leucocyte adhesion molecule (ELAM-1; E-selectin) with 3 endogenous and 5 additional NF-κB binding sites (pNFIt2; InvivoGen) and the CAG promoter, providing high and ubiquitous expression levels were used as references.
2.2 | Generation of SEAP reporter constructs

All constructs were based on a CAG-SEAP reporter plasmid consisting of the pcDNA3.1-hygro\(^*\) (Thermo Fisher) plasmid backbone, a CAG promoter driven secreted embryonic alkaline phosphatase gene (SEAP) and a bovine growth hormone polyadenylation signal. Endogenous promoters were PCR amplified by QS high-fidelity polymerase (New England Biolabs) or GoTaq G2 polymerase (Promega) according to the manufacturer’s instructions using genomic DNA, isolated from the human immortalized mesenchymal stem cell line SCP1 or from primary porcine kidney fibroblasts as template. Primer sequences are shown in Table S1. The promoter sequences were either first subcloned into pJet1.2/blunt (Thermo Fisher) or used directly to replace the CAG promoter of the CAG-SEAP reporter plasmid.

2.3 | Modification of promoter sequence

The 2.9 kb hCCL2 promoter (hCCL2-2892) was reduced in size to 210 bp (bp −145 to +65; Transcript ID: ENST00000225831.4) (hCCL2-210). It contains a CAAT motif, two AP-1 sites and binding sites for SP-1 and NF-κB.\(^{24}\) Addition of the sequence −2,834 to 2,519, which contains two distal NF-κB sites,\(^{24}\) to hCCL2-210 resulted in a 529 bp promoter (hCCL2-529).

Nine variants of the pA20 promoter were generated by sequential insertion of different clusters of SP-1 and/or NF-κB binding sites into plasmid pA20-SEAP. NF-κB sequences were inserted at position −404 and/or downstream of both endogenous NF-κB binding sites at position +52. The SP-1 cluster was introduced at position −101, upstream of the endogenous SP-1 site (Transcript ID: ENSSSCT00000034426.2). All modifications were performed using the NEBuilder HiFi Assembly Kit (New England Biolabs).

2.4 | Cloning of dual-luciferase constructs

Inducible and reference promoters were PCR amplified and cloned into the SnaB1 restriction site of pSL1180-psiCHECK plasmid, based on a pSL1180 backbone (Amersham) and the Nhel, BamHI digested fragment of psiCHECK2 (Promega) containing a dual-luciferase construct. Subsequently, a SV40-driven hygromycin resistance cassette was cloned into all plasmids by NEBuilder HiFi Assembly Kit (New England Biolabs). The final dual-luciferase plasmids contain cDNA sequences encoding Renilla luciferase (Rluc), driven by one of the inducible promoters, and Firefly luciferase (Fluc) driven by the HSV-TK promoter as an internal control for data normalization.

2.5 | Cell culture

Porcine kidney fibroblasts and Porcine Kidney-15 (PK-15)\(^{25-29}\) cells were cultivated in antibiotic-free medium (DMEM, 10%-20% FCS, 1 mmol/L sodium pyruvate, 1× NEAA, 2 mmol/L Ala/Glu) at 37°C in a CO\(_2\) enriched (5%), humidified atmosphere. Medium was changed every 2-3 days. Cells were transfected (4 μg plasmid per 10 cm dish) using Lipofectamine 2000 (Thermo Fisher) at 25%-60% confluence according to the manufacturer’s instructions and selected with antibiotics starting 24-48 hours after transfection for up to 9 days using 600 respectively 1200 μg/mL Hygromycin B (PanReac AppliChem).

2.6 | SEAP reporter assay

Secreted embryonic alkaline phosphatase activity in cell culture supernatants was determined using the SEAP Reporter Assay Kit (InvivoGen) according to the manufacturer’s instructions. The optical density at \(λ = 405\) nm was measured for 120 minutes at 15 minutes intervals using a Multiskan Ex ELISA reader (Thermo Fisher). Collection of cell culture supernatants for SEAP reporter assays was conducted 72 hours after cytokine addition. Supernatants were stored up to 14 days at −20°C.

