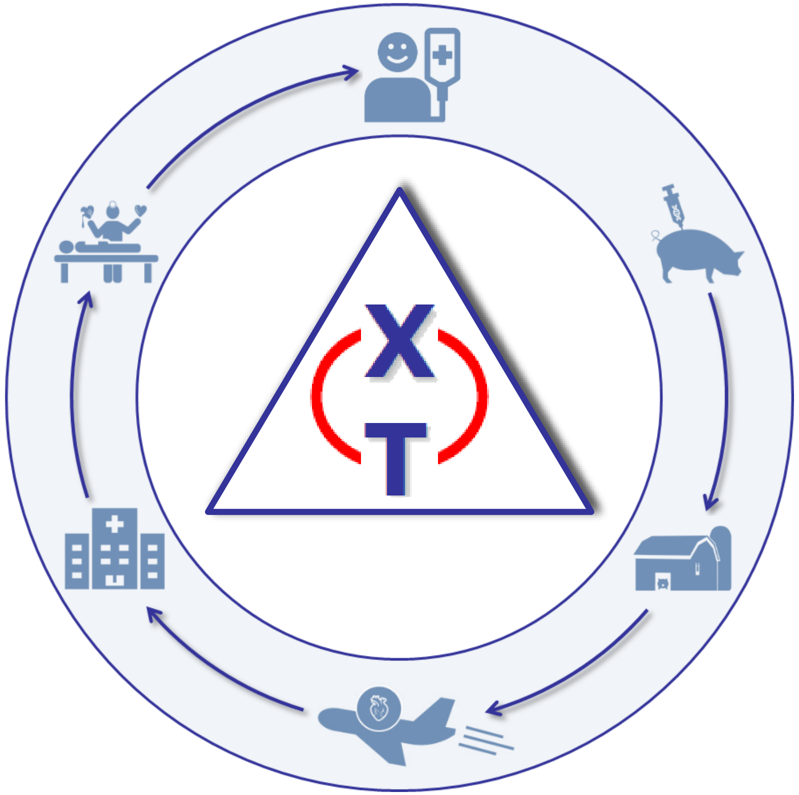
Cellular, Tissue, and Gene Therapies Advisory Committee Meeting

Source animals with intentional genomic modifications



Eckhard Wolf

Gene Center, LMU Munich

LMU

LUDWIG-
MAXIMILIANS-
UNIVERSITÄT
MÜNCHEN

GENE CENTER MUNICH
MOLECULAR ANIMAL BREEDING
AND BIOTECHNOLOGY

LMU

LUDWIG-
MAXIMILIANS-
UNIVERSITÄT
MÜNCHEN

CENTER FOR INNOVATIVE
MEDICAL MODELS

CiMM

Disclosures

- Spokesperson DFG SFB/TR 127 *Biology of xenogeneic cell, tissue and organ transplantation – from bench to bedside*

- Co-founder and Scientific Director of MWM Biomodels GmbH, Tiefenbach

- Co-founder of *XT*ransplant GmbH, Starnberg

- SAB Member, Defymed, Strasbourg

Anatomical and physiological similarities of pigs with humans

BODY DIMENSION

human-like size and weight

CARDIOVASCULAR SYSTEM

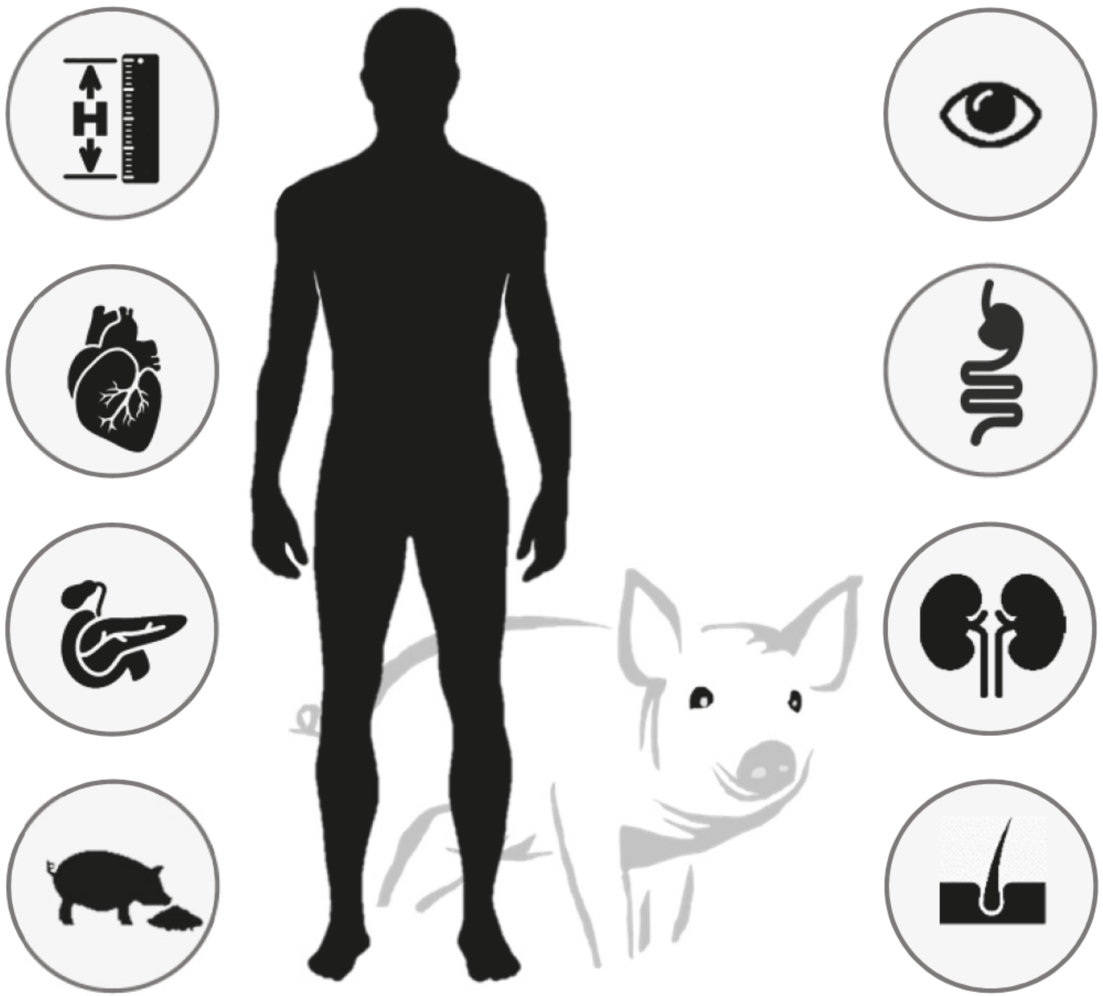
heart size, heart-to-body mass ratio, coronary anatomy, susceptible to diet-induced hypercholesterolemia

PANCREAS

islet structure, vascularization, beta-cell mass, structure of insulin (only 1 aa different)

CIRCADIAN RHYTHM

nutritional intake, day-night-cycle



EYES

retinal vasculature, cone photoreceptors in outer retina and macular area, retinal and scleral thickness

GASTROINTESTINAL TRACT

organ structure, eating behavior, omnivorous

KIDNEYS

size, multilobular and multipapillary, similar vascularization patterns

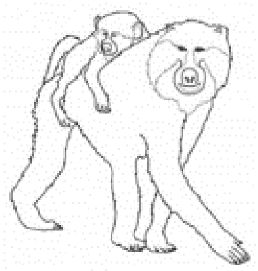
SKIN

anatomy and pharmacokinetic characteristics

short generation interval ● large litters ● breeding under DPF conditions ● genetic engineering

What is the best donor species for xenotransplantation?

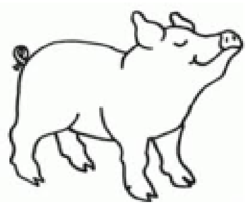
Concordant



Advantages:
- anatomical similarities
- physiological compatibility
- rejection resembles allotransplants (?)

Disadvantages:
- long gestation period
- single offspring
- organ size unsuitable for human adults
- transmission of infections
- high costs
- ethical considerations

Discordant



Advantages:
- anatomical and physiological similarities
- short gestation period (114 days)
- large litters, rapid growth of offspring
- organ size suitable for human adults
- designated pathogen-free breeding (DPF)
- **genetic modification well established**

Disadvantages:
- hyperacute / humoral rejection
- physiological incompatibilities
- porcine endogenous retroviruses (PERV)

Genetic modifications of source pigs for xenotransplantation

Deletion of sugar moieties of pig cells with pre-formed recipients' antibodies

GGTA1-KO, CMAH-KO, B4GALNT2-KO, B4GALNT2L-KO

Complement regulation by human complement-regulatory gene expression

hCD46-tg, hCD55-tg, hCD59-tg, hC1-INH-tg

Coagulation regulation by human coagulation-regulatory gene expression

hTBM-tg, hEPCR-tg, hTFPI-tg, hCD39-tg, hCD73-tg

Prevention of cell-mediated rejection

LEA29Y-tg, hCTLA4-Ig-tg, pCTLA4-Ig-tg, SLA class I-KO, CIITA-DN-tg, hTRAIL-tg, PD-L1-tg
HLA-E/b2M-tg, hCD47-tg

Expression of anti-inflammatory proteins

A20-tg, hHO-1-tg, shTNFRI-Fc-tg

Reduction of growth

GHR-KO

Reduction/elimination of the risk of PERV transmission

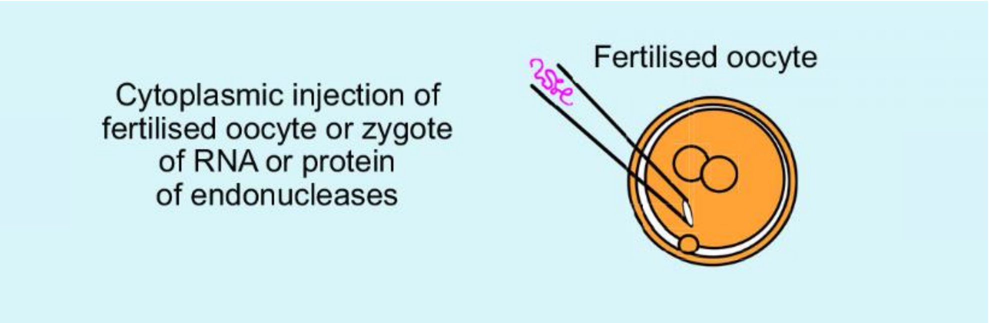
Knockdown of PERV expression, genome-wide KO of the PERV pol gene

Reviews:
Kemter et al., Curr Diab Rep 18, 103 (2018)
Kemter et al., Curr Opin Genet Dev 64, 60-65 (2020)

Transposon based transgenesis

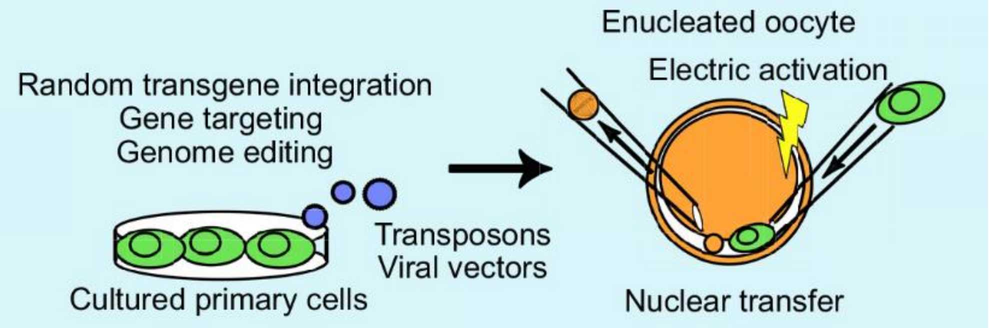
2008 — Adeno-associated viru

2006 — ICSI-mediated gene tra



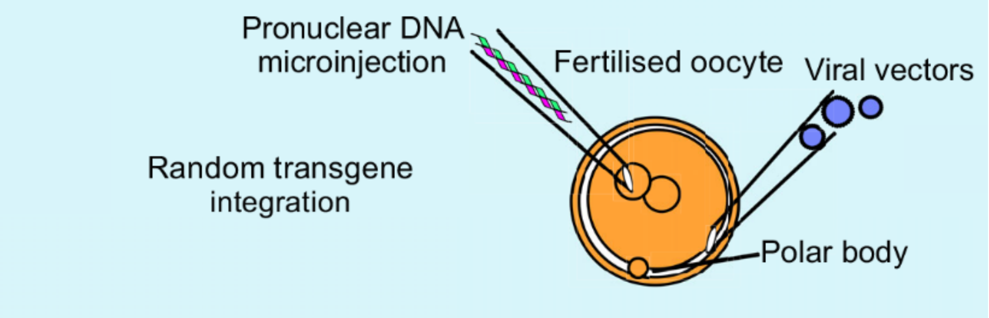
2003 — Lentiviral gene transfe

2002 — Gene targeting in soma



2002 — Sperm-mediated gene

1985 — Pronuclear DNA micro



Dmochewitz & Wolf, Animal Frontiers 5, 50-56 (2015)

Disease Models & Mechanisms (2018) 11, dmm030783

Efficiency of porcine somatic cell nuclear transfer: data analysis with over 200,000 reconstructed embryos

TABLE 1. EFFECT OF DONOR CELL TYPE AND GENE MODIFICATION ON THE OVERALL EFFICIENCY OF HANDMADE CLONING

Donor cell	*No. of reconstructed embryos*	*No. of transferred blastocysts*	*No. of recipients*	*No. of pregnant recipients*	*No. of farrowed recipients*	*Litter size*	*No. of piglets*	*No. of piglets born alive*	*No. of abnormalities*	*Efficiency (%)*
FF	14897	2799	31	23	17	1–5	46	42	5	1.50[a]
AF	54892	15402	159	92	54	1–12	198	132	112	0.86[b,c]
AP	12263	3304	33	25	13	1–7	41	34	10	1.03[a,b]
BM	7617	1984	21	9	7	2–6	27	18	14	0.91[a,b,c]
CAF	5279	1450	17	9	7	1–8	32	23	9	1.59[a]
CFF	4322	970	11	8	6	1–4	17	3	16	0.31[c]
TFF	104312	31456	311	199	139	1–13	626	509	158	1.62[a]
KOFF	24648	7192	73	42	23	1–10	77	49	30	0.68[b,c]

Efficiency was calculated by piglets born alive/transferred blastocyst; within the same column, values with different superscript letters (a, b, and c) were significantly different ($p < 0.05$).

FF, fetal fibroblast; AF, adult fibroblast; AP, adult preadipocyte; BM, adult blood mesenchymal cell; CAF, cloned adult fibroblast; CFF, cloned fetal fibroblast; TFF, transgenic fetal fibroblast; KOFF, gene knockout fetal fibroblast.

Liu et al., Cellular Reprogramming 17, 463-471 (2015)

Somatic cell nuclear transfer (cloning) may induce epigenetic variation

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 276, No. 43, Issue of October 26, pp. 39980–39984, 2001
Printed in U.S.A.

