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.



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.



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.



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.



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.



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 multitransgenic 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.



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.



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.



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-tobaboon 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

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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-BHQ	1
pGAPDH probe	HEX-CCACCAACCCCAGCAAGAG-BHQ1	
JVHEVProbe	FAM-TGATTCTCAGCCCTTCGC-BHQ1	
PCMV real probe	FAM-CAGGGCGGCGGTCGAGCTC-TAMRA	

CD46-001/007	TATCAGATCCACTTATTATTACTAGAGGCTPATTACTTAATTGCTGAAGAAATTCCTAATAT	CD46-002/004	TATCAGATGCACTTATTTGTAATGCGGGTTATTACTTGGTGAAGAAATTCTATAT	CD46-005/006	TATCAGATGCACTTATTTCTAGGGGTATTACTTAATTGCGGAGGAAATTCTATAT
seq	GCACTTATTTGTAATGAGGGCTPATTACTTAATTGCTGAGGAAGAAATTCTAATAT	seq	ACCAGTTATTGT-ATGAGGGTTATTACTTAATTGGTGAAGAAATTCTAAT	seg	GCACTTAATTGAGGGTTATTACTTAATTGCGGAGAAAATTCTATAT
CD46-001/007	TGTGAACTTAAAGGATCAGTAGCAATTTGGAGCGGTAAGCCCCCCAATATGTGAAAAGGTT	CD46-002/004	TOTGAACTTAAAGGATCAGTAGCAATTTGGGAGCGGTAAGCCCCCAATATGTGAAAAGGTT	CD46-005/006	TGTGAACTTAAAGGATCAGTAGCAATTTGGAGCGGGTAAGCCCCCCAATATGTGAAAAGGTT
seg	TGTCAACTTTAAAGGATCAGTAGCAATTTGGGAGCGGGTAAGGCCCCCCAATTATGTGAAAAGGTT	seg	TGTGAACTTAAAGGATCAGTAGCAATTTGGGAGCGGGTAAGCCCCCCAATATGTGAAAAGGTT	seq	TGTGAACTTAAAGGATCAGTAGCAATTTGGAGCGGGTAAGCCCCCCAATATGTGAAAAGGTT
CD46-001/007 seq	TTGTGTACACCACCTCCAAAAATAAAAAATGGGAAAAACACACCTTTAGTGAAGTAGAAGTA TTGTGTCACCACCTCCAAAAAATAAAAAAAGGGAAAAACACACCTTTAGTGAAGTAGTA TTGTGTCACCACCTCCAAAAAAAAAA	CD46-002/004 seg	TTGF0FRCACCCCCCCAAAAATAAAAAGGGAAAACACACCTFF76F0AAGFAGAAGFA TTGF0FRCACCCCCCCAAAAATAAAAAAGGGAAAACACACCTFT76F0AGAAGFAGAAGFA	CD46-005/006 seq	TTGTGTACACCTCCCAAAAATAAAAAAGGGAAAACACACGTTTAGTGAAGTAGAAGTA TTGTGTACACCACCTCCCAAAAAAAAAA
CD46-001/007	TTTGAGTAFCTTGATGCAGTAACTTATAGTTGATCCTGGACCTGGACCAGAATCCATT	CD46-002/004	TTTGAGTATCTIGATGCGGTAACTTATAGTTGTGATCCTGCACCGGATCGATC	CD46-005/006	TTTGAGTANCTTGANGCAGTAACTTAIAGTNGTGANCCTGGACCAGGACCAGATCCATTT
seg	TTTGAGTAFCTTGATGCAGTAACTTATAGTTGATGCATGCAGGACCAGGACCAGATCCATTT	seg		seq	TTTGAGTANCTTGANGCAGTAACTTAIAGTNGTGANCCTGGACCAGGACCAGATCCATTT
CD46-001/007 seq	TCACTTATTGGAGAGAGACACGATTATTGTGGTGAGAATTCAGTGTGGAGAGGGGGGGG	CD46-002/004 seg	TCACTTATTOGAGAGAGACACGATTATTGTGGTGACAATTCAGTGTGGAGTCGTGCT TCACTTATTGGGAGAGAGAGACACGACAATTATGGTGAAATTCAGTGGTGGTGCT ****************************	CD46-005/006 seq	**************************************