2.7 | Animal welfare

Animal experiments were approved by the Government of Upper Bavaria and performed according to the German Animal Welfare Act and European Union Normative for Care and Use of Experimental Animals.

2.8 | Xenotransplant of porcine cells

Xenotransplant of unmodified PK-15: \(2 × 10^6\) cells were resuspended in 10 μL PBS and injected subcutaneously (s.c.) or intramuscularly (i.m) into the hind legs of immunodeficient NOD-Scid-IL2 receptor gamma\(^{null}\) (NSG) mice using a 31G needle.

Xenotransplant of PK-15 cells transfected with the dual-luciferase constructs: \(1 × 10^5\) cells were resuspended in 10 μL PBS and injected subcutaneously into the hind leg using a 31G needle. Cell clones were selected with comparable levels of Firefly luciferase activity to minimize the effects of plasmid copy number variation and/or position effect (Figure S1). Eight days after transplantation, splenocytes isolated from NOD mice (\(1 × 10^7\) in 100 μL PBS) were transferred via the tail vein of the NSG mice to reconstitute the immune system. Splenocyte transfer was not performed for the control group.

2.9 | In vivo bioluminescence imaging

Ten days after splenocyte transfer, in vivo bioluminescence imaging and tissue collection were conducted. Mice were anaesthetized with 3.0% isoflurane in a whole-body chamber and narcosis was
maintained with 2.5% isoflurane within the imaging system (IVIS Lumina LT series III; PerkinElmer). To evaluate Renilla luciferase activity, 100 µL of VivRen substrate (Promega) (0.3 µg/µL in PBS) was injected into the tail vein and photon emission was detected after 1 minute (exposure time: 60 seconds). One hour after the injection into the tail vein and photon emission was detected after exposure time: 60 seconds), and mice were subsequently euthanized intraperitoneally. Firefly luciferase activity was measured 7 minutes after substrate injection (exposure time: 60 seconds), and mice were subsequently euthanized by cervical dislocation. Promoter induction was calculated by radiance total flux photons/sec [Mean (Renilla Luciferase without spleno/Firefly Luciferase spleno)] / [Mean (Renilla Luciferase without spleno/Firefly Luciferase without spleno)]. Luminescence values below 5.0 x 10^3 p/s were excluded from analyses due to the approximation to the background signal.

### 2.10 Immunohistochemistry

Murine hind legs were fixed without skin for 3-4 days in 4% paraformaldehyde. After removing the thighbone, samples were embedded in paraffin. Histological sections were prepared using a Microm Cool-Cut microtome (Histoserve) prior to deparaffinization. Microscope slides with the tissue sections were incubated three times in Roticlear (Carl Roth) for 5 minutes and then washed twice in 100% EtOH, 95% EtOH, 80% EtOH and H2O for 5 minutes. For antigen unmasking, tissue slides were boiled for 10 minutes in 10 mmol/L citrate buffer (pH 6.0) and cooled to room temperature. Slides were washed three times in H2O for 5 minutes. Endogenous peroxidases were inactivated by 3% H2O2 for 10 minutes. After three 5 minutes washes in H2O, slides were drained and 90 µL of the primary antibody dilution was added (Biotin anti-mouse CD45, Sigma-Aldrich). Sections were covered and incubated overnight at 4°C. Slides were washed in PBS for 5 minutes, covered with VECTASTAIN Elite ABC Reagent (Vector laboratories) and incubated for 30 minutes. After washing for 5 minutes in PBS, sections were stained with peroxidase substrate (TMB substrate; Thermo Fisher). Counterstaining was performed by incubating in haematotoxilin (Vector laboratories) for 10 seconds. Slides were washed twice in H2O for 5 minutes, twice in 95% EtOH for 10 seconds and finally in 100% EtOH. For fixation, the stained tissue sections were mounted with Eukitt quick-hardening mounting media (Sigma-Aldrich).

### 2.11 Statistical analysis

Mean values of triplicate measurements of the control and the studied group were compared using T test and R script (The R project for statistical computing, version 3.2.1). For all groups, three technical replicates of stably transfected cell pools were analysed.