Typical Demethylation Events in Cloned Pig Embryos

CLUES ON SPECIES-SPECIFIC DIFFERENCES IN EPIGENETIC REPROGRAMMING OF A CLONED DONOR GENOME*

Received for publication, July 12, 2001, and in revised form, August 24, 2001
Published, JBC Papers in Press, August 27, 2001, DOI 10.1074/jbc.M106516200

Yong-Kook Kang‡, Deog-Bon Koo‡, Jung Sun Park‡, Young-Hee Choi‡, Ha-Na Kim‡, Won-Kyong Chang§, Kyung-Kwang Lee‡, and Yong-Mahn Han‡¶

From the ‡Animal Developmental Biotechnology Laboratory, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yusong, Taejon 305-600, South Korea and the §National Livestock Research Institute, Suwon 441-350, Korea

Li *et al. BMC Genomics* 2014, **15**:811
http://www.biomedcentral.com/1471-2164/15/811

BMC Genomics

RESEARCH ARTICLE — Open Access

Dysregulation of genome-wide gene expression and DNA methylation in abnormal cloned piglets

Guanglei Li[1], Qitao Jia[2], Jianguo Zhao[2], Xinyun Li[1], Mei Yu[1], Melissa S Samuel[3], Shuhong Zhao[1], Randall S Prather[3] and Changchun Li[1*]

frontiers in Genetics

ORIGINAL RESEARCH
published: 20 February 2020
doi: 10.3389/fgene.2020.00023

Whole-Genome Methylation Analysis Reveals Epigenetic Variation in Cloned and Donor Pigs

Mengfen Wang[1,2], Shuaifei Feng[1], Guanjun Ma[1,2], Yiliang Miao[1], Bo Zuo[1], Jinxue Ruan[1], Shuhong Zhao[1], Haiyan Wang[1,3], Xiaoyong Du[1,2,3] and Xiangdong Liu[1,2*]*

[1] Key Laboratory of Swine Genetics and Breeding of Ministry of Agriculture & Key Laboratory of Agriculture Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Animal Science, Huazhong Agricultural University, Wuhan, China, [2] Key Lab of Swine Healthy Breeding of Ministry of Agriculture and Rural Affairs, Guangxi Yangxiang Co., Ltd., Guigang, China, [3] Hubei Key Laboratory of Agricultural Bioinformatics, College of Informatics, Huazhong Agricultural University, Wuhan, China

Stem Cell Reports
Article
ISSCR
OPEN ACCESS

TDG is a pig-specific epigenetic regulator with insensitivity to H3K9 and H3K27 demethylation in nuclear transfer embryos

Xin Liu,[1,2,6] Lu Chen,[3,6] Tao Wang,[1,2,6] Jilong Zhou,[1,2] Zhekun Li,[1,2] Guowei Bu,[1,2] Jingjing Zhang,[1,2] Shuyuan Yin,[1,2] Danya Wu,[1,2] Chengli Dou,[1,2] Tian Xu,[1,2] Hainan He,[1,2] Wei Zhu,[1,2] Longtao Yu,[1,2] Zhiting Liu,[1,2] Xia Zhang,[1,4] Zhen-Xia Chen,[3,*] and Yi-Liang Miao[1,2,4,5,*]

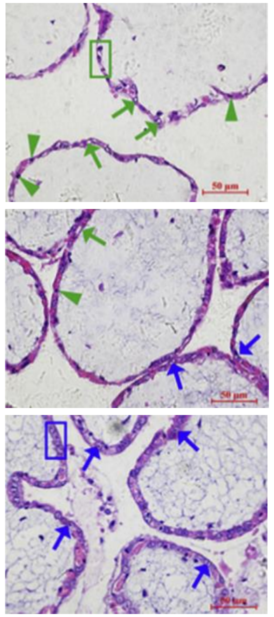
[1]Institute of Stem Cell and Regenerative Biology, College of Animal Science and Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China
[2]Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction (Huazhong Agricultural University), Ministry of Education, Wuhan 430070, China
[3]Hubei Key Laboratory of Agricultural Bioinformatics, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
[4]The Cooperative Innovation Center for Sustainable Pig Production, Wuhan 430070, China
[5]Hubei Hongshan Laboratory, Wuhan 430070, China
[6]These authors contributed equally
*Correspondence: zhen-xia.chen@mail.hzau.edu.cn (Z.-X.C.), miaoyl@mail.hzau.edu.cn (Y.-L.M.)
https://doi.org/10.1016/j.stemcr.2021.09.012

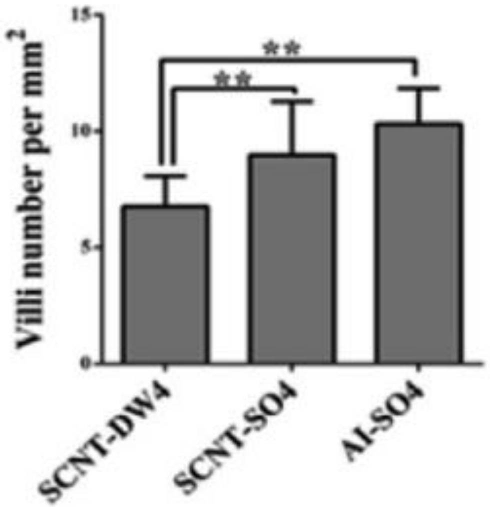
Morphological alterations and aberrant gene expression in placentas of cloned piglets

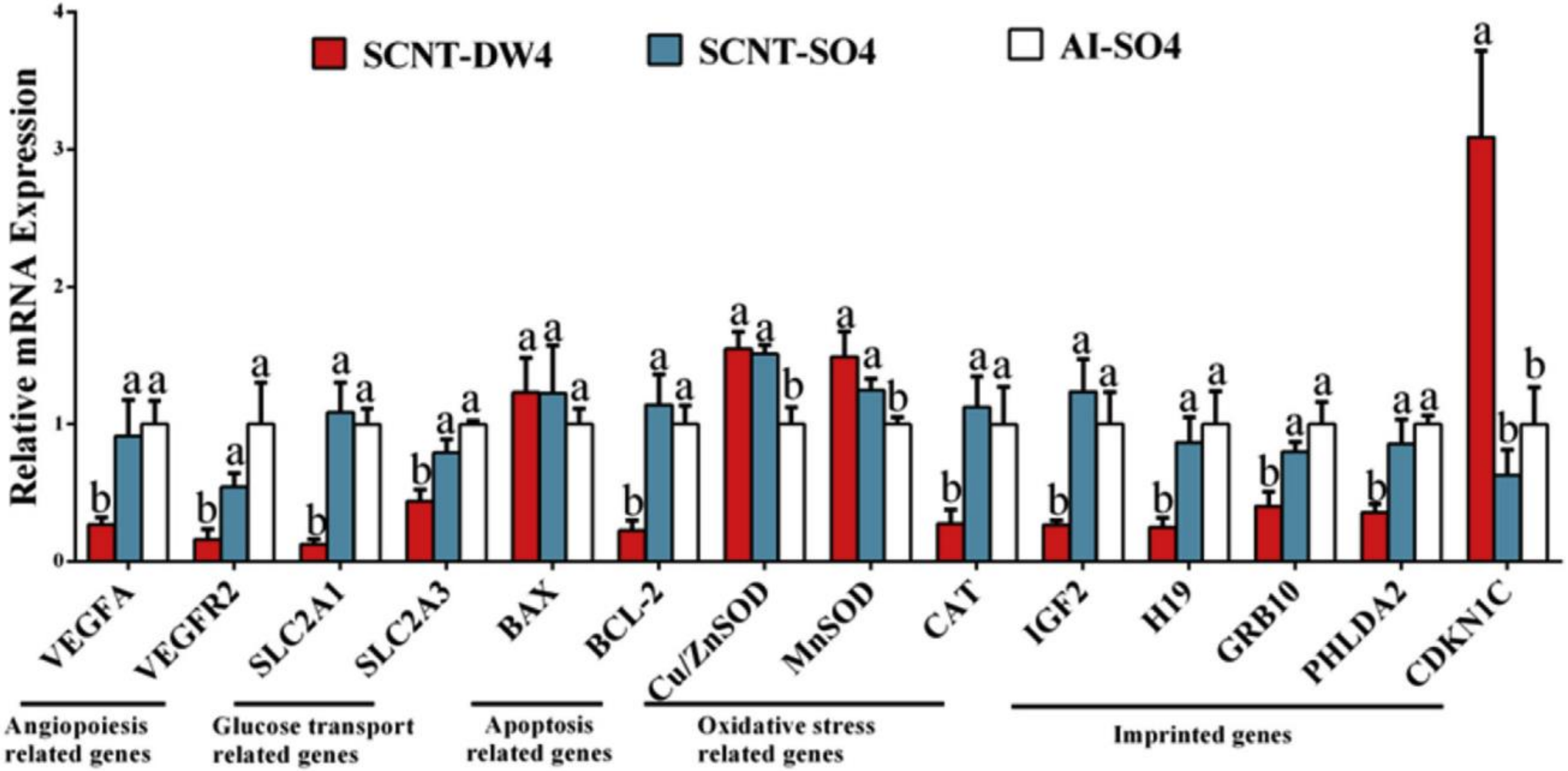
SCNT-DW4

SCNT-SO4

AI-SO4

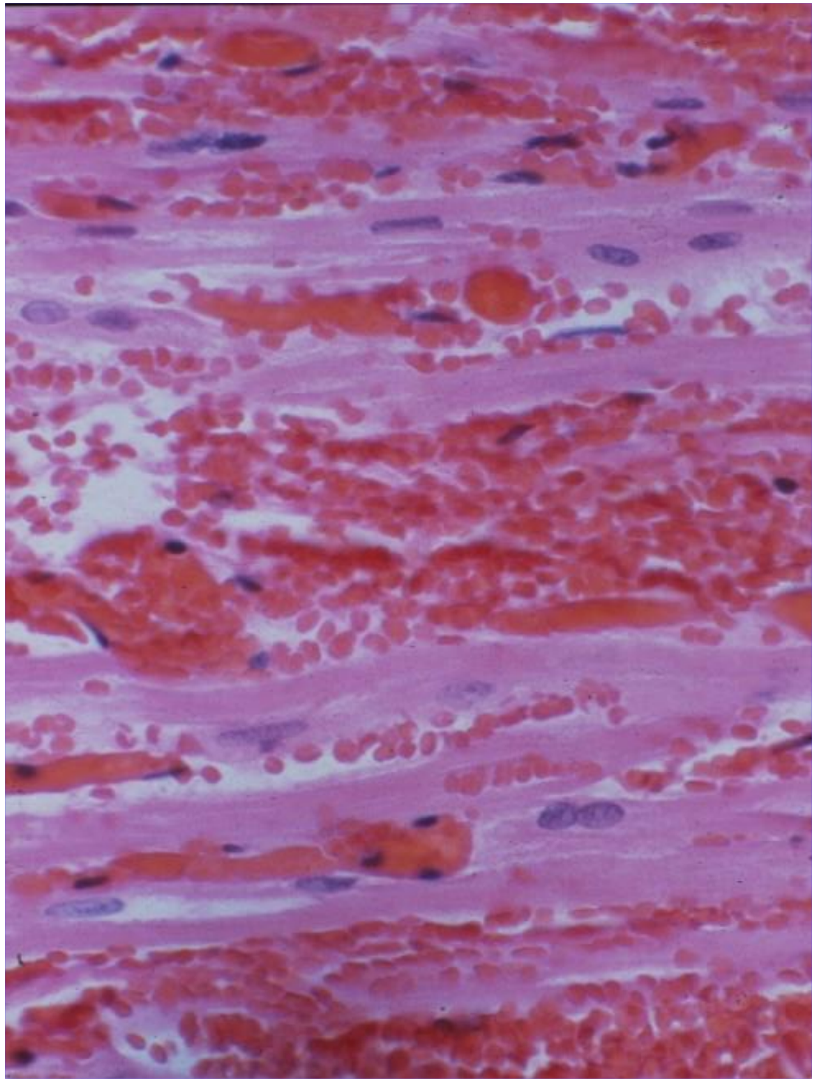


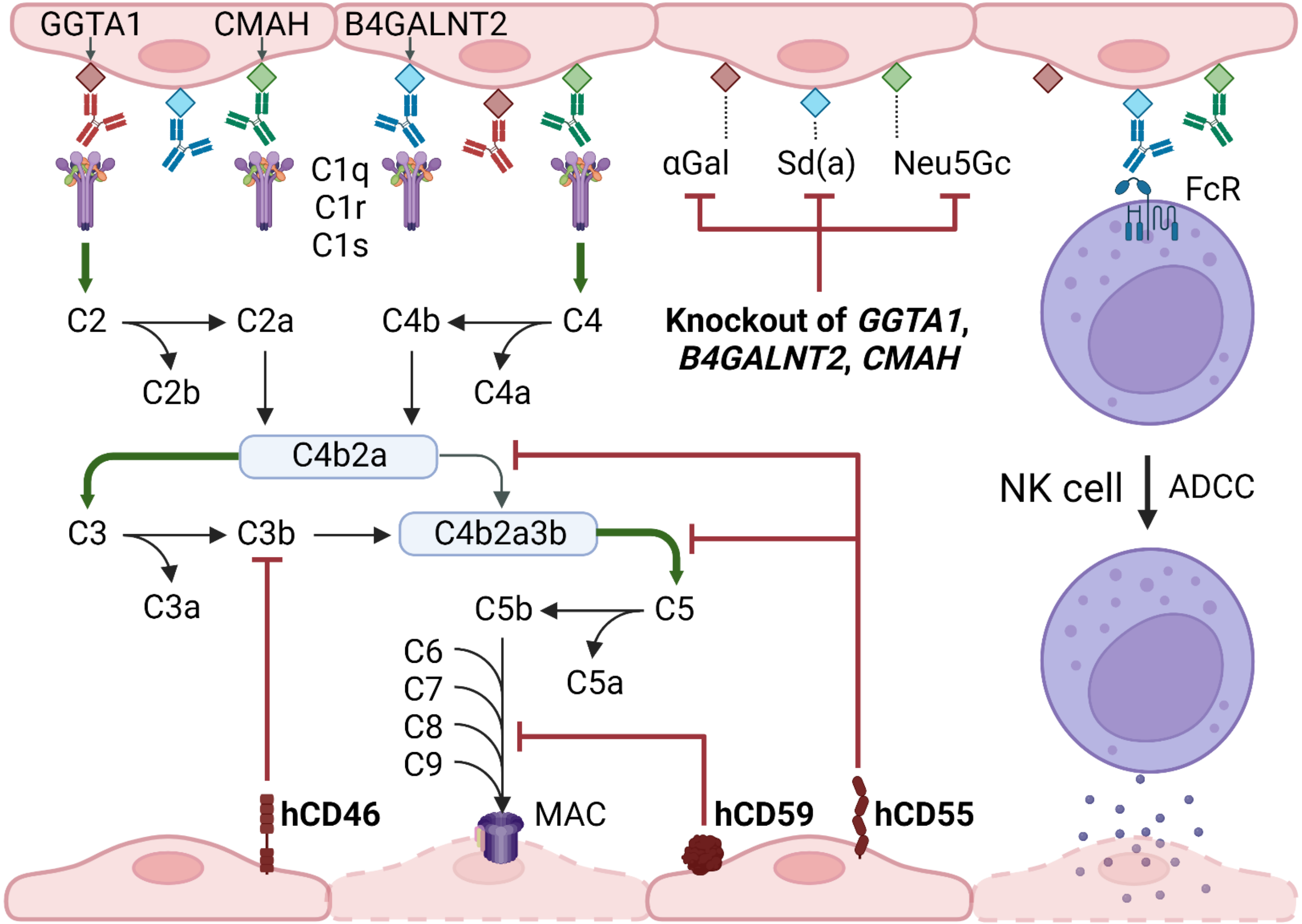




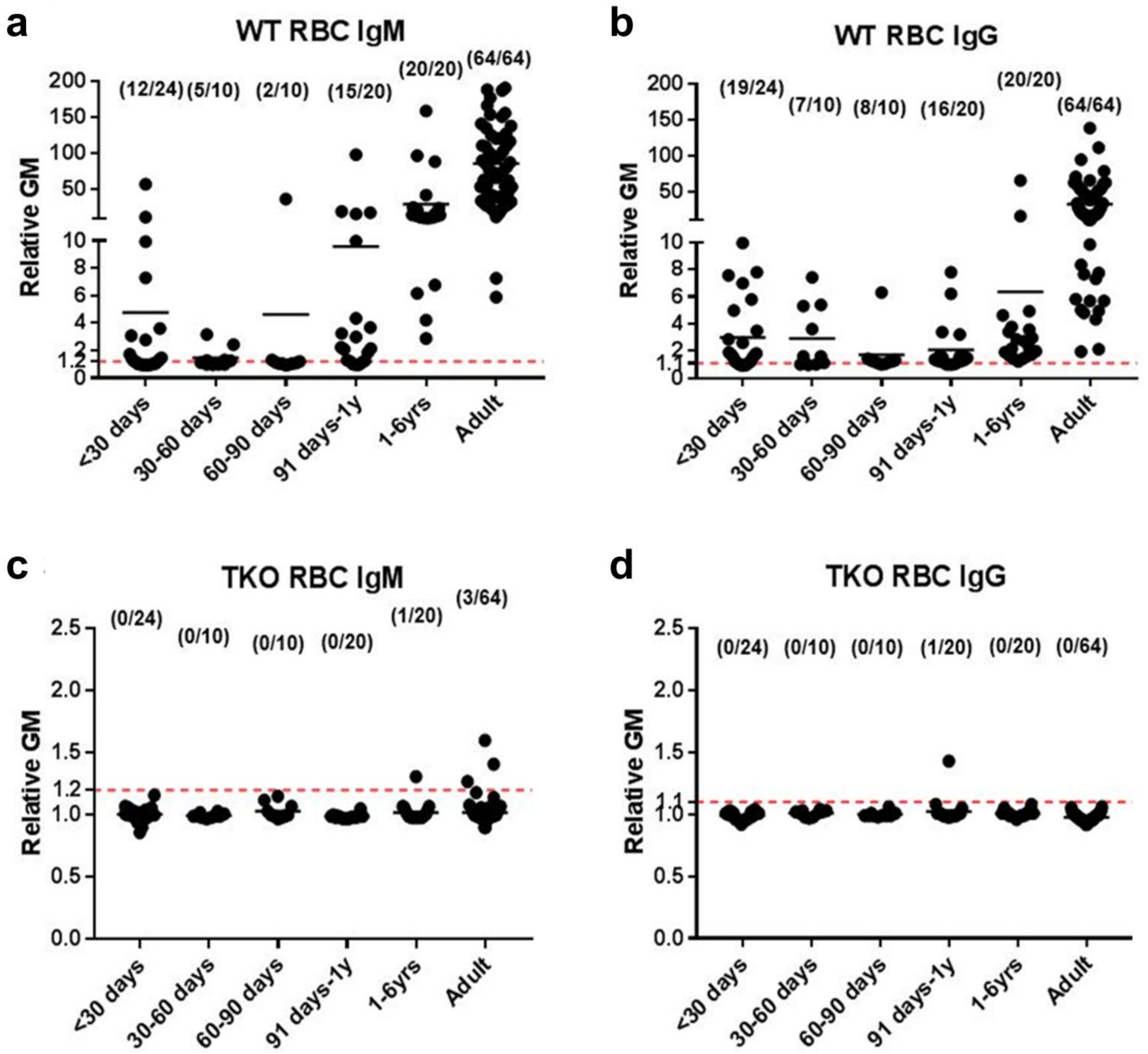
Ao et al., Placenta 57, 94-101 (2017)

