## Supplementary Information

**Efficient production of multi-modified pigs for xenotransplantation by 'combineering', gene stacking and gene editing.**

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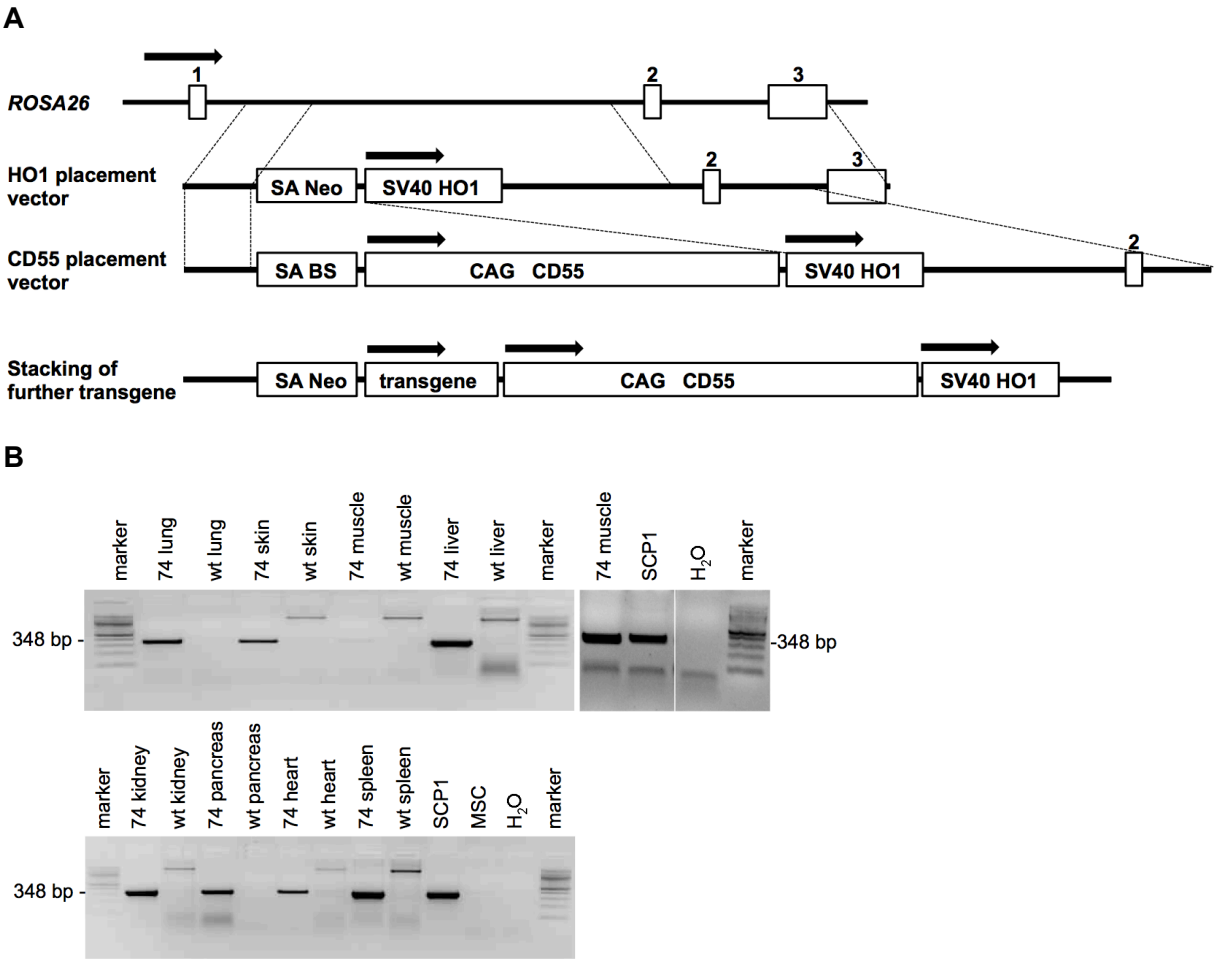
**Supplementary Figures 1 - 8**
**Supplementary Tables 1 and 2**
**Supplementary Sequence Files 1 and 2**

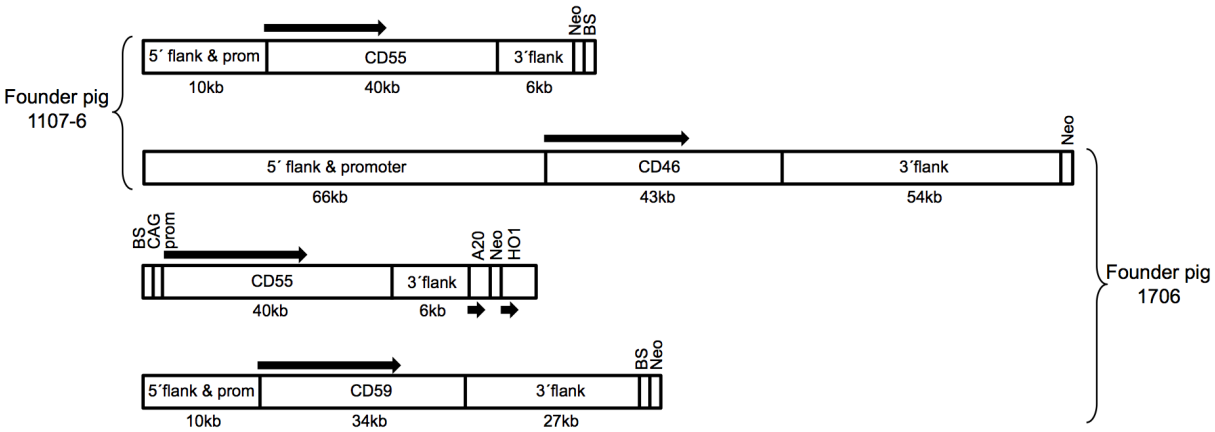



**Supplementary Figure 1**

**Gene stacking.**

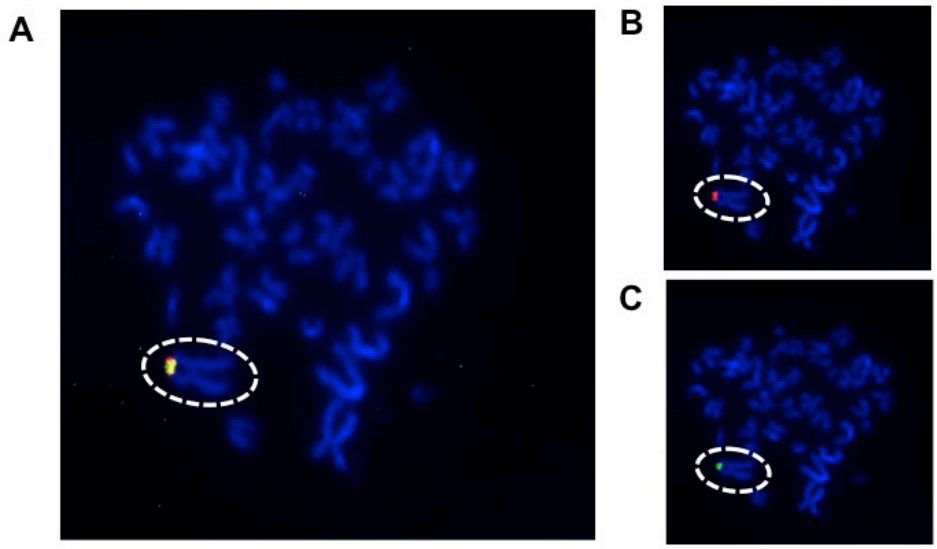
A. Top, Porcine *ROSA26* locus, exons are indicated by boxes and numbered as indicated. Upper middle, Placement of SV40 driven HO1 cassette into *ROSA26*. Lower middle, Retargeting to place a CAG driven CD55 minigene adjacent to HO1. Bottom, Sequential retargeting to stack further transgenes and exchange the drug resistance marker. Dotted lines indicate regions of homology in targeting vectors. SA splice acceptor. Transcription is indicated by arrows.

B. RT-PCR analysis of HO1 expression in organs of pig 74, which carries the SV40-driven HO1 cDNA placed at the ROSA26 locus, compared to wild-type pig organs as shown. MSC, primary porcine mesenchymal stem cells; human MSC line SCP1 used as a positive control; water PCR control, as indicated. The size of the 348 bp diagnostic fragment is marked. Note: The 74 muscle lane was underloaded in the left panel, an extra panel on the right confirms HO1 expression in muscle.