CD46-001/007 seq	CCAGAGTGTAAGTGGTCAAATGTCGATTTCCAGTAGTGGAAAATGGAAAACAGATATCA CCAGAGTGTAAGTGGTCAAATGTCGATTTCCAGAAAATGGAAAAACGGAAAACAGATATCA	CD46-002/004 seq	CCAGGGTGAAAGTGGTCAAATGTCGATTTCCAGTAGTCGAAAATGGGAAAGGGTATCA CCAGAGTTTAAAGTGGTCAAATGTCGATTTCCAGTAGTGGGAAAAAGGATAACG	CD46-005/006	**************************************
CD46-001/007 seg	GGATTTGGAAAAAATTTTACTACAAACAACAACAGAGATAGGGTTTTGGATGGGATAAAGGGTTTT GGATTTGGAAAAAATTTTGCTACAAGCAACAACAGTTTGGATGGGATAAAGGGTTTT ***************	CD46-002/004 seg	GGATTTGGAAAAAATTTTACTACAAAGCAACAGTATATGTTTGAATGGGATAAGGGTTT GGATTTGGAAAAAATTTTACTACAAAGCAACAACAGTAATGTTTGAATGGGATAAGGGTTTT	cD46-005/006	CONTRACTOR OF A CONTRACTOR OF
CD46-001/007	TACCTCGATGGCAGCGACACATTGTCTGTGACAGTAACAGTACTTGGGATCCCCCGAGT	CD46-002/004	TACCTCGATGGCAGCGACAATTGTCTGTGACAGTAACAGTACTTGGGATCCCCCAGTT	seg	GGATTTGGAAAAATTTTACTACAAGGAAAGCATTATGTTTGGATGGA
seg	TACCTCGATGGCAGGCAACAATTGTCTGTGACAGTAACTAAC	seq	TACCTCGATGGCAGCGACACAATTGTCTGTGACAGTAACAGTAACTTGGGATCCCCCAGTT	CD46_005/006	
CD46-001/007	CCAAAGTGTCTTAAAGTGCTGCTCCATCTAGTACAAAACCTCCAGCTTTGAGTCATTCA	CD46-002/004	CCAAAGTGTCTTAAAGTGTCGACTTCCTTCCACTACAAAATCTCCAGGGTCCAGTGCCTCA	0000	TACCTCGATGGCACGGACAAATGTCTGTGACAGTAACAGTAACAGTACTGGGATCCCCCCAGT
seq	CCAAAGTGGTGTGGT	seq	CCAAAGTGTCTTAAGTGTCGACTTCTTCCACTACAAAATCTCCCAGGGTCCAGTGCCTCA		************************************
CD46-001/007 seg	GTGTCGACTTCTACCACAAAATCTCCAGGGTCCAGTGCGTCAGGGCCTAGCT GTGTCCACTTACAAAAATCTCCAGGGTCCAGTGCGGTCCAGGGGTCCTAGT GTGTCCACTTACAAAAAATCTCCAGGGGTCCAGTGGGTCCAGGGGTCCTAGGGCTTACT	CD46-002/004 seg	GGTCCTAGGCCTACTTACAAGCCTCCAGTCTCAAATTATCCAGGATATCCTAAACCTGAG GGTCCTAGGCCTACTTACAAGCCTCCAGTCTCAAAATTATCCAGGATATCCTAAAACCTGAG GGTCCTAGGCCTACTTACAAGCCTCCAGTCTCAAAATTAATCCAGGATAATCCTAAACCTGAG	CD46-005/006 seg	CCAAGGTCTTTAAGGTCCTAGGCCTACTTACAAGCTCCAGTCTCAATTATCCAGGA CCAAGGTCTTTAAGGTCTTAGGCCTACTTACAAGCCTCCAGGTTTATCCCAGGA ********************************
CD46-001/007 seq	TACAAGCCTCCGGTCTCAAATTATCCAGGATATCCTAAACCTGAGGAAGGA	CD46-002/004 seg	GAAGAATACTTGACAOTTTGGATGTTTGGGOTCATTGCTGGGATACTTGCCATAGTT GAAGGAATACTTGACAOTTTGGATGTTTGGGATGTTGCTGGGATAGTTGTTGCCATAGTT	cD46-005/006 seq	TATCCTAAACCTGAGGAAGGAATACTTGACACTTTGGATGTTTGGGTCATTGCTGTGTGT TATCCTAAACCTGAGGAAGAATACTTGACAGTTTGGATGTTTGGGTCATTGCTGCTGATT TATCCTAAACCTGAGGAAGAATACTTGACAGTTTGGATGTTTGGGTCATTGCTGCTGATT
CD46-001/007	agtytggangtytgggrgattgrgattgrystygggangtgggggrgggggrggggrggggg	CD46-002/004	gttggagttgcagtaatttgtgtgtgtgcccgtacagatatatcttcaaaggaggaagaaa	CD46-005/006	GTTATTGCCATAGTTGCAGAGTGCAGTAATTGTGTGGTGGTCGCGTACAGATATCTTCAA
seg	Agtytgggrgatgggggrgatggrgattggrgatgggggggggg	seg	gttggagttgcagtaatttgtgttgtg	seg	GTTATTGCCATAGTTGGGGGGGGGG
CD46-001/007	ATTYGTGTGTCGCGTACATATCTTCAAGGAGGAAGAAGAAGGAGGAAGAAAGGCACATACCTAACT	CD46-002/004	GGGAAAGCAGATGGTGGAGCTGAATATGCCACTTACCAGCTAAATCAACCACCACCAGCA	CD46-005/006	AGGAGGAAGAA-AGGCACATACCTAACTGATGAGGACCCACAGAGAAGTAAAATTAC
seq	ATTYGTGTGTCGCCCGTACAAAACATCTTCAAAGGAAGAAGAAAAAAAGG	seg	GGCAAA	seq	AGGAGGAGAAAAAAAGGCA