Strategies to overcome hyperacute pig-to-human xenotransplant rejection



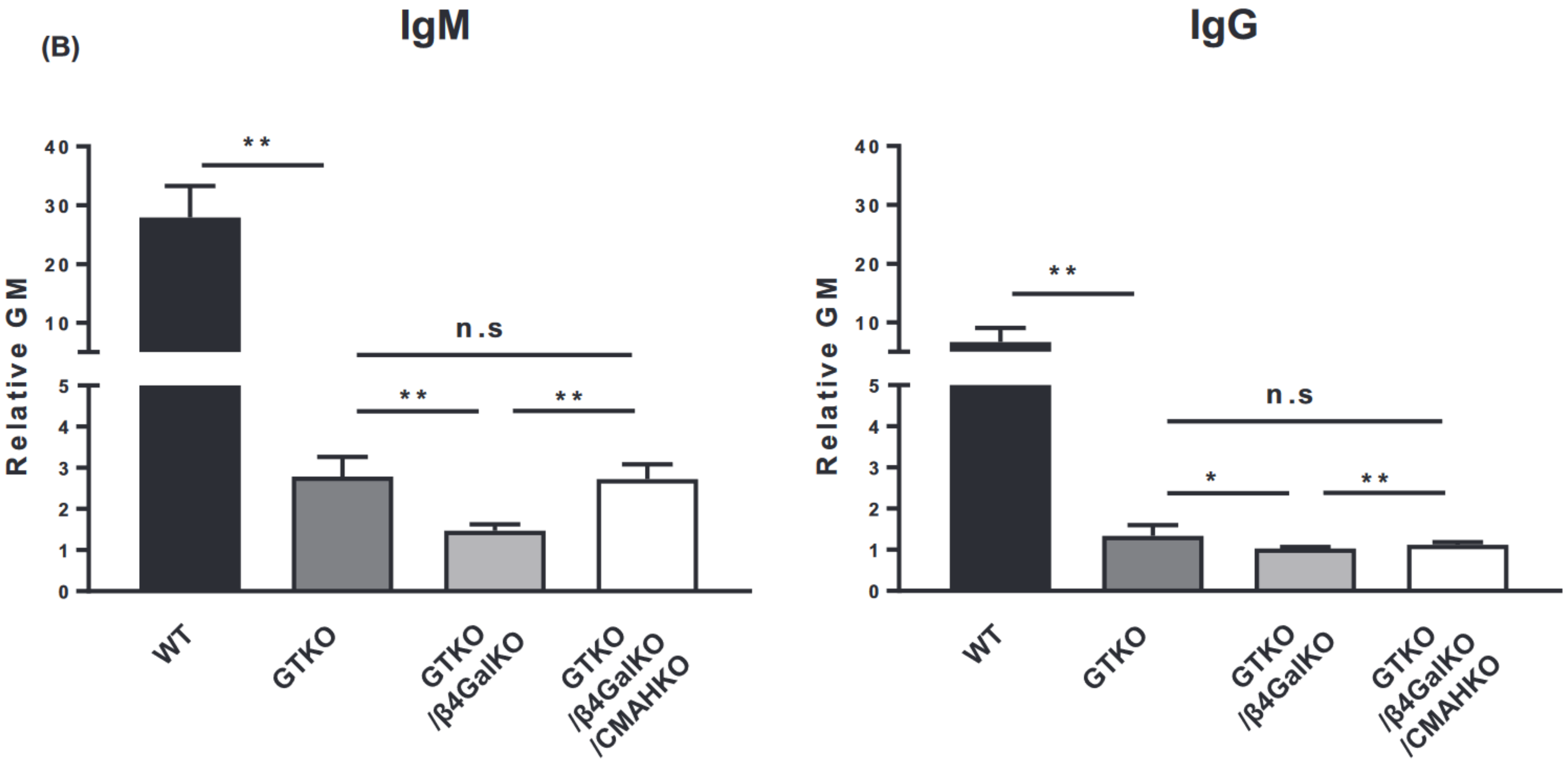


Geometric mean (GM) binding and age correlation of human serum IgM and IgG antibodies to WT and TKO pig red blood cells (RBC)



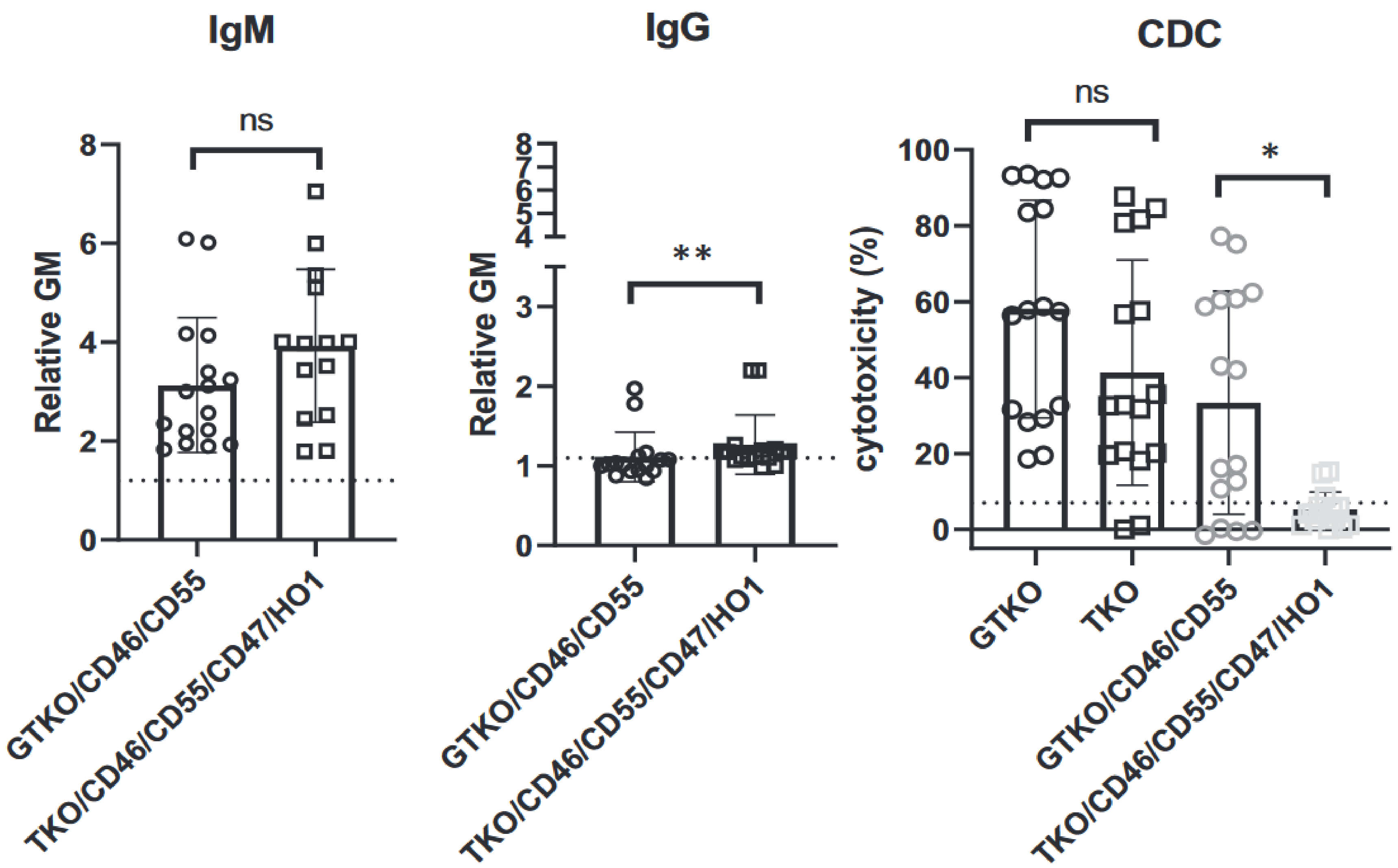
Li et al., Ann Thorac Surg 109, 1268-1273 (2020)

Deletion of Neu5Gc (CMAH-KO) in pig cells appears to expose a 4th xenoantigen against which baboons have natural antibodies



Yamamoto et al., Xenotransplantation 27, e12596 (2020)

Expression of additional protective human transgenes reduces complement-dependent cytotoxicity (CDC) against TKO pig cells



Yamamoto et al., Xenotransplantation 28, e12658 (2021)

Kidney transplantation from triple-knockout pigs expressing multiple human proteins in cynomolgus macaques

Gene	TKO-A	TKO-B
GGTA1	KO	KO
CMAH	KO	KO
B4GALNT2	KO	KO
CD46	Low	High
CD55	Low	High
CD59	Low	High
HLA-E/B2M	High	Mod
CD47	High	Mod
PDL1	High	—

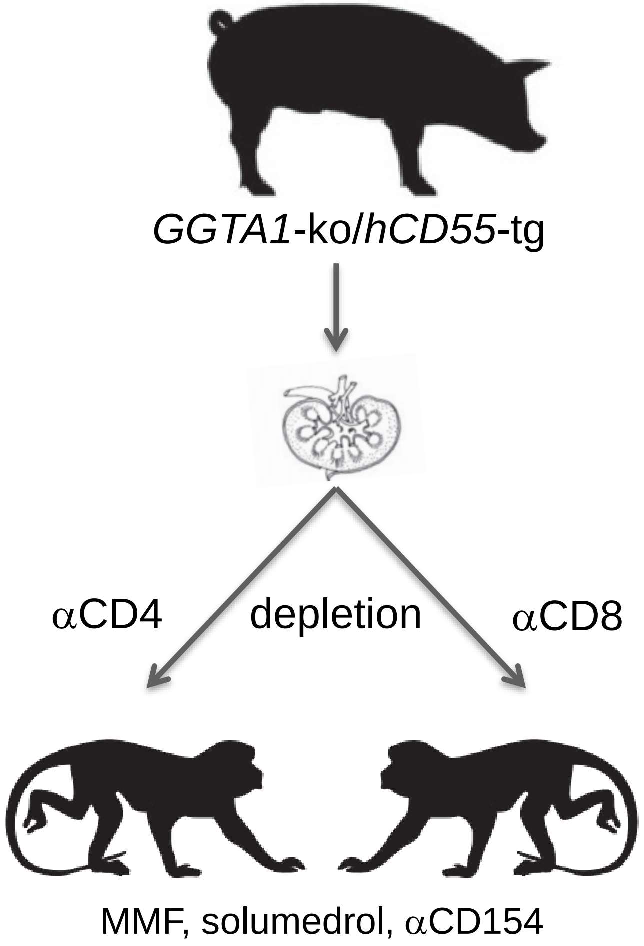
Survival time

TKO-A
2 and 61 days

TKO-B
15, 20, 71, 135, 265, and 316 days

Ma et al., Am J Transplant 22, 46-57 (2022)

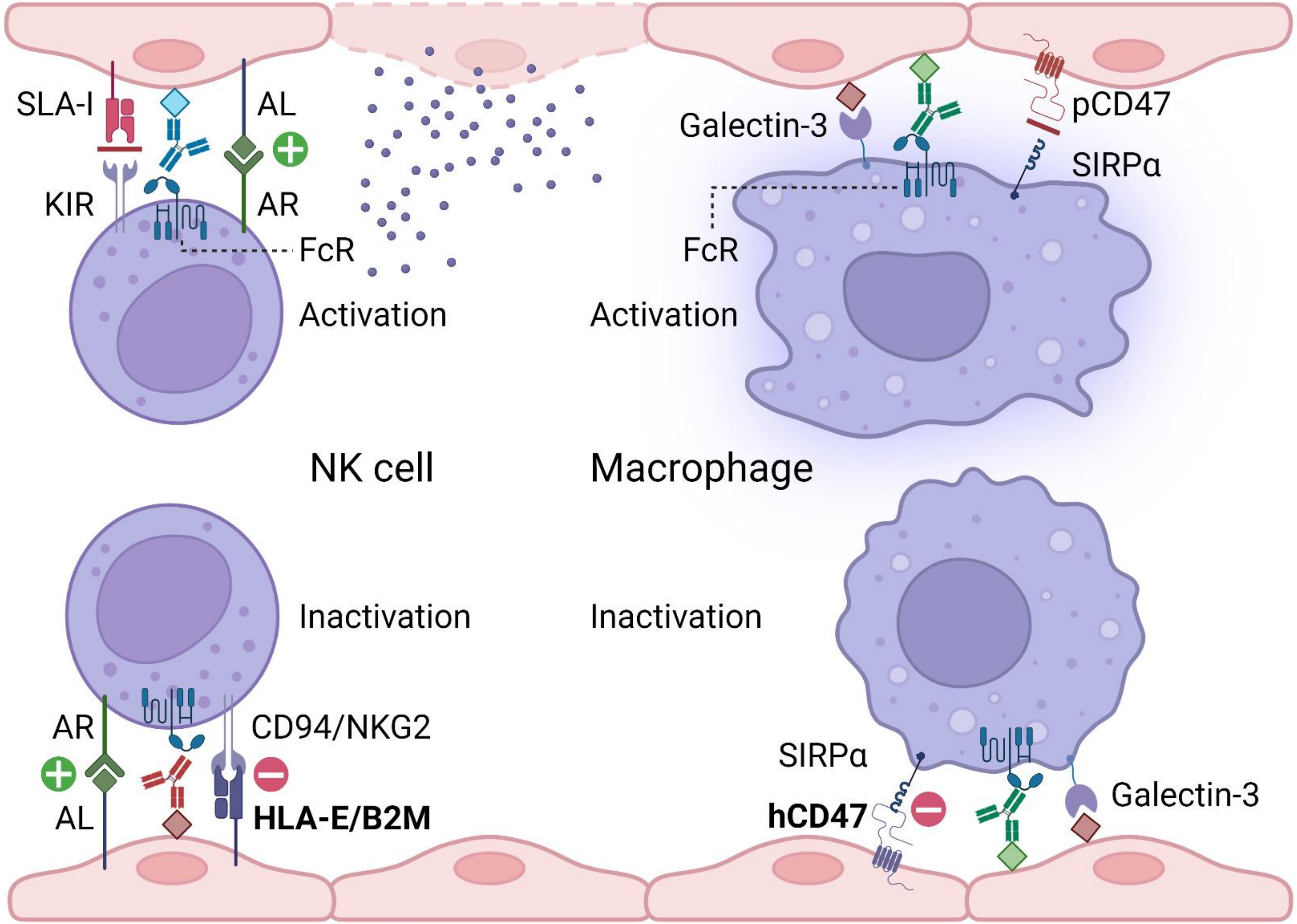
Long-term survival of pig-to-rhesus macaque kidney xenografts after CD4$^+$ T cell depletion