### 3 RESULTS

The ideal promoter candidate for implementing the Smart Graft approach has low basal promoter activity, in the best case almost close to zero, and high inducibility upon cytokine release. We first identified human and porcine promoters containing cytokine responsive elements. This included three human (TNF alpha-induced protein 1—hTNFAIP1, Vascular cell adhesion molecule 1—hVCAM1, C-C Motif Chemokine Ligand 2—hCCL2), one porcine (TNF alpha-induced protein 3—pA20) and the modified proximal E-selectin promoter (ELAM) containing 3 endogenous and 5 additional NF-κB binding sites. The CAG promoter was chosen as a positive control for high and ubiquitous transgene expression.

SEAP reporter constructs were generated for all 5 promoters. Reporter constructs were transfected into porcine cells, and SEAP expression was analysed after cytokine challenge. In contrast to the ELAM, hCCL2 and pA20 promoters, the hTNFAIP1 and hVCAM1 promoters showed minimal and non-significant inducibility upon increasing concentrations of TNFα (see Figures 1 and S2). Therefore, the hCCL2 and pA20 promoters were chosen for further modifications.

The hCCL2-2892 promoter was reduced in size to remove elements which may provide a negative effect on expression while retaining important regulatory elements for induction. The hCCL2-210 promoter contained only the proximal NF-κB and SP-1 site, the hCCL2-529 promoter included the two distal NF-κB sites which were now located close to the proximal NF-κB site. Both shorter CCL2 promoter variants had a deletion of the AP-1 and the CCAAT enhancer binding protein beta (C/EBPβ) sites. AP-1 sites were shown to bind AP-1 proteins as well as CAMP responsive element binding proteins (CREBs). CREBs are transcription factors essential for gene regulation but were shown to downregulate transcription in response to TNFα and other inflammatory cytokines. Removing the AP-1 site could thus improve TNFα responsiveness. Figure 2 shows the modified versions of the human CCL2 promoter.

The pA20 promoter was modified (a) by adding 3, 7 or 9 NF-κB sites (pA20 + 3NF to +9NF) to optimize inducibility, (b) to increase basal and inducible expression 4 or 8 SP-1 binding sites (pA20 + 4SP to +8SP) were inserted. SP-1 binding sites were described to elevate gene expression in response to TNFα, and (c) by combining 4 additional SP-1 sites with 3 to 13 NF-κB sites (pA20 + 3NF + 4SP to +13NF + 4SP). Schematic diagrams of the modified A20 promoters are shown in Figure 3.

#### 3.1 Modified promoters show improved inducibility

Primary porcine kidney fibroblasts were stably transfected with SEAP reporter plasmids. Cell pools were then treated with either increasing amounts of human TNFα (0.1, 1, 10 or 30 ng/mL, Figure 1) or with human TNFα (30 ng/mL), human IL1β (50 ng/mL) or IFNγ.
The 17 promoter variants tested provide a broad range of activity: CAG and hCCL2-529 had the highest basal activity followed by the ELAM, hCCL2-2892 and hCCL2-210 promoter. The lowest basal activity was obtained for the pA20 promoter and its variants. Only for two promoters (pA20 + 3NF and pA20 + 13NF + 4SP) basal SEAP expression was comparable to that obtained from the ELAM promoter. Figure 1 shows basal promoter activity levels and their upregulation upon increasing concentrations of hTNFα.

All promoters except hVCAM1 and hTNFAIP1 as mentioned above showed a significant response to hTNFα (Figure S2). Induction of the human TNFAIP1 promoter by hIL1β was low but significant (Figure S2). The ELAM promoter provided the highest inducibilities for both cytokines, hTNFα and hIL1β. Addition of NF-κB binding sites to the pA20 promoter clearly increased inducibility by hTNFα. Moreover, these also introduced sensitivity to hIL1β that was not observed for the unmodified pA20 promoter or pA20 with additional SP-1 sites only (Figure S2). Also, a slight upregulation of the CAG promoter by hTNFα was observed. By in silico analysis, four potential NF-κB binding sites were identified at positions -245, -286, -904 and -1492 (Figure 4), which would explain this finding.

The promoters pA20, pA20 + 3NF, hCCL2-210, hCCL2-529 were also tested for responsiveness to human interferon gamma (IFNγ).
Human IFNγ has been shown to be ineffective on porcine cells\textsuperscript{43} and therefore served as a negative control. The tested promoter constructs had no or only a negligible response to IFNγ (Figure S2).