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

:D55-001 leg	GGAGAGCACTCTATTATTGTACTGTGATGATGAAGGAGAGAGTGGAGTGGCCCACCA 	CD55-009 seg	GGAGAGCACTCTATTTTTTTTTGTACTGTGAATGATGATGAGGAGAGTGGAGTGGACCCACCA
:D55-001 eq	CCTGAATGCAGAGAAAATCTCTAACTTCCAAGGTCCCACCAACAGGTTCAGAAACCTACC CCTGAATGCAGAGAAAATCTCTTACTTCCAAGGTCCCACCAACAGGTTCAGAAACCTACC ******************************	CD55-009 seg	CCTGAATGCAGGGGAAAATCTCCTAACTTCCAAGGTCCCACCACGAGGTTCAGGAACCTACC CCTGAATGCAGGGGAAAATCTCCTAACTTCCAAGGTCCCACCACCAGGGTTCAGGAAGCCTACC *********************************
:D55-001 leg	ACAGTAAATGTTCCAACTACAGAAGTCTCACCAACTTCTCAGAAAACCACCACAAAAACC ACAGTAAATGTTCCAACTACAGAAGTCTCACCAACTTCTCAGAAAAACCACCACAAAAACC *****************	CD55-009 seq	ACAGTAAATGTTCCAACTACAGAAGTCTCACCAACTTCTCAGAAAACCACCACAAAAACC ACAGTAAATGTTCCAACTACAGAAGTCTCCACCAACTTCTCAGAAAACCACACAAAAACC *******************
:D55-001 leg	ACCACACCAAATGCTCAAGCAACACGGGGGTACACCTGTTTCCAGGACAACCAAGCATTTT ACCACACAAATGCTCAAGCAACACGGGGGTACACCTGTTTTCCAGGACAACCAAGCATTTT *********************************	CD55-009 seg	ACCACCAAATGCTCAAGCAACACGGGGTACACCTGTTTCCAGGACAACCAAGCATTT ACCACCCAAATGCTCAAGCAACACGGAGTACACCTGTTTTCCAGGACAACCAAGCATTTT *********************************
:D55-001 leg	CATGAAACAACCCCAAATAAAGGAAGTGGAACCACTTCAGGTACTACCGGTCTTCTATCT CATGAAACAACCACAAATAAAGGAAGTGGAACCACTTCAGGTACTACCCGGTCTTCTATCT *****************************	CD55-009 seg	CATGAAACAACCCCCAAATAAAGGAAGTGGAACCACTTCAGGTACTACCGGTCTTCTATCT CATGAAACAACCCCCAAATAAAGGGAAGTGGGAACCACTTCAGGTACTACCGGTCTTCTATCT *****************************
:D55-001 eq	GGGCACGCGTGTTTCACGTTGACAGGTTTGCTTGGGACGCTAGTAACCATGGGCTTGCTG GGGCACACGTGTTTCACGTTGACAGGTTTGCTTGGGACGCTAGTAACCATGGGCTTGCTG ****************************	CD55-009 seg	<pre>GGTTCTCGTCCTGTCACCCAGGCTGGTAGTGGTGGTGTGTGATCGTAGCTCACTGCAGTCT GGTTCTCGTCCTGTCCCCAGGCTGGTATGCGGTGGTGTGTGT</pre>
:D55-001 eq	ACTTAG ACTT ****	CD55-009 seg	CGAACTCCTGGGTTCAAGCGATCCTTCCACTTCAGCCTCCCAAGTAGCTGGTACTACAGG CGAACTCCTGGGGTTCCAAGGCGATCCTTCCACTTCAGCCTCCCCAAGTAGCTGGGTACTACAGG **********************************
		CD55-009 seg	GCACACGTGTTTTCACGTTGACAGGTTTTGCTTGGGACGCCTAGTAACCATGGGCTTGCTGAC GCACACGTGTTTTCACGTTGACAGGTTTGCTTGGGACGCTAGTAACCATGGGCTTGCTGAC ************************************
		CD55-009 seq	TTAGCCAAAGAAGATTAAGAAGAAAATACACACAAGTATACAGACTGTTCCTAGTTTCT TA

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