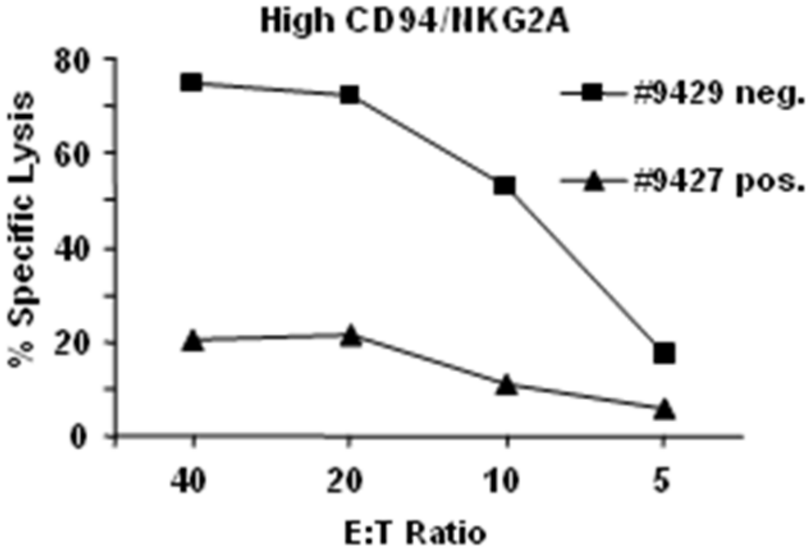


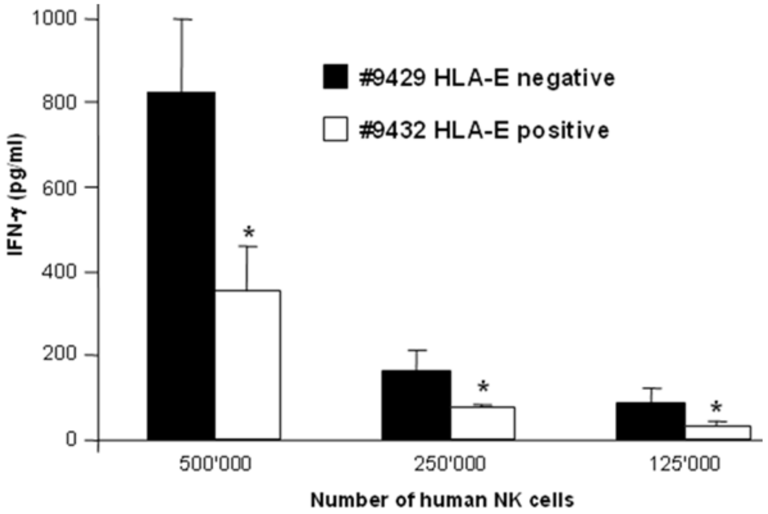
Treatment group	Donor ID	Donor weight (kg)	Recipient weight (kg)	Recipient pretrans-plant IgG mean fluorescent intensity (MFI)	Cold ischemia time (min)	Survival (d)
High titer	D1	16.8	3.60	8983	195	6
αCD4$^+$αCD8$^+$ anti-CD154	D1	16.8	3.65	2960	90	310
	D2	15.0	3.20	1041	93	160
	D3	25.5	4.42	1699	45	406
			3.70	1049	147	18
	D4	35.8	4.04	2273	51	115
			4.11	966	180	>400
αCD4$^+$ anti-CD154	D5	25.0	3.92	1195	200	499
	D6	30.6	4.07	1340	235	414
	D7	37.0	5.87	1446	62	>70
αCD8$^+$ anti-CD154	D5	25.0	5.18	1969	40	15
	D6	30.6	4.41	2159	150	6
	D7	37.0	8.58	979	132	6

Kim et al., Am J Transplant 19, 2174-2185 (2019)

Strategies to overcome rejection by NK cells and macrophages

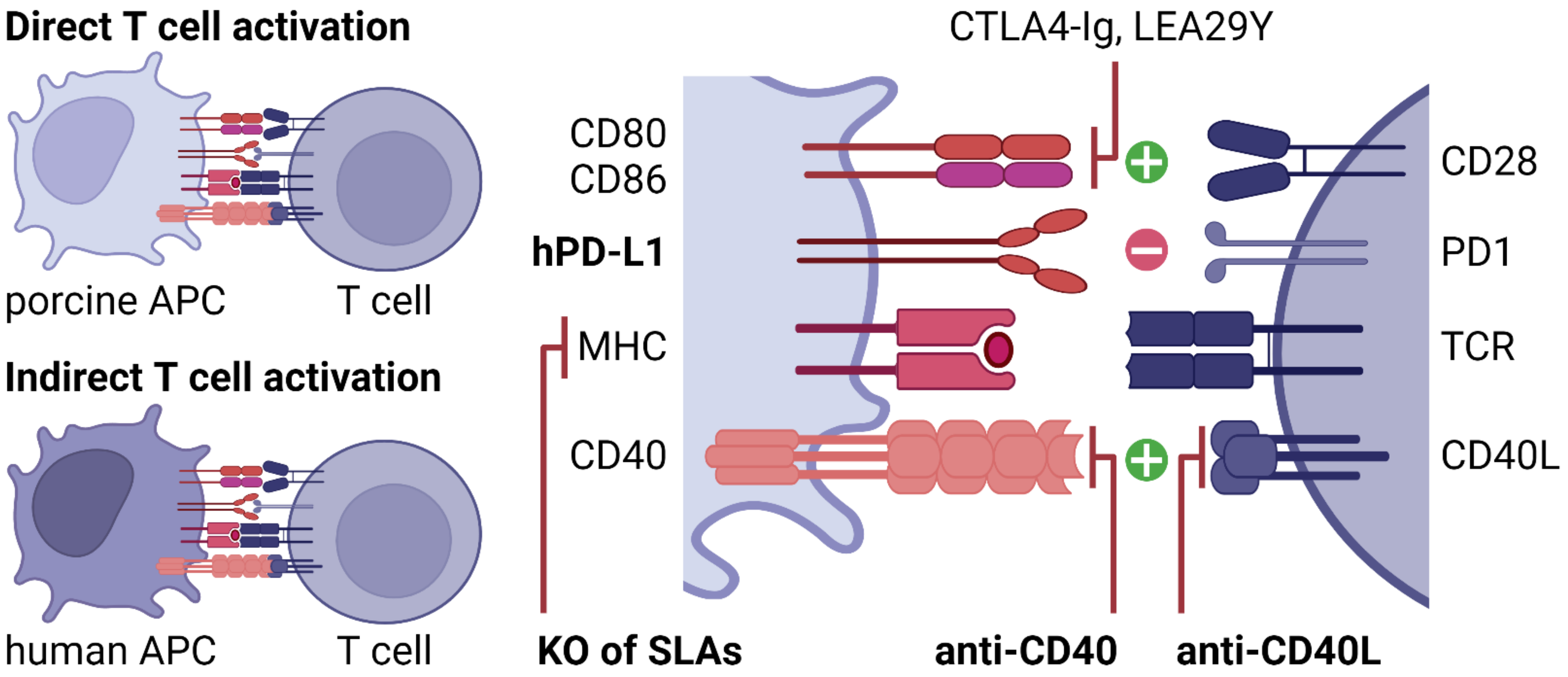




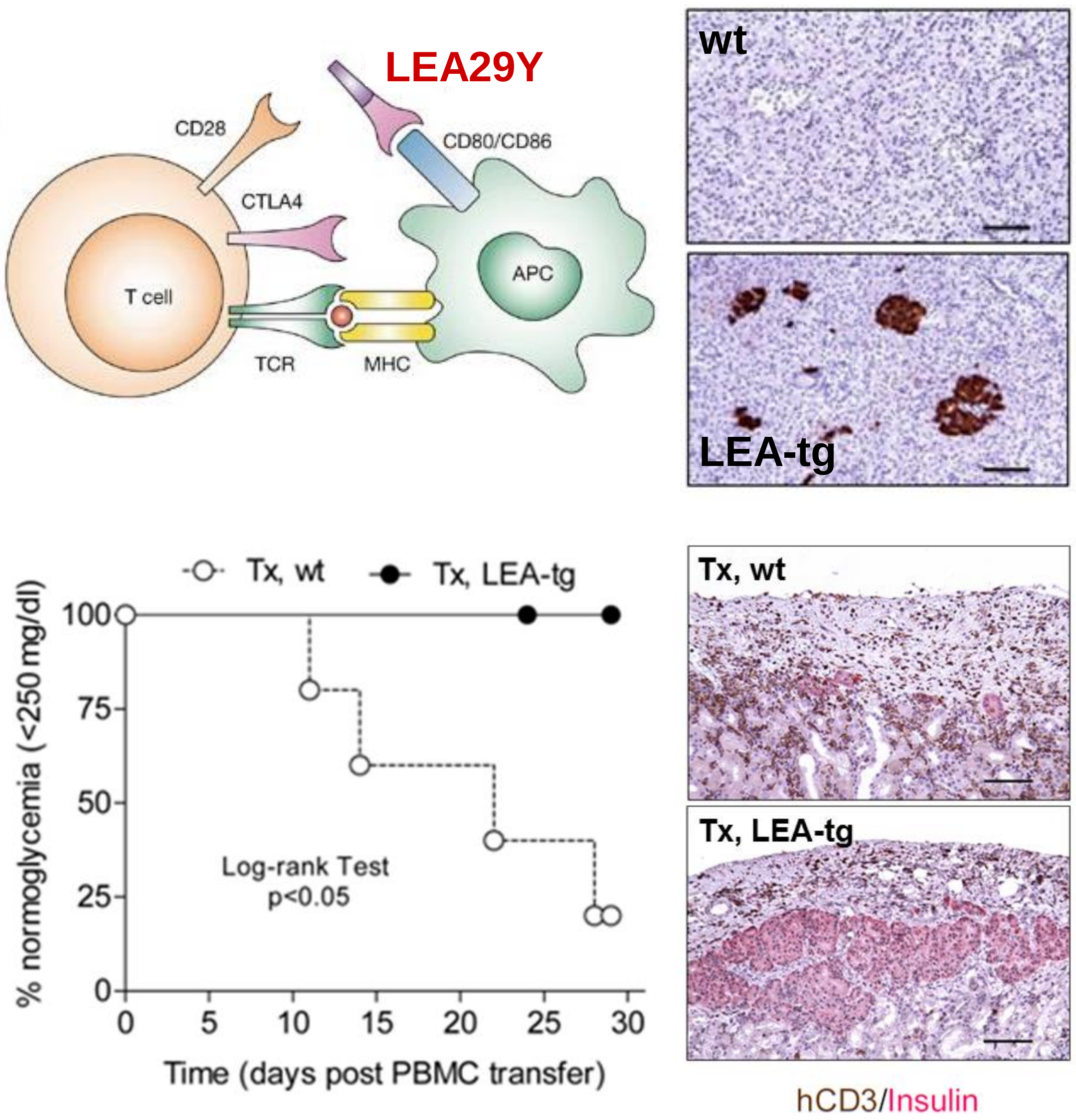


Weiss et al., Transplantation 87, 35–43 (2009)

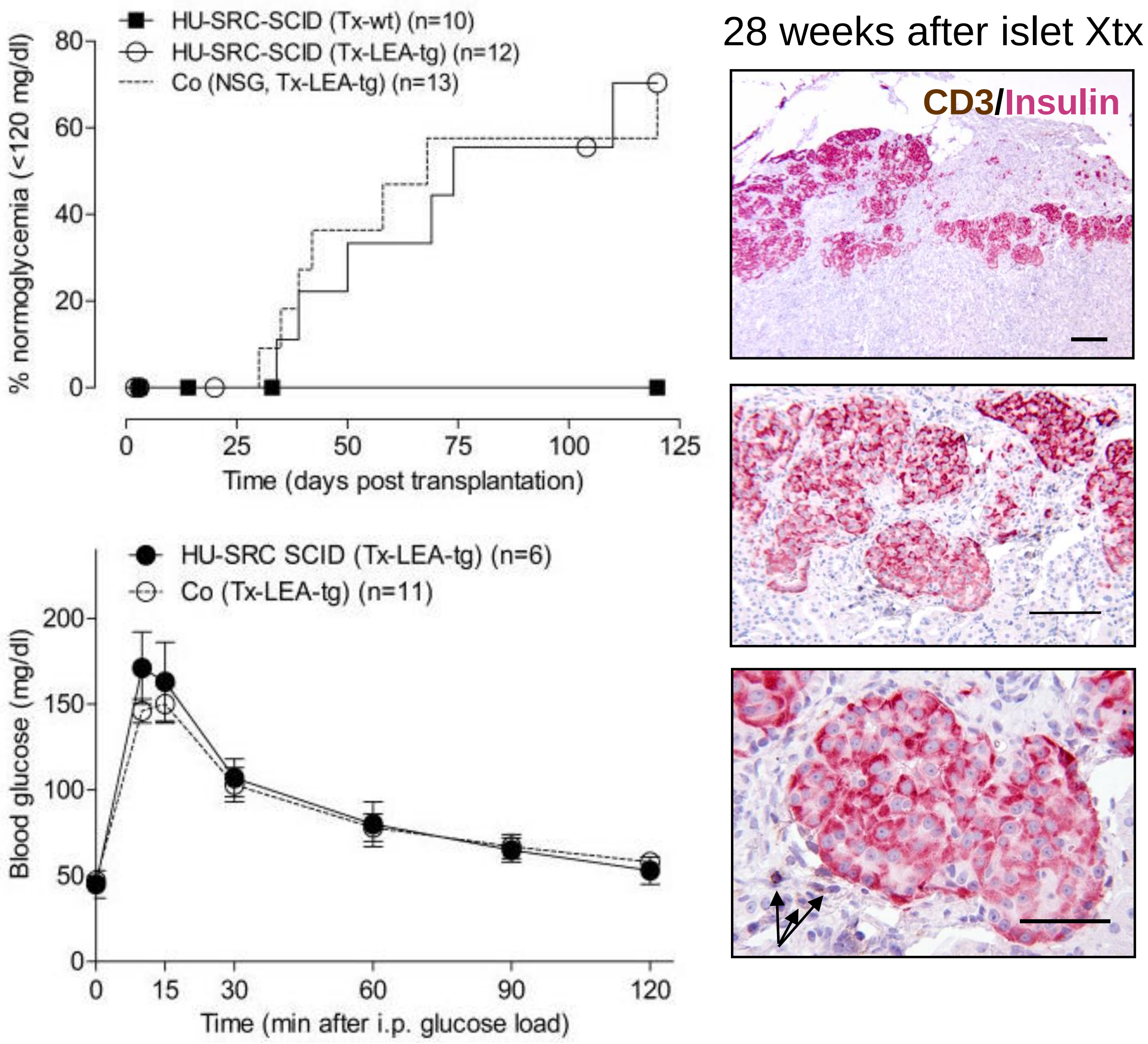
Strategies to overcome pig-to-human xenotransplant rejection



Protection of porcine xeno-islets by local expression of LEA29Y

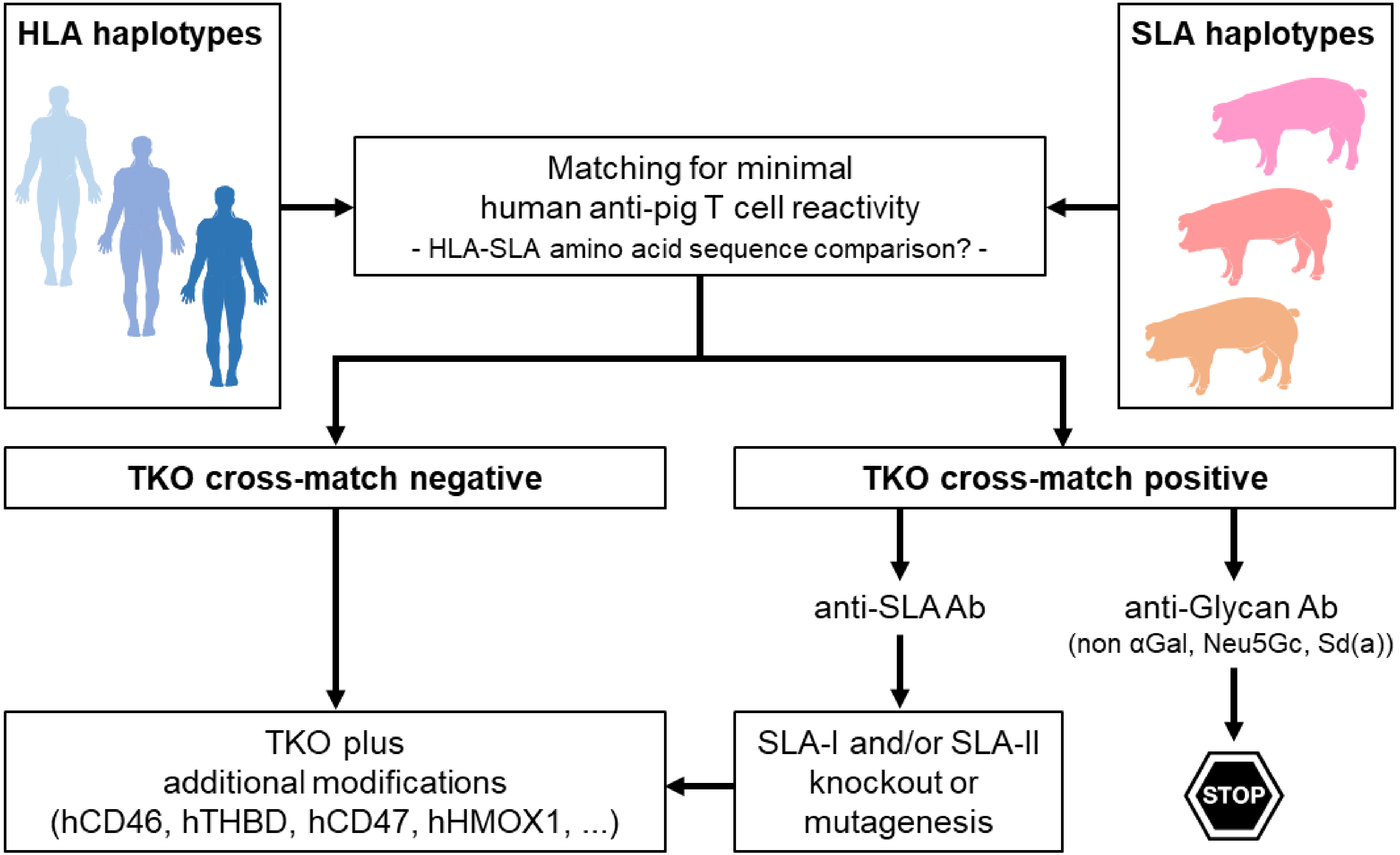


Klymiuk et al., Diabetes 61, 1527-1532 (2012)



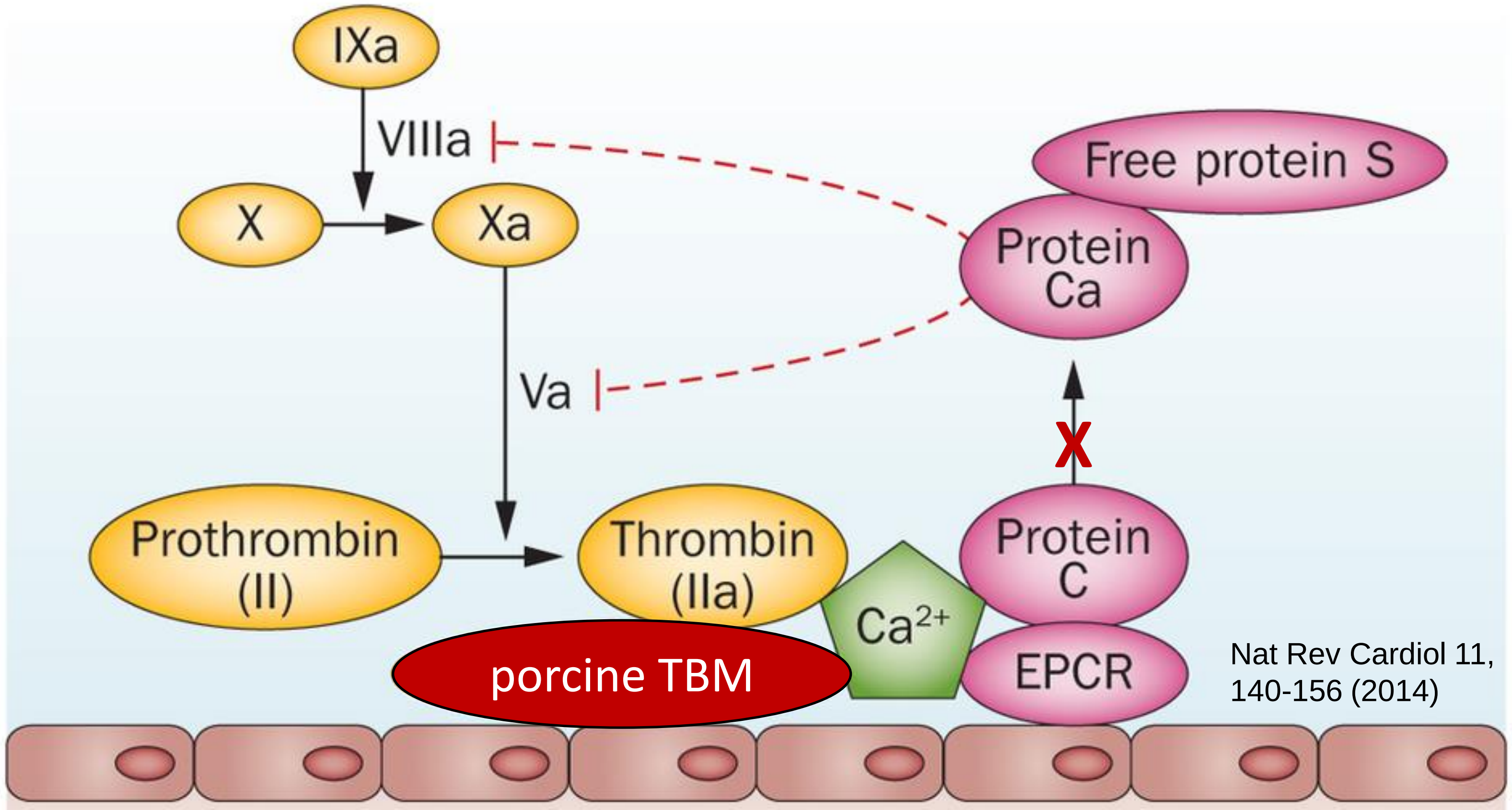
Wolf-van Bürck et al., Sci Rep 7, 3572 (2017)

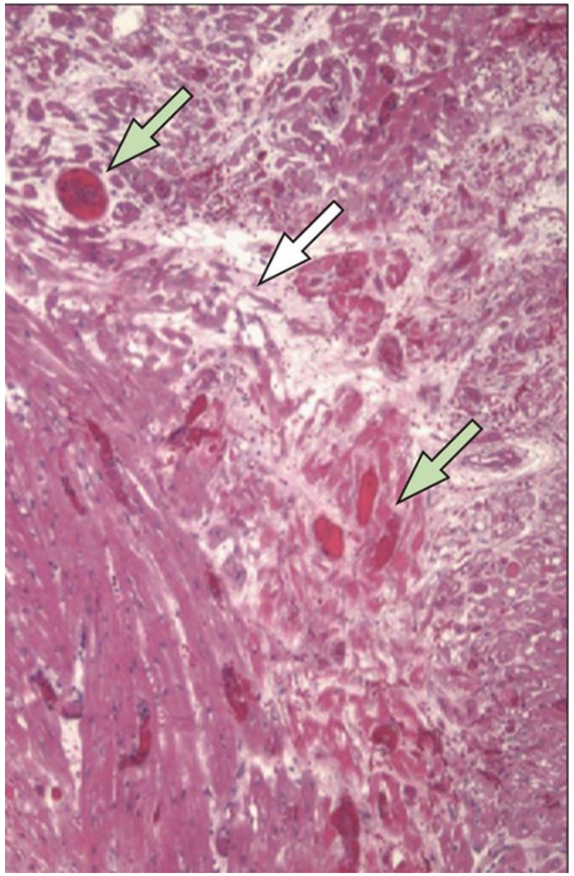
The role of SLAs in xenotransplantation



Reichart et al., Transplantation 105, 1930-1943 (2021)

Xenogeneic coagulation disorder

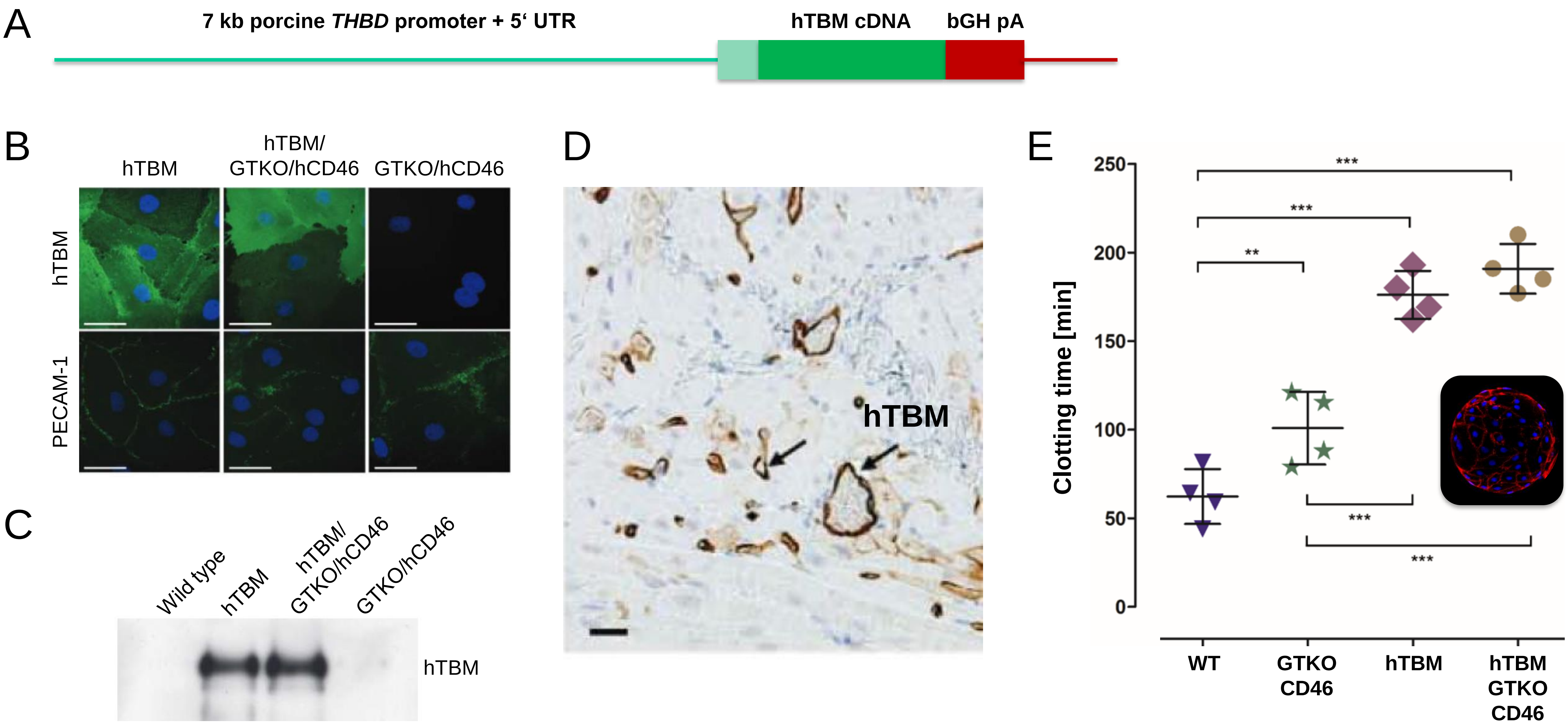




Porcine thrombomodulin binds human thrombin, but is a poor cofactor for the activation of human protein C

Cowan & d'Apice, Immunology and Cell Biology 87, 203–208 (2009)

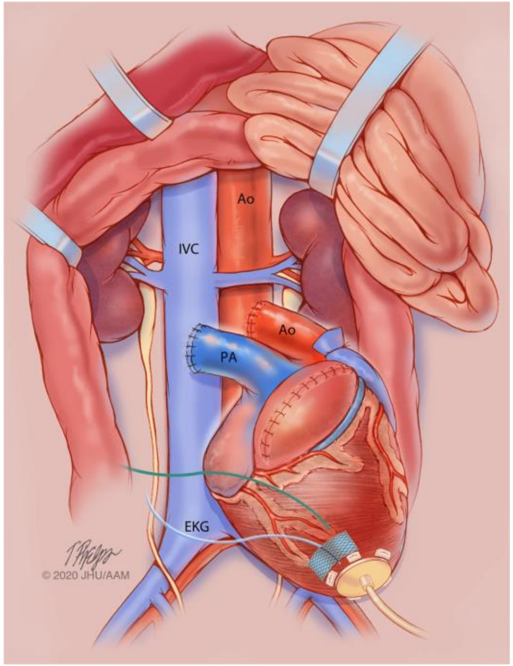
Human thrombomodulin expression in transgenic pigs



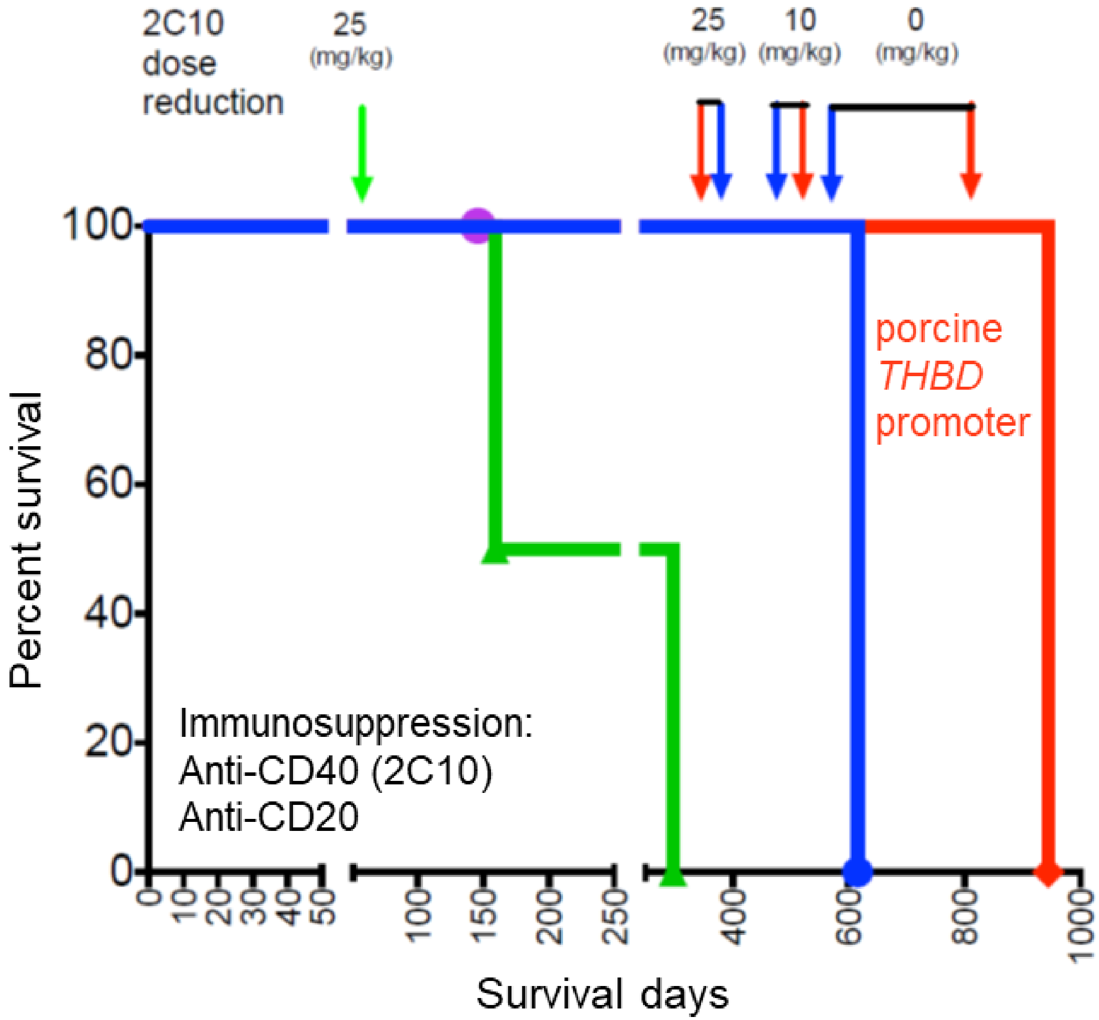
Wuensch et al., Transplantation 97, 138-147 (2014)

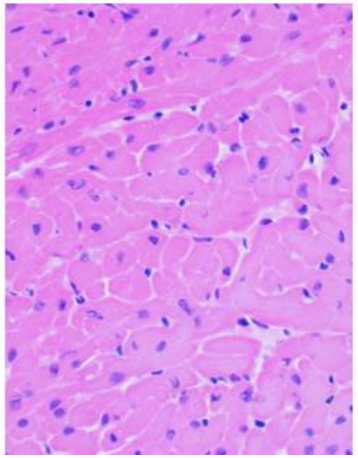
Heterotopic abdominal cardiac xenotransplantation in baboons

https://www.nature.com/articles/s41598-020-66430-x



GGTA1 knockout
hCD46 transgenic
hTHBD transgenic





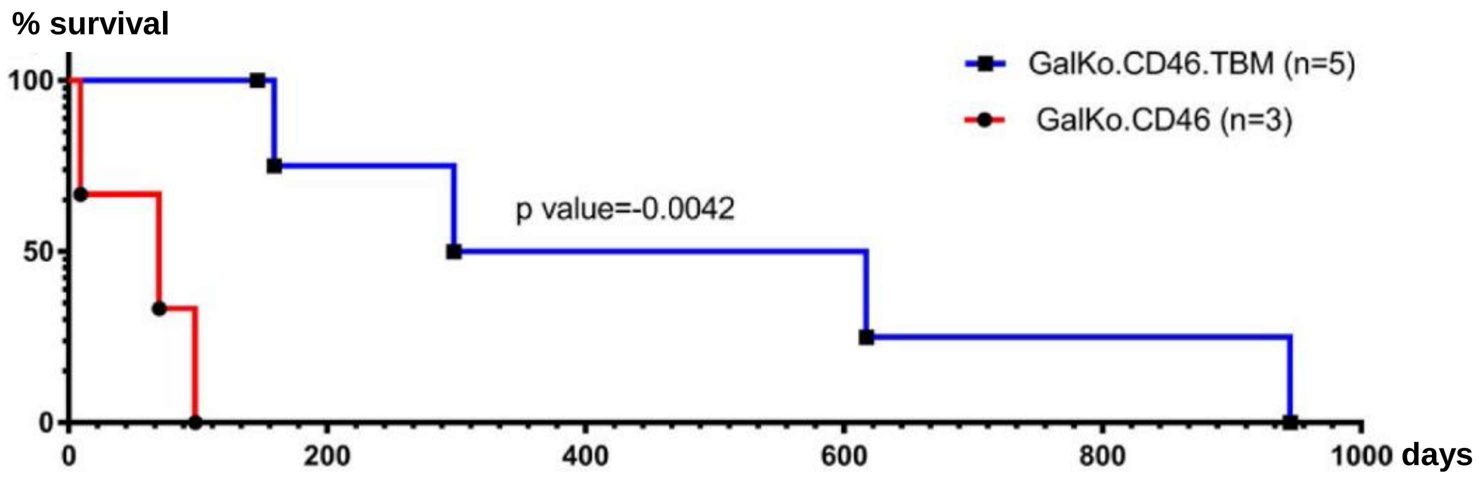
Biopsy day 640

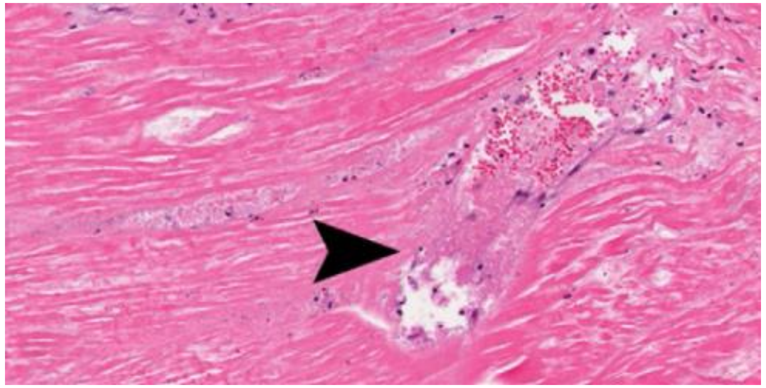
Mohiuddin et al., Nat Commun 7, 11138 (2016)

Expression of human thrombomodulin was key to success

Cardiac xenografts show reduced survival in the absence of transgenic human thrombomodulin expression in donor pigs

- Results from a preclinical pig-to-baboon heterotopic cardiac xenotransplantation model suggest that a three-pronged approach is successful in extending xenograft survival:
  (a) α-1,3-galactosyl transferase gene knockout pigs (GTKO) to prevent Gal-specific antibody-mediated rejection;
  (b) transgenic expression of hCD46 and hTBM to avoid complement activation and coagulation dysregulation; and
  (c) effective induction and maintenance of immunomodulation (co-stimulation blockade of CD40-CD40L pathways with anti-CD40 (2C10R4) monoclonal antibody (mAb).
- **Xenografts from pigs without hTBM expression (GTKO.CD46) underwent rejection at an early time point (median 70 days)** despite utilization of our previously reported successful immunosuppression regimen …

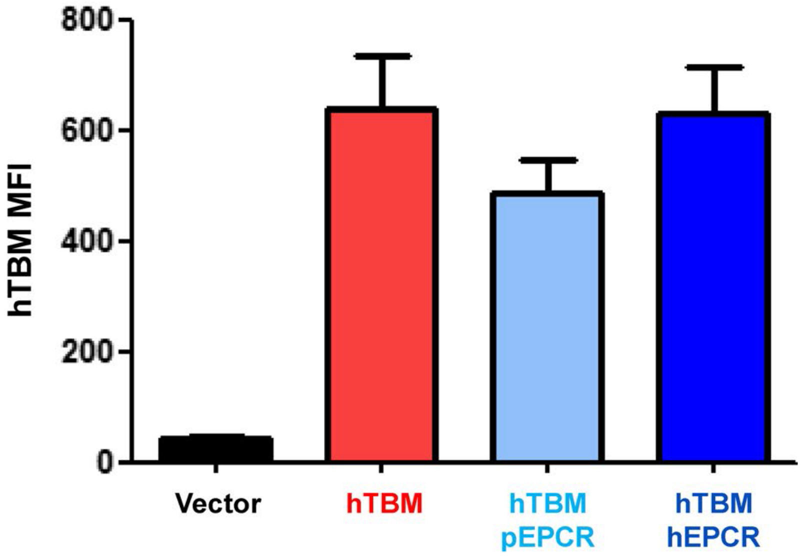


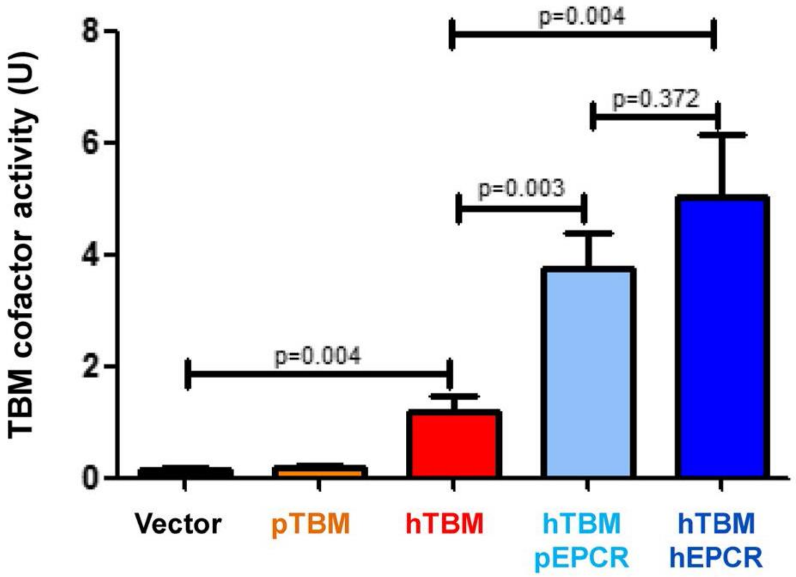


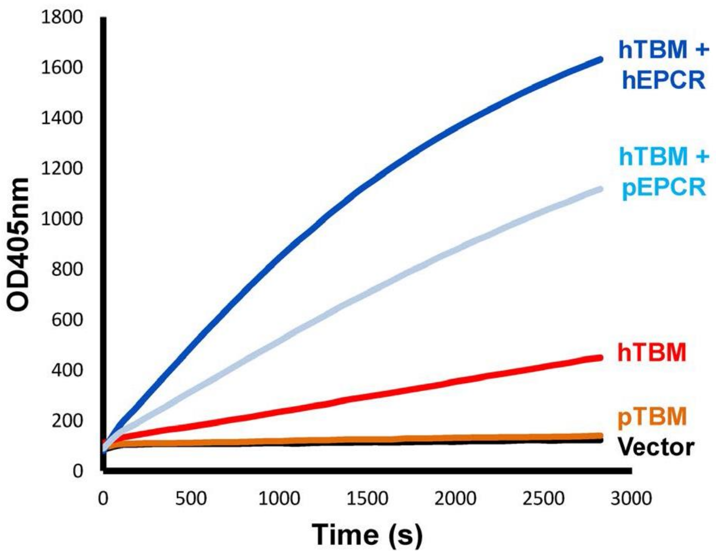
Singh et al., Xenotransplantation. 2018 Oct 5:e12465. doi: 10.1111/xen.12465.

Pig EPCR is functionally compatible with the human protein C pathway

Transient transfection experiments in primate COS-7 cells

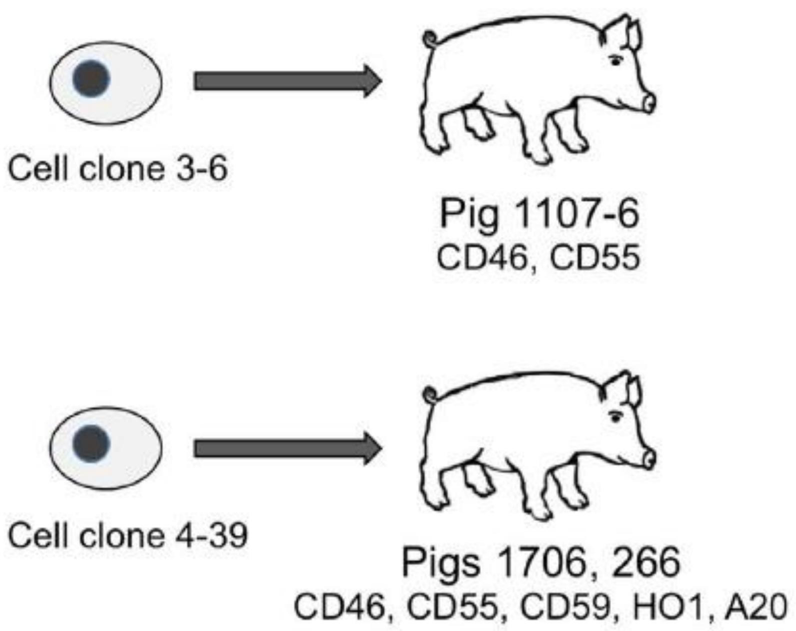


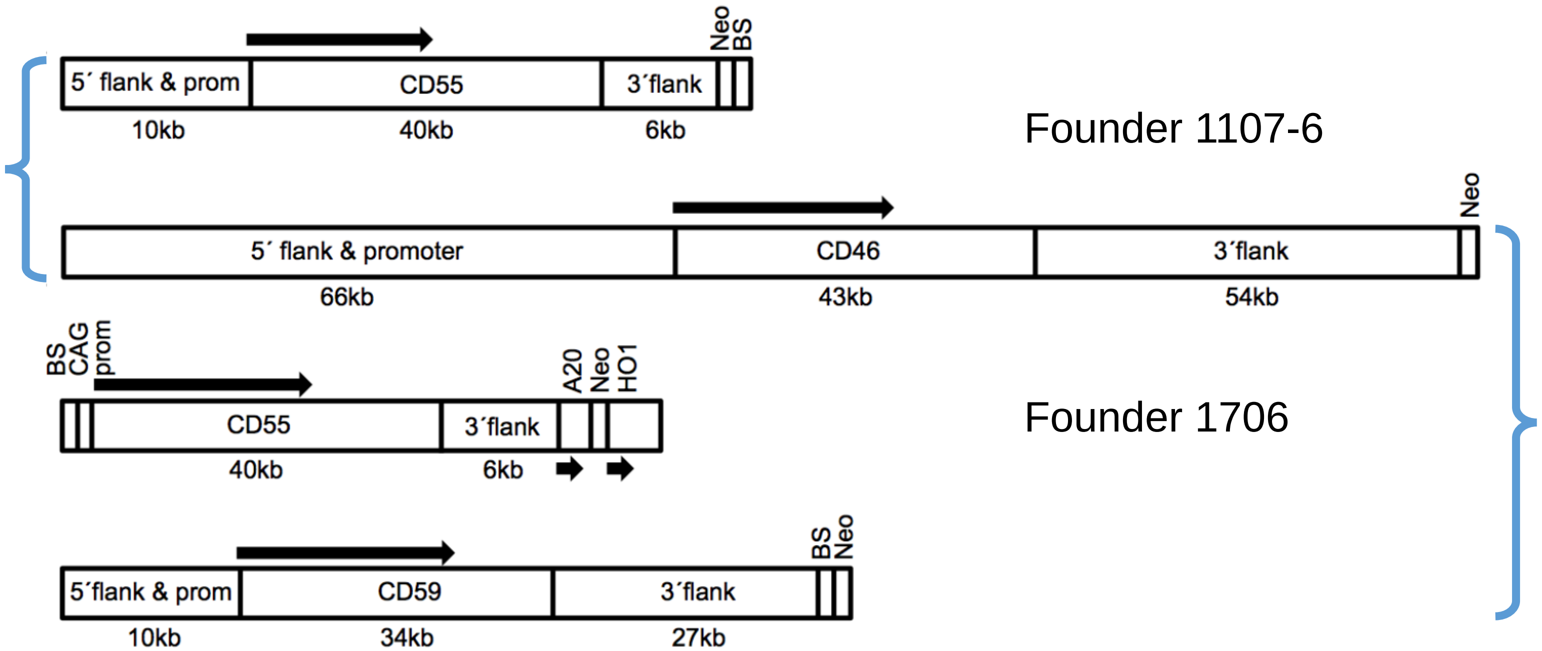


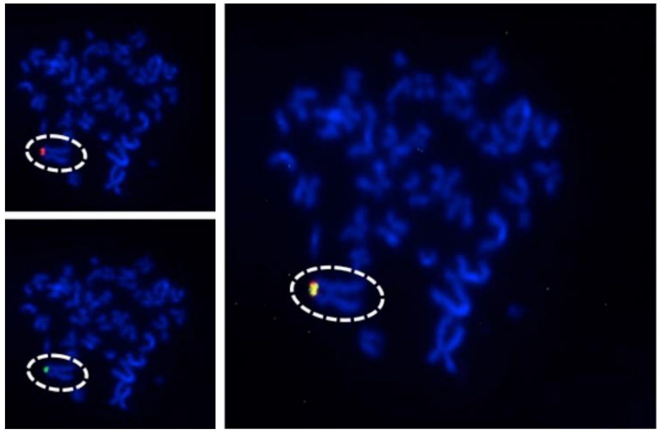


Salvaris et al., Xenotransplantation 27, e12557 (2020)

Efficient production of multi-modified pigs for xenotransplantation by transgene ‘combineering’



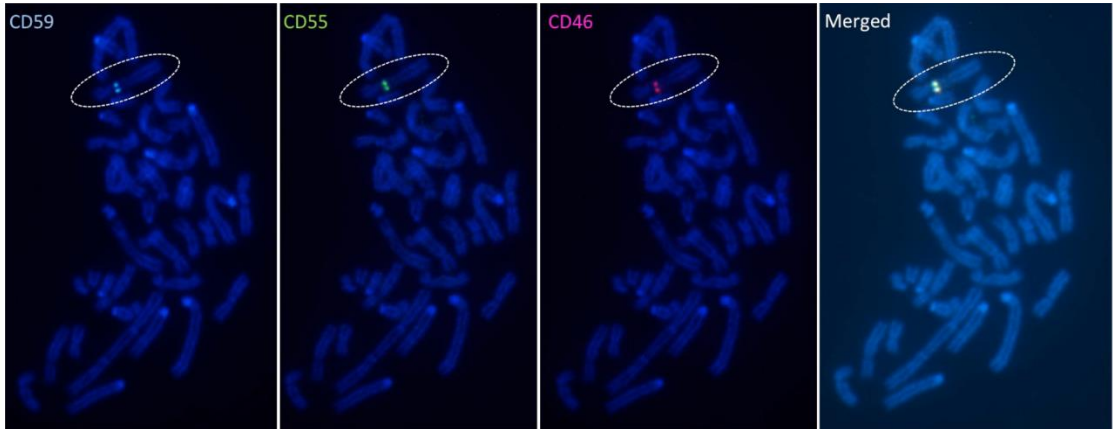




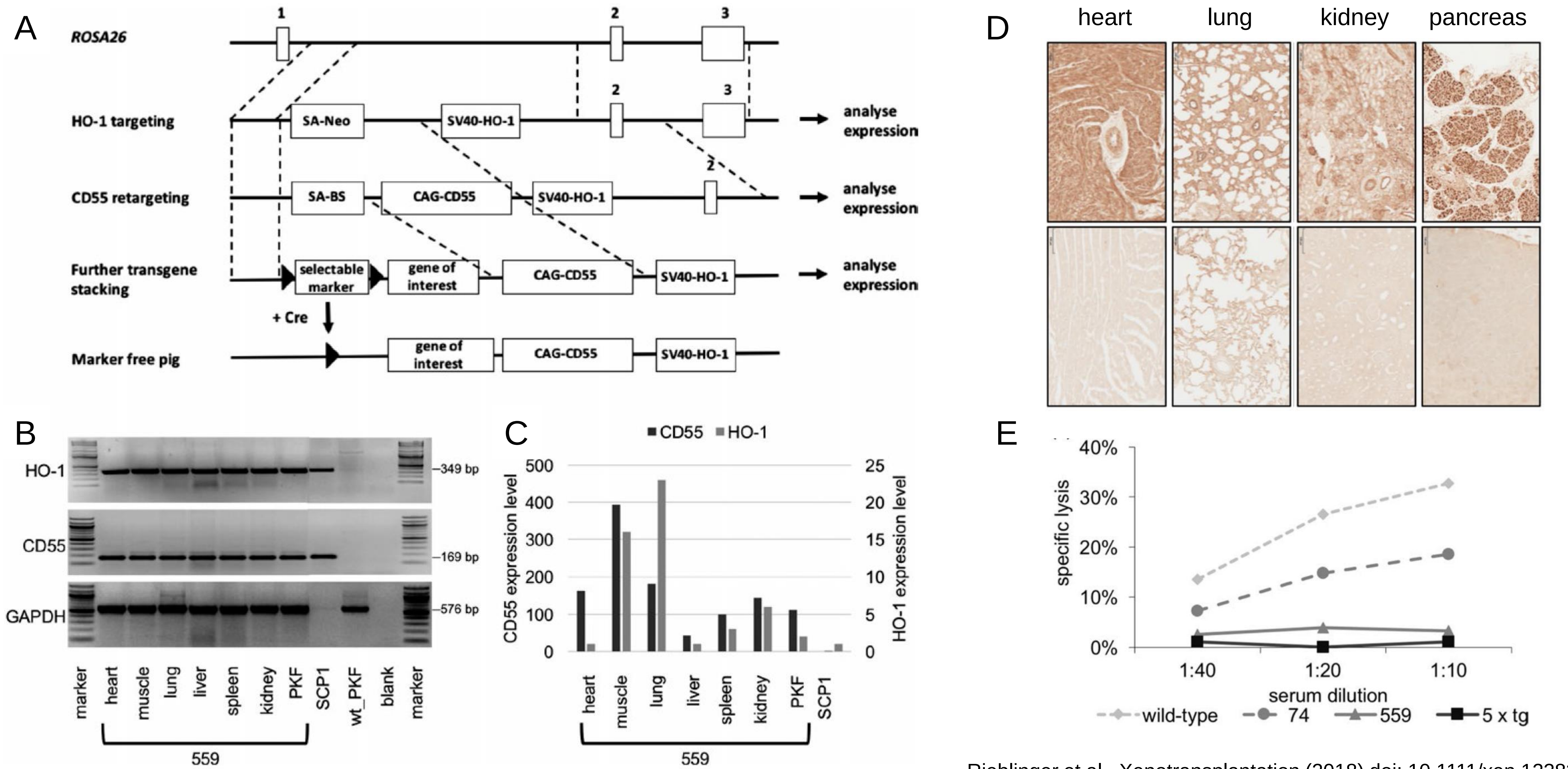
← Founder 1107-6

Founder 1706 →

Fischer et al.,
Sci Rep 6,
29081 (2016)

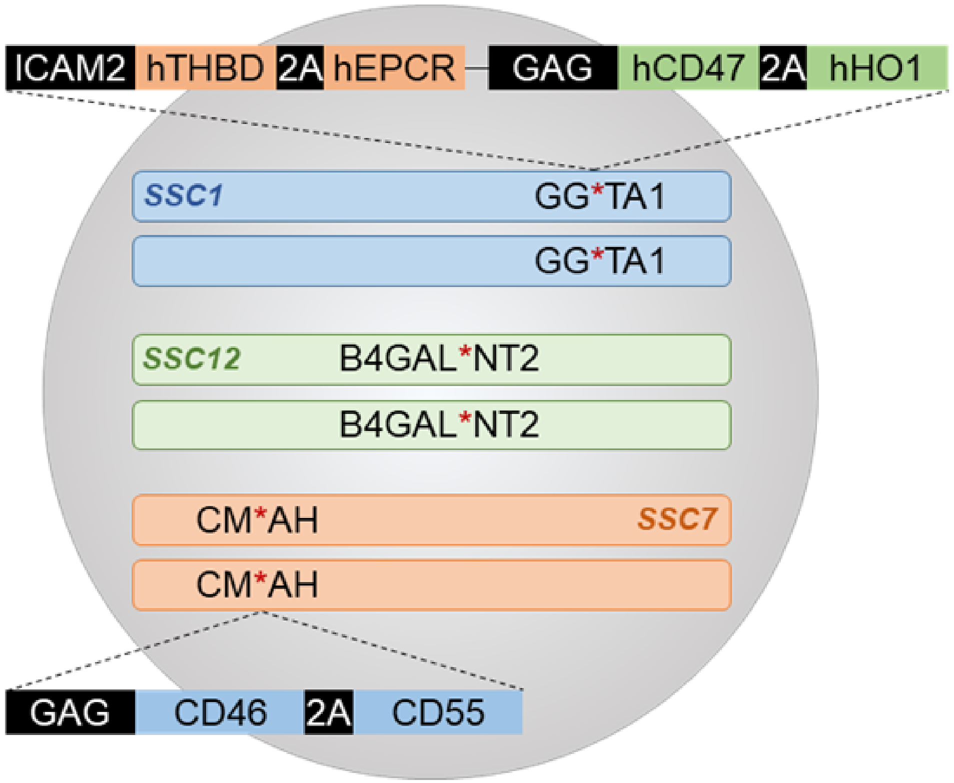


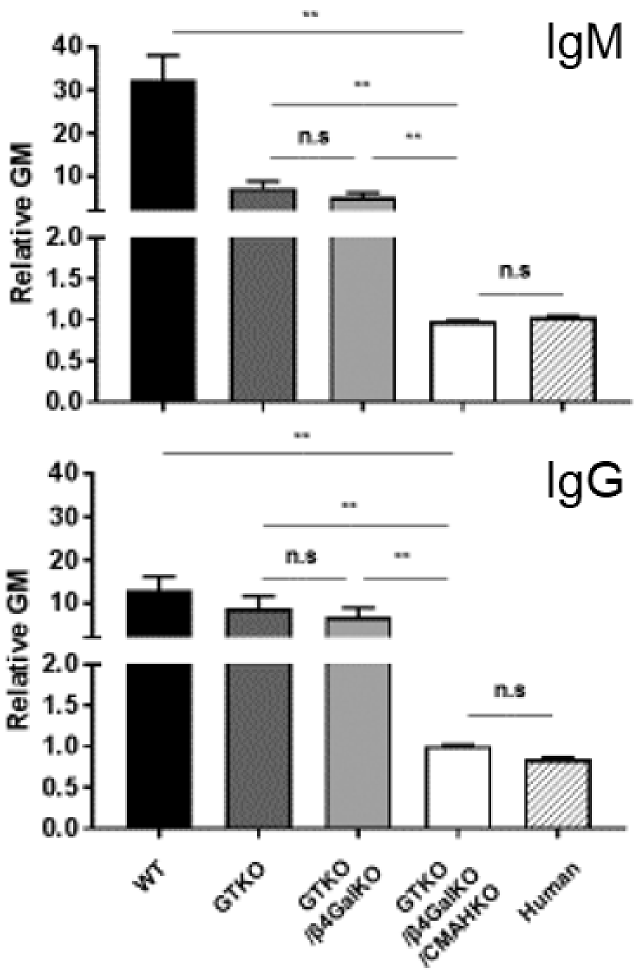
Transgene stacking into the *ROSA26* locus

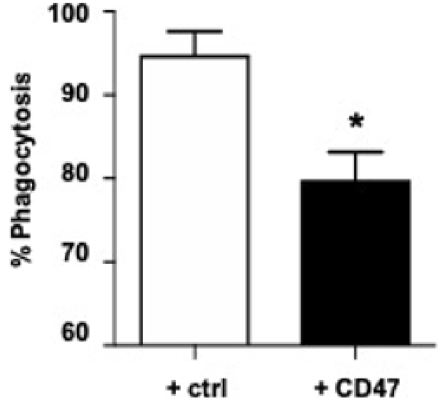


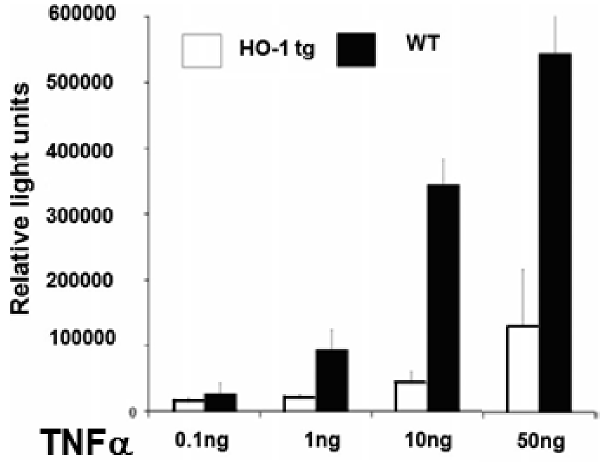
Rieblinger et al., Xenotransplantation (2018) doi: 10.1111/xen.12382

Nine genetic modifications suggested for xeno-organ donor pigs



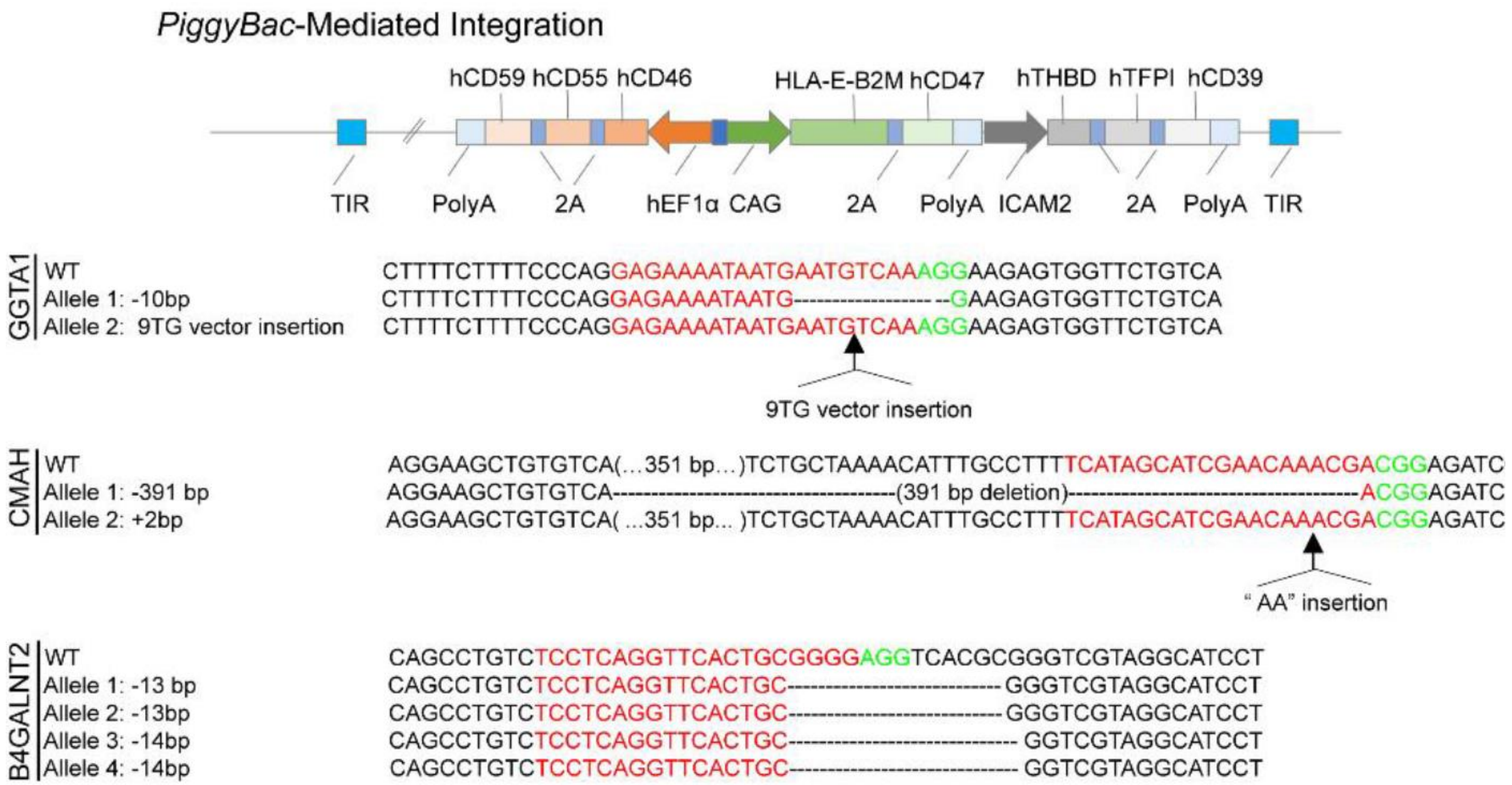


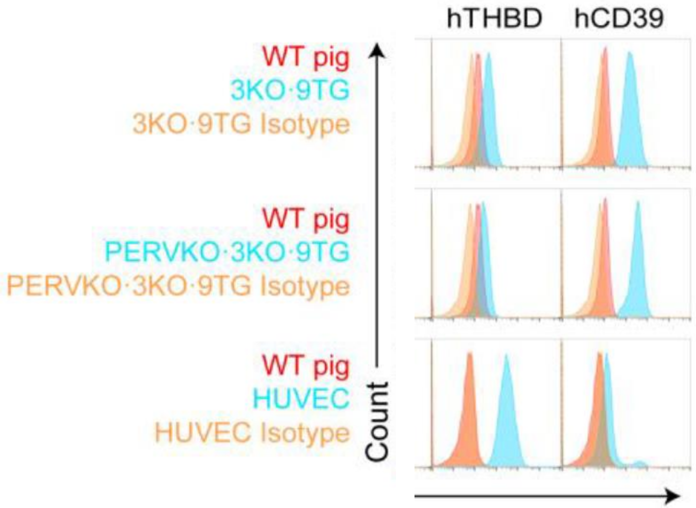




Cooper et al., Xenotransplantation 26, e12516 (2019)

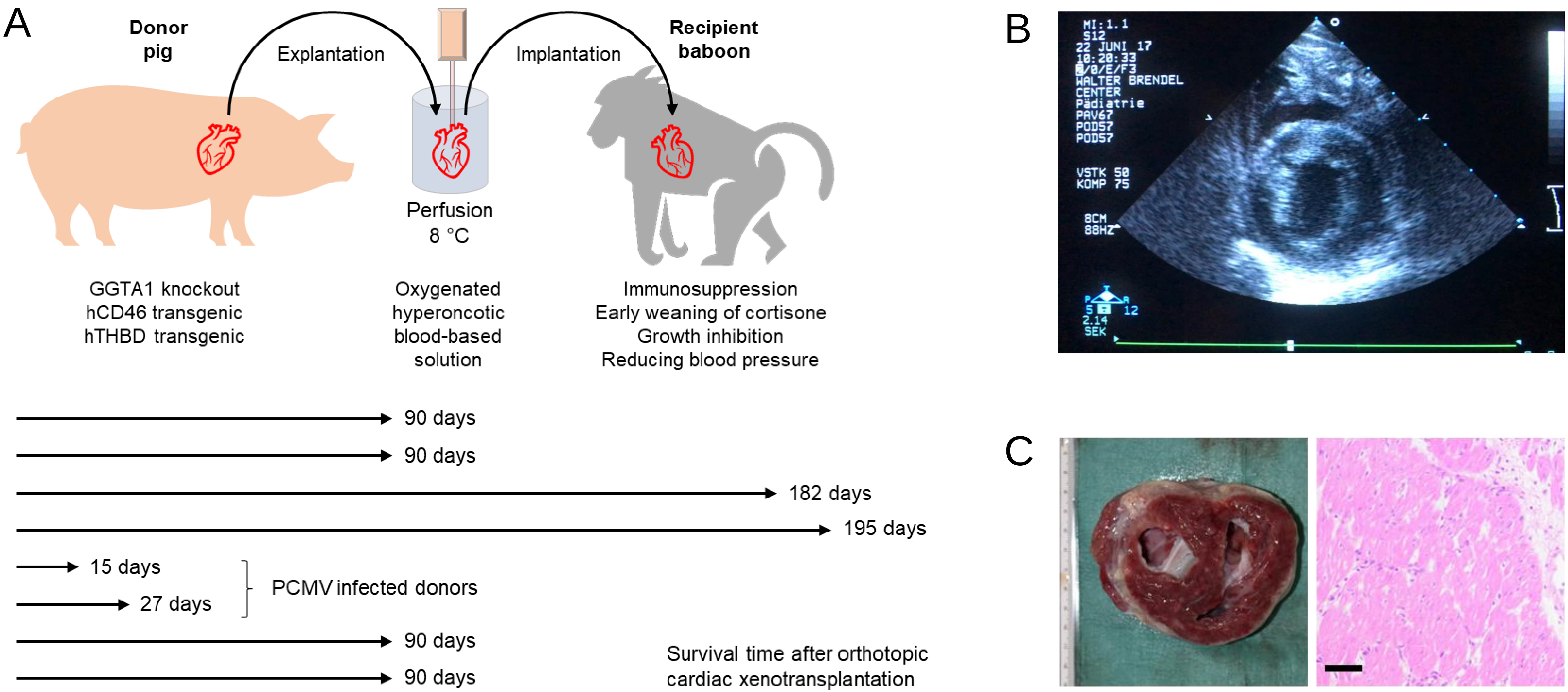
Extensive pig germline genome engineering





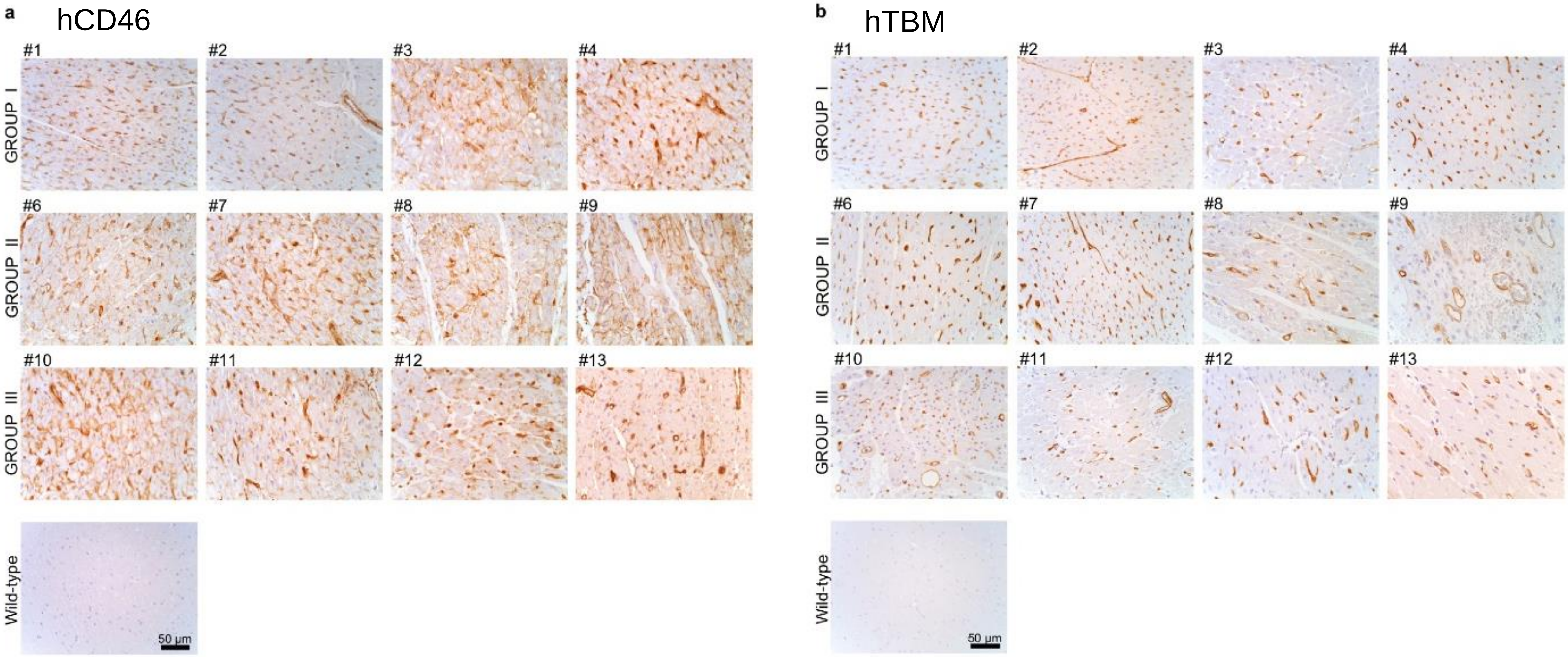
Yue et al., Nat Biomed Eng 5, 134-143 (2021)

Consistent success in life-supporting cardiac xenotransplantation



Längin et al., Nature 564, 430-433 (2018); Reichart et al., J Heart Lung Transplant 39, 751-757 (2020); Reichart et al., Transplantation 105, 1930-1943 (2021)

Consistency of transgene expression (level, localization)

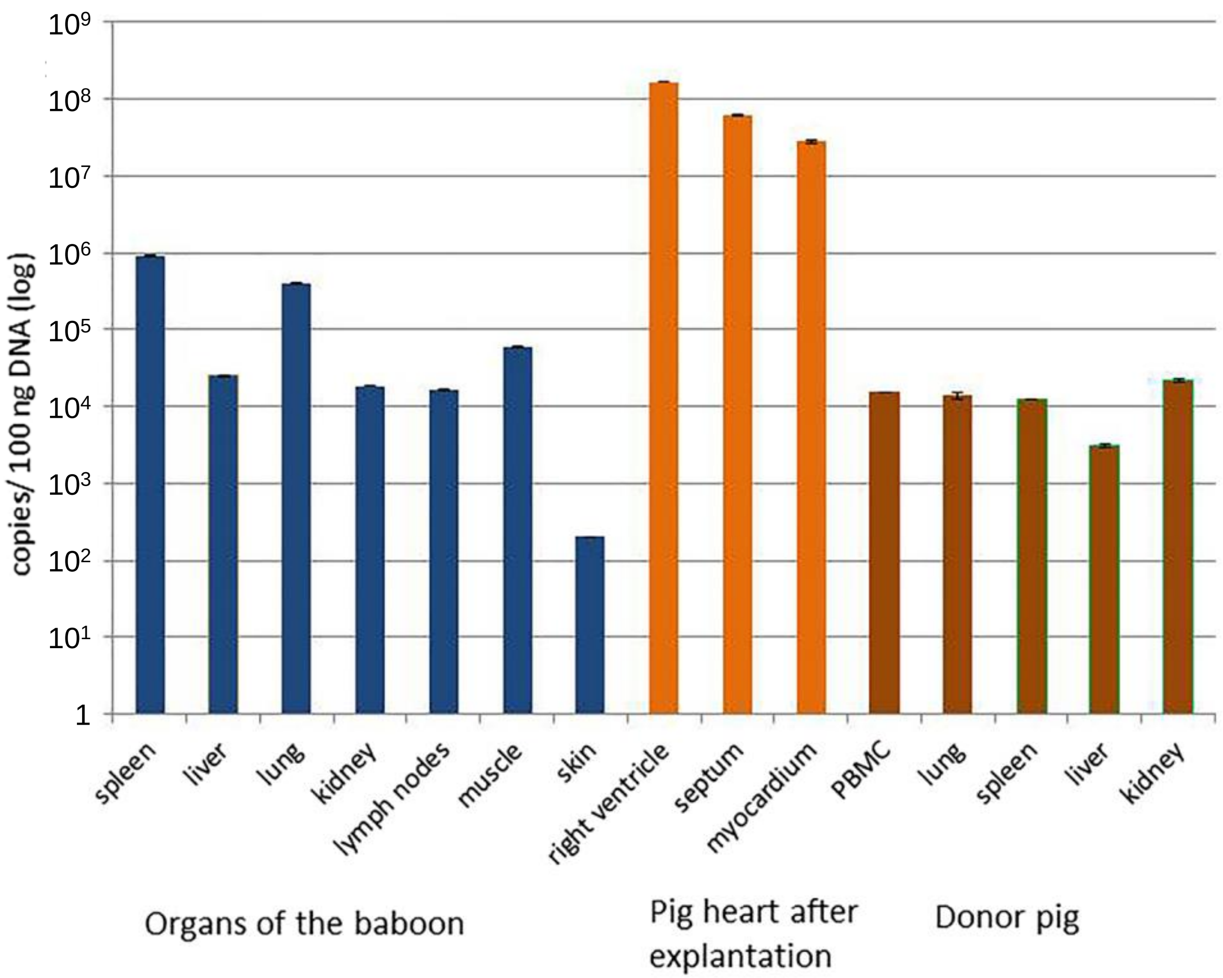


Längin, Mayr, Reichart et al., Nature. 2018 Dec 5. doi: 10.1038/s41586-018-0765-z. [Epub ahead of print]

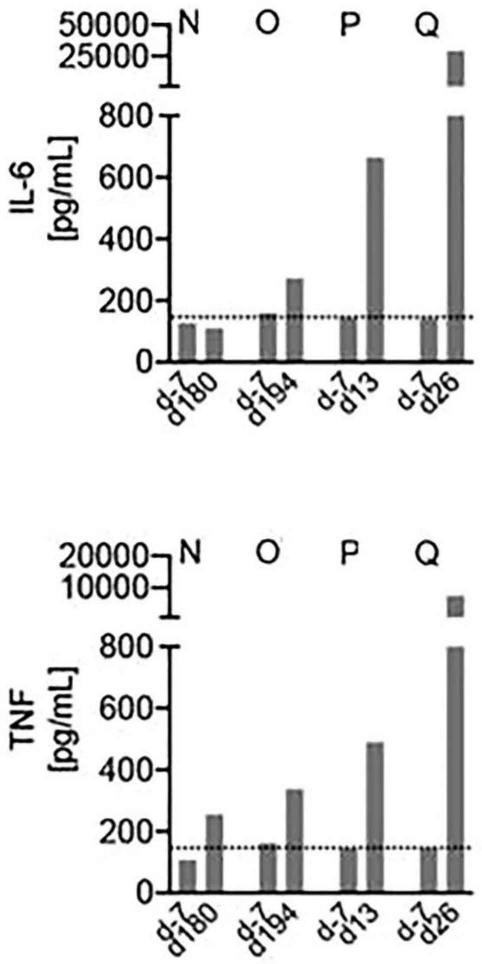
PCMV infection shortens the survival of porcine xenotransplants

Animal	Survival	PCMV
Pig 5528		-
Baboon J	90 days	-
Pig 5415		-
Baboon K	50 days	-
Pig 5420		-
Baboon L	90 days	-
Pig 5807		-
Baboon N	182 days	-
Pig 5803		-
Baboon O	195 days	-
Pig 6249		++
Baboon P	15 days	+++
Pig 6253		+
Baboon Q	27 days	++

Viral load (copies per 100 ng DNA) in different organs of baboon P after explantation of the pig heart

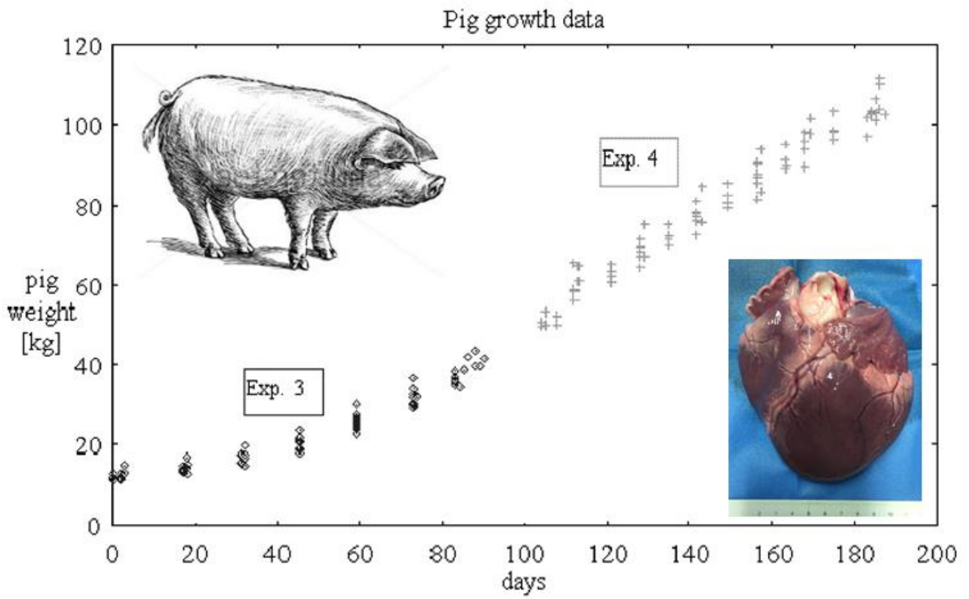