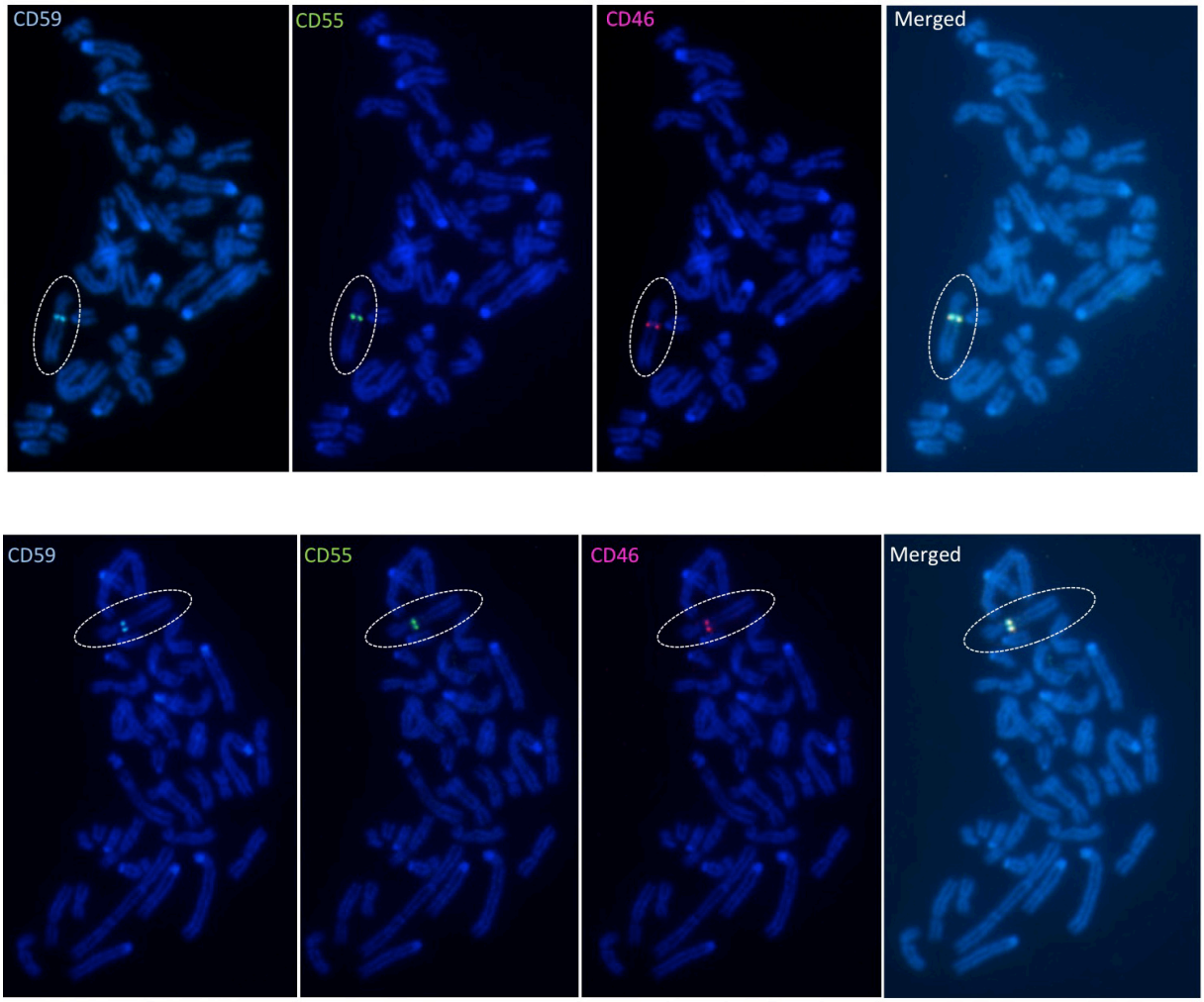

**Supplementary Figure 2**

**Outline structure of constructs containing genomic complement regulator sequences and endothelial protection cDNA expression cassettes.** Transcription is indicated by arrows and the position of neomycin and blasticidin selection cassettes shown. The combination of constructs in each of the founders generated by 'combineering' is indicated by brackets.