These results confirm that a significant increase in inducibility can be achieved by modifying the hCCL2 and pA20 promoters and that in vitro the A20 promoters pA20 + 3NF and pA20 + 13NF + 4SP achieved similar activities compared to the ELAM promoter, while activity of the induced hCCL2-529 promoter nearly reached levels obtained for the CAG promoter.

### 3.2 Promoter induction is reversible

To avoid chronic systemic immunosuppression of pigs and human patients, the inducible promoters should become activated shortly after cytokine release, maintain expression during inflammation at high levels and importantly reduce expression to basal levels afterwards. Therefore, the time necessary for promoter deactivation was determined. After removal of hTNFα from the culture medium, all promoters showed a decrease in activity. Basal activity levels were

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**FIGURE 3** Structure of the modified pA20 promoter variants. Top: endogenous pA20 promoter. Nucleotide positions refer to pA20 transcript ID ENSSSCT00000034426.2. Endogenous promoter elements are marked as black boxes. Restriction sites relevant for cloning are indicated as black circles. Below: pA20 promoter variants featuring different clusters of added transcription factor binding sites. Orange and green boxes indicate additional NF-κB and SP-1 binding sites.
reached within 24 hours for the ELAM and pA20 promoters and 72-96 hours for all other promoters.

3.3 | Mouse xenograft model

To test the smart graft strategy in vivo, we chose a xenograft model in which immortalized porcine cells, which express a luciferase reporter gene under the control of one of the inducible promoters, are transplanted into immune-deficient mice. After the porcine cells have engrafted, the murine immune system is reconstituted by splenocyte transfer. Immune cell infiltration of the xenograft and the upregulation of the inducible promoters can then be analysed. The workflow is shown in Figure 5. Prior to this, we had to confirm that murine TNFα and IL1β were able to bind to the corresponding receptors on porcine cells to induce promoter activity and that these cells were capable to engraft and form tumours in a short period of time.

3.4 | PK15 cells are suitable for engraftment and tumour formation

PK-15 cells were tested for the transplantation and engraftment in NSG mice. PK-15 cells were stably engrafted after im and s.c.
injection, resulting in large tumours of approx. 5 mm in diameter after 4 weeks. Moreover, we could show that after reconstitution of the immune system, the tumours were infiltrated by immune cells, a prerequisite for cytokine release (Figure 6).

### 3.5 Promoters constructs in PK15 cells respond to murine TNFα and IL1β

Cells, stably transfected with a SEAP reporter construct under the control of the modified pA20 + 13NF + 4SP promoter were tested for the response to murine TNFα and murine IL1β (Figure S3). For both cytokines, a significant promoter induction was obtained. In case of murine IL1β, the response was considerably weaker than after challenge with human IL1β.

### 3.6 Inducible promoters are activated by in vivo immune response

PK15 cells were transfected with the dual expression vector for Renilla luciferase under the control of the inducible promoters (CAG, ELAM, hCCL2-529, pA20 and pA20 + 3NF) and Firefly luciferase under the control of the Herpes simplex virus thymidine kinase promoter (HSV-TK). The latter enabled standardization of expression. The time course for xenotransplantation of cells, splenocyte transfer and in vivo bioimaging is outlined in Figure 5. Data from 4-6 mice were evaluated to ensure data reproducibility.

Optimal time points for bioluminescence measurements of Renilla luciferase was tested for the promoters CAG, ELAM, pA20 and pA20 + 3NF. Time points for Firefly luciferase measurements were tested for the promoters pA20 and pA20 + 3NF (Figure S4). Tissue collection was performed to confirm tumour formation and immune cell infiltration (Figure 7).

In the absence of immune cells, all promoters showed some basal activity (control group) which was increased after reconstitution of the immune system and immune cell-dependent cytokine release. The CAG promoter showed an increase in activity by 4.2-fold. The ELAM promoter was induced by a factor of 1.5, the hCCL2-529 promoter by a factor of 2.0. The non-modified pA20 promoter showed an inducibility by a factor of 2.7. Modification of the pA20 promoter by addition of 3 NF-κB binding sites resulted in a strong increase in inducibility by a factor of 9.7 (Figure 8). These are promising data for the implementation of the Smart Graft strategy for xenotransplantation.