**Supplementary Figure 3**

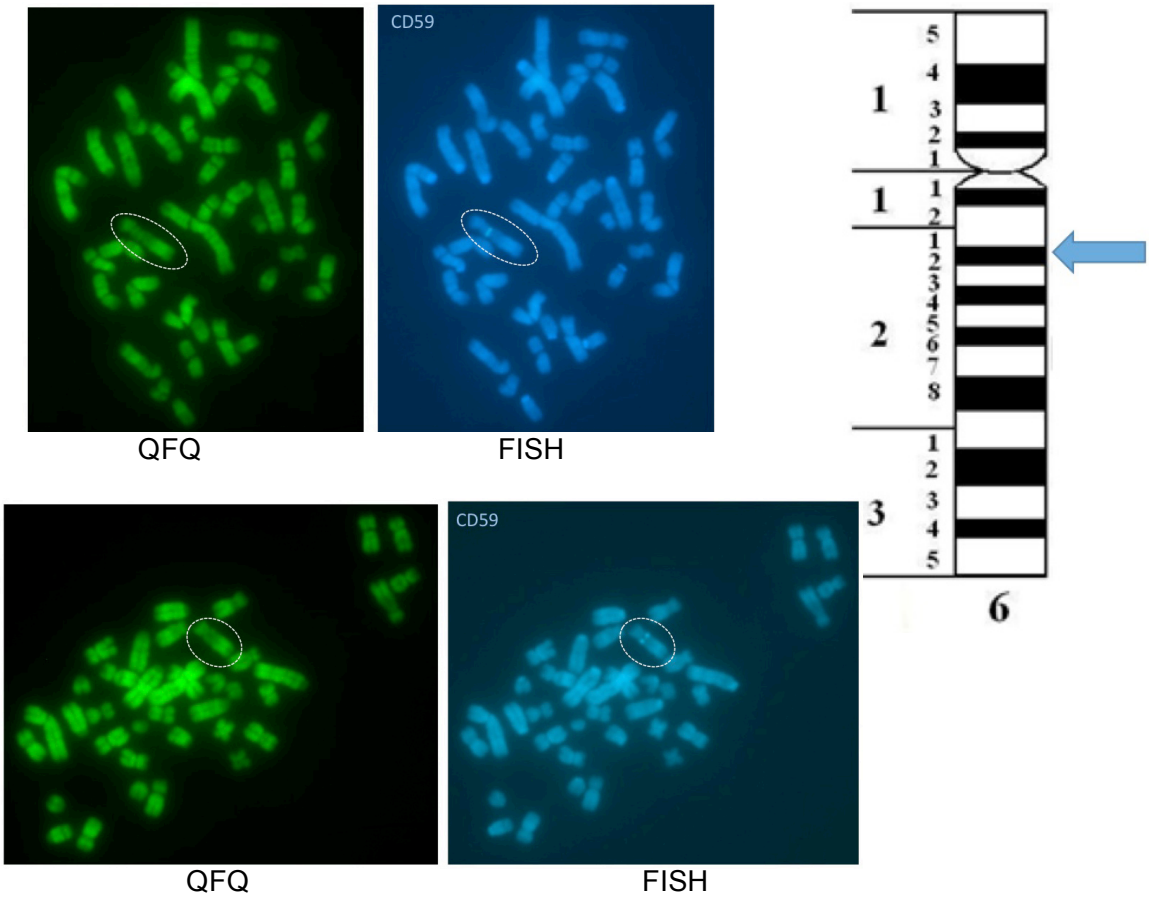
**A, Fluorescent in situ hybridisation analysis of piglet 1107-6.** Overlay of CD46 and CD55 signals. **B,** CD46 (Cy3.5) signal. **C,** CD55 (fluorescein) signal.




**Supplementary Figure 4 A**

**Fluorescent in situ hybridisation analysis of multi-transgenic piglet 1706.**

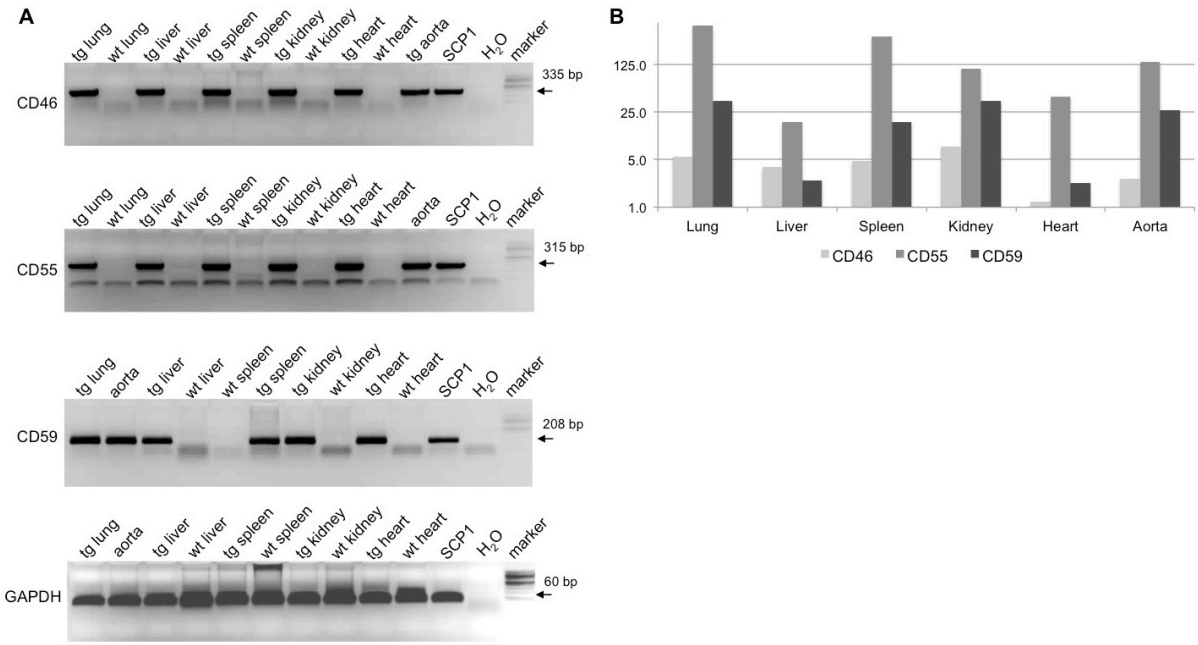
Sample metaphase spreads with CD46 (Cy3.5), CD55 (fluorescein), CD59 (DEAC) signals and merged overlay, as indicated.




**Supplementary Figure 4 B**

**Q banding and fluorescent in situ hybridisation analysis of multi-transgenic piglet 1706.**

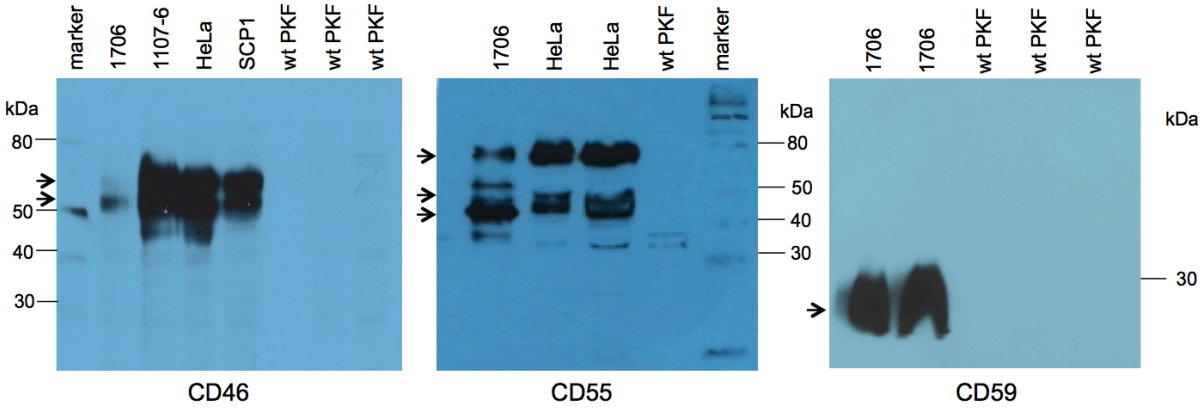
Sample metaphase spreads showing localisation of CD59 on porcine chromosome 6 q2 2.




**Supplementary Figure 5**

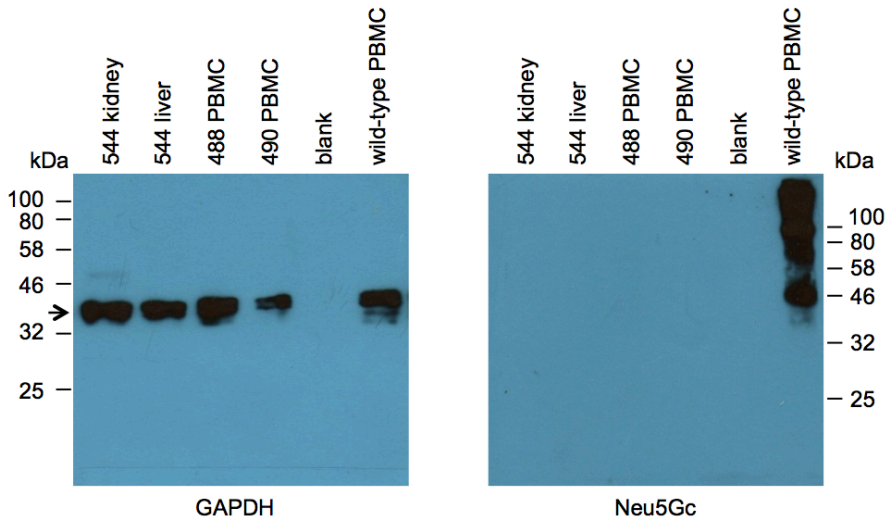
**A, RT-PCR analysis of CD46, CD55 and CD59 expression in multi-transgenic piglet 1706 organs**. 1706 multi-transgenic (tg) and wild-type (wt) lung, aorta, liver, spleen, kidney and heart as indicated. Human MSC line SCP1 was used as a positive control.

**B, Q-RT-PCR analysis of CD46, CD55 and CD59 expression in organs of multi-transgenic piglet 1706.** Expression values are shown relative to those in human MSC line SCP1 (expression=1). Please note that the y-axis indicates 5-fold differences of expression.




**Supplementary Figure 6**

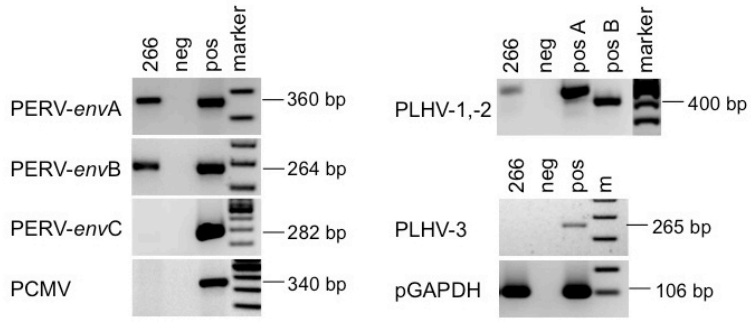
**Western blot analysis of complement regulator expression in PKF of multi-transgenic piglet 1706.** Detection of CD46, CD55 and CD59 in cell types as indicated.




**Supplementary Figure 7**

**Western blot analysis of CMAH deficient pigs.** Shown are organs from *CMAH/GGTA1* double knockout multi-transgenic male piglet 544 and blood cells from *CMAH/GGTA1* double knockout non-transgenic females 488 and 490. PBMC peripheral blood mononuclear cells. Note that wild-type PBMC show a range of proteins carrying N-glycolylneuraminic acid epitopes.

**Supplementary Figure 8**




**PCR analysis of porcine endogenous retroviruses (PERV), porcine cytomegalovirus (PCMV) and porcine lymphotropic herpesvirus (PLHV) in multi-transgenic cells of pig 266 (recloned pig 1706)**. Human 239 cells were used as negative control. Positive control A for PLHV-1, positive control B for PLHV-2, M, marker, the size of the amplicon is indicated.

**Microbiological analysis of founder pig 266**

The possibility of activating porcine endogenous retroviruses (PERV) has long been recognized, although evidence is growing that the risk posed is probably negligible (1). Nevertheless, following the precautionary principle, we examined the founder multi-transgenic pig 226 for PERVs. PERV-A and PERV-B are present in all pigs, whereas the PERV-C load varies by breed (2) and is of concern because it can recombine with PERV-A to generate infectious recombinant PERV-A/C (3). We found that the genome of founder 266 was free of PERV-C (Suppl. Fig. 8).

Risks posed by other agents are clearer. PCMV, a herpes virus, reduces survival of pig-to-baboon and pig-to-cynomolgus kidney transplants (3-5). Real time PCR analysis revealed that it was absent in the founder 266 genome.

Real-time PCR detected HEV sequences in 266, but blood analysis revealed no antibodies that would indicate exposure to infectious particles. Any risk posed by HEV genotype 3, the version present in pigs, is also unclear but means of eliminating it have been developed (1).

Porcine lymphotropic herpesvirus-1 (PLHV1) was detected in 266 serum, but PLHV2 and PLHV3 were not. PLHV1 is a γ-herpesvirus associated with post-transplant lymphoproliferative disorder in pigs (6), however the importance of these viruses for xenotransplantation is currently not known.

Also confirmed absent in 266 were: influenza virus, porcine parvovirus, porcine reproductive and respiratory syndrome virus, salmonella, porcine circovirus 2, actinobacillus pleuropneumoniae, mycoplasma hyopneumoniae (enzootic pneumonia), lawsonia intracellularis (porcine intestinal adenomatosus), chlamydia and leptospira.

**Methods for Suppl Fig 8**

DNA was isolated from EDTA blood using the DNAeasy blood and tissue kit (Qiagen, Germany), RNA was isolated from serum using the RNAeasy Minikit (Qiagen, Germany), according to the manufacturer´s instructions. PCR, real-time PCR and real-time RT-PCR for PERV-A, PERV-B, PERV-C, HEV, PCMV and PLHV1, 2, 3 and Western blot analysis for antibodies against HEV were performed as described (7) (Suppl. Table 2). Duplex real-time PCR to detect PCMV with primers specific for PCMV and porcine GAPDH as a reference was also carried out (Suppl. Table 2) using TaqMan Universal PCR 2x Mastermix (Life Technologies, USA) according to the manufacturer´s instructions. PCR to detect PERV-C were performed using the Kapa2G robust PCR Kit (Peqlab, Germany). Screening for other infectious agents was carried out by the Tiergesundheitsdienst Bayern e.V. (Bavarian Animal Health Service).

**References - Suppl Fig 8**

1. Denner, J. Xenotransplantation and hepatitis E virus. *Xenotransplantation* **22**, 167-173 (2015).

2. Denner, J. and Tönjes, R.R. Infection barriers to successful xenotransplantation focusing on porcine endogenous retroviruses. *Clin Microbiol Re*v. **25**, 318-343 (2012).

3. Denner, J. Xenotransplantation and porcine cytomegalovirus (PCMV). *Xenotransplantation* **22**, 329-335 (2015).

4. Yamada, K. *et al*. Porcine cytomegalovirus infection is associated with early rejection of kidney grafts in a pig to baboon xenotransplantation model. *Transplantation* **98**, 411-418 (2014).

5. Sekijima, M., *et al*. Results of life-supporting galactosyltransferase knockout kidneys in cynomolgus monkeys using two different sources of galactosyltransferase knockout swine. *Transplantatio*n **98**, 419-426 (2014).

6. Doucette, K., *et al*. Gene expression of porcine lymphotrophic herpesvirus-1 in miniature swine with post transplant lymphoproliferative disorder. *Transplantation* **83**, 87-90 (2007).

7. Plotzki, E., *et al*. Virus safety of islet cell transplantation from transgenic pigs to marmosets. *Virus Res.* **204**, 95-102 (2015).

**Supplementary Table 1**

**Primer combinations for: RT-PCR analysis of transgene expression; screening of cell clones for *GGTA1* and *CMAH* gene inactivation; and junction PCR analysis of HO1 and CD55 transgene stacking at porcine *ROSA26* locus.**

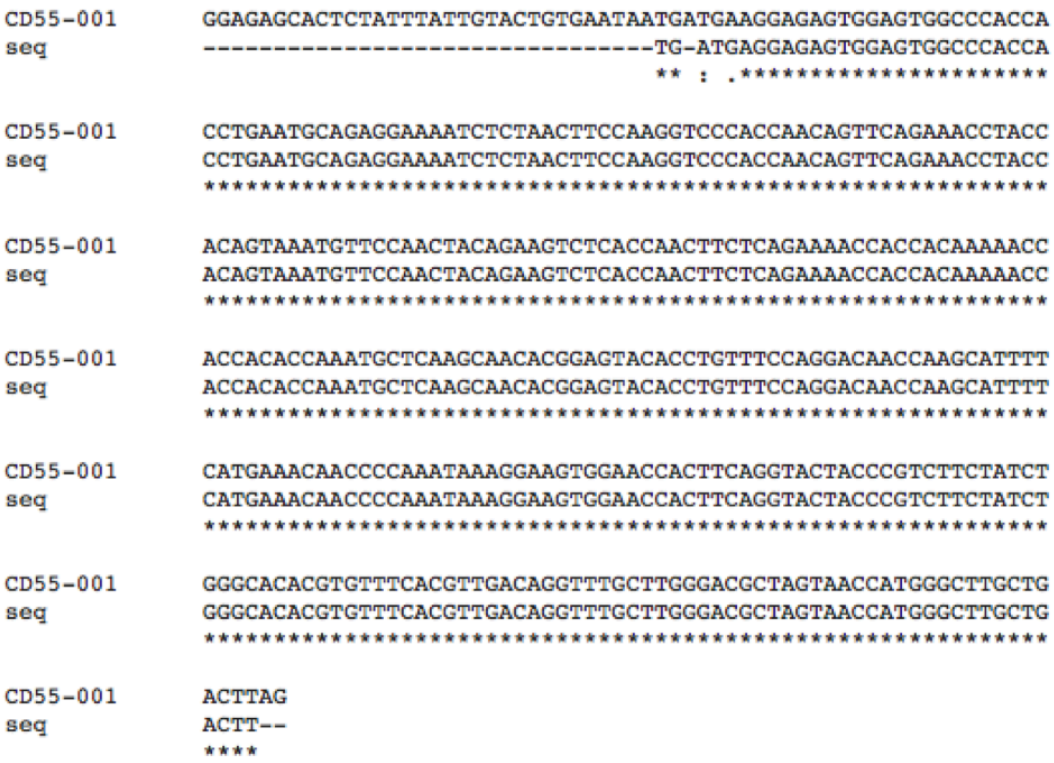
| | Forward 5´-3´ | Reverse 5´-3´ | Size |
|---|---|---|---|
| CD46 | CAAGCAGTCCCTGCAAATGG | GCAGCACGACTCCACACTGA | 335 bp |
| CD55 | TTGTCCAGCACCACCACAAA | CGTGTTGCTTGAGCATTTGG | 315 bp |
| CD59 | GGAGTTGAGACCTACTTCACAG | CTTTGGTAATGAGACACGCAT | 208 bp |
| A20 | TGGGACTCCAGAAAACAAGG | GTCCTTTTGGCCTCATGAAA | 231 bp |
| HO1 | GATGGAGCGTCCGCAACC | CTTCACATAGCGCTGCATGGC | 348 bp |
| GAPDH | GGCGTGAACCATGAGAAGTATG | GGTGCAGGAGGCATTGCT | 60 bp |
| GAPDH | TGGTGAAGGTCGGAGTGAAC | ATGAGGTCCACCACCCTGTT | 535 bp |
| CD46 splice variants | CAAGCAGTCCCTGCAAATGG | TGCCTTTCTTCTTCCTCCTTTGA | |
| CD55 splice variants | GGATTCACCATGATTGGAGAGCACTC | AAGTCAGCAAGCCCATGGTTACTAGC | |
| GGTA1 screening | AAGACCATCGGGGAGCACAT | GGCTTTCATCATGCCACTCG | 346 bp |
| CMAH screening | TGCCGTAAACAAAGAGGGATT | TTGTCTGCTGGGTGGGATTC | 357 bp |
| ROSA26 HO1 5' junction | TATGGGCGGGATTCTTTTGC | GGAGCGGCGATACCGTAAAG | 3.4 Kb |
| ROSA26 HO1 3' junction | GCCCCAGGATTTGTCAGAGG | CAGGTGGAAAGCTACCCTAGCC | 6.9 Kb |
| ROSA26 CD55 5' junction | TATGGGCGGGATTCTTTTGC | CGGCTGTCCATCACTGTCCT | 3.0 Kb |
| ROSA26 CD55 3' junction | TCCCACCAACAGTTCAGAAACCT | CAGGTGGAAAGCTACCCTAGCC | 9.3 Kb |

## Supplementary Table 2

## PCR and real-time PCR primers and probes used for microbiological analysis

| | Forward 5´-3´ | Reverse 5´-3´ |
|---|---|---|
| PervenvA | TGGAAAGATTGGCAACAGCG | AGTGATGTTAGGCTCAGTGG |
| PervenvB | TTCTCCTTTGTCAATTCCGG | TACTTTATCGGGTCCCACTG |
| PervenvC | CTGACCTGGATTAGAACTGG | ATGTTAGAGGATGGTCCTGG |
| envC real | CCCCAACCCAAGGACCAG | AAGTTTTGCCCCCATTTTAGT |
| pCyclo real | TGCTTTCACAGAATAATTCCAGGATTTA | GACTTGCCACCAGTGCCATTA |
| pGAPDH | ACATGGCCTCCAAGGAGTAAGA | GATCGAGTTGGGGCTGTGACT |
| JVHEV | GGTGGTTTCTGGGGTGAC | TGATTCTCAGCCCTTCGC |
| PCMV real | GTTCTGGGATTCCGAGGTTG | ACTTCGTCGCAGCTCATCTGA |
| PCMV 199 | ACGAGAAAGATATTCTGACGGTGCA | TCTAGACGAAAGGACATTGTTGATA |
| PLHV-1,-2 747 | CAYGGTAGTATTTATTCAGACA | GATATCCTGGTACATTGGAAAG |
| PLHV-3 905 | ACAAGAGCCTTAGGGTTCCAAACT | GTGTCCAGTGTTGTAATGGATGCC |
| envC probe | FAM-CTCTAACATAACTTCTGGATCAGACCC-BHQ1 | |
| pCyclo probe | Cy5-TGCCAGGGTGGTGACTTCACACGCC-BHQ1 | |
| pGAPDH probe | HEX-CCACCAACCCCAGCAAGAG-BHQ1 | |
| JVHEVProbe | FAM-TGATTCTCAGCCCTTCGC-BHQ1 | |
| PCMV real probe | FAM-CAGGGCGGCGGTCGAGCTC-TAMRA | |


**Supplementary DNA sequence 1**
Cloned RT-PCR CD46 splice variants from piglet 1706 aligned with ENSEMBL reference sequences for CD46 variants 001/007, 002/004 and 005/006, as indicated





**Supplementary DNA sequence 2**

Cloned RT-PCR CD55 splice variants from piglet 1706 aligned with ENSEMBL reference sequences for CD55 variants 001 (gDAF) and 009 (sDAF), as indicated