### 4 DISCUSSION

Here, we describe a potential means of providing high expression of T-cell regulatory genes in xenografts in response to rejection.
mounted by the recipient. We identified promoters and promoter elements activated by the inflammation-associated cytokines TNFα, IL1β and IFNγ.44–46 TNFα and IL1β cause NF-κB signalling. A modified version of the E-selectin promoter with three endogenous and five additional NF-κB binding sites (ELAM) was tested and also a variety of human and porcine promoters containing NF-κB binding sites. Of the newly tested promoters, human CCL2 and porcine A20 showed significant induction by TNFα with medium (hCCL2) or low (pA20) basal activity.

CCL2 is a chemokine secreted by macrophages during viral infection and plays a role in the regulation of migration and infiltration of monocytes and macrophages.57,48 Others have reported NF-κB-dependent activation of the hCCL2 promoter.24 Here, we show that this response can be significantly increased by deleting promoter elements responsible for cytokine-dependent downregulation and re-locating the NF-κB binding sites closer to the core promoter region.

A20 is a zinc finger protein which is TNFα inducible.49 The addition of NF-κB sites to the pA20 promoter increased inducibility by TNFα and conferred sensitivity to IL1β. Some pA20 promoter modifications increased basal expression, and expression after induction. It is currently unknown how much basal expression of T-cell regulatory genes can be tolerated without causing immune-suppression and what levels are necessary to suppress rejection. We thus generated a variety of inducible promoters with low to high basal and inducible activity.

As expected, the CAG promoter showed the highest basal activity and highest absolute expression levels of all promoters analysed. It’s activity was further increased 4-fold by the inflammatory in vivo response. These results accord with data from CAG-driven hPD-L1 transgenic primary fibroblasts that showed >2.5-fold increase in expression after 48 hours incubation with TNFα,50 and is most likely due to the presence of four potential NF-κB binding sites. The highest fold induction in vivo was obtained with the pA20 promoter with three additional NF-κB sites (almost 10-fold), while hCCL2 and ELAM had 2-fold and CAG 4-fold. Importantly porcine A20 displayed relatively low basal expression levels, making it suitable for LEA29Y expression in donor pigs. In all instances, expression reversed after cytokine removal.

While others have generated cytokine reporter mice,51,52 ours is the first systematic comparison of inducible promoters both in

Figure 7 Sections of graft sites after immune cell infiltration. Shown in brown: CD45 + immune cells. Morphology of tumors formed by PK-15 cells, expressing inducible promoters (A) CAG, (B) ELAM, (C) hCCL2-529, (D) pA20, (E) pA20 + 3NF. Scale bar: 500 μm

Figure 8 Promoter in vivo inducibility in the xenograft mouse model. Shown is the x-fold upregulation of promoter activity most likely due to cytokine release compared to the non-treated control group as mean ± SD. N (CAG) = 5. N (ELAM) = 5. N (hCCL2-529) = 6. N (pA20) = 4. N (pA20 + 3NF) = 4
vitro and in vivo in a xenograft setting. It included native and novel semi-synthetic promoters and achieved nearly 10-fold inducibility. While our goal is the dynamic expression of immunomodulatory genes, similar strategies can be envisaged to include not only response elements for inflammation, but also apoptosis. We successfully verified the Smart Graft approach in the mouse model using a reporter gene. We now have to confirm that similar induction levels can be obtained for other genes, such as immune regulatory genes, that these are expressed in the relevant tissues and that the induction response occurs fast enough to ensure xenogene-organ protection. The next step will be the production of a Smart Graft pig with inducible expression of T-cell regulatory genes or inducible expression of tissue protective genes such as IL10 or TGFβ for xenotransplantation.

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CONFLICT OF INTEREST
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
AF, NS and KF performed cloning of the constructs and cell culture experiments. JS and LWB performed the transplantation, splenocyte transfer and histology. AF, KM, DW and KF performed bioimaging and histology. AF, JS, PK, AS and KF co-wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors agreed to the final version.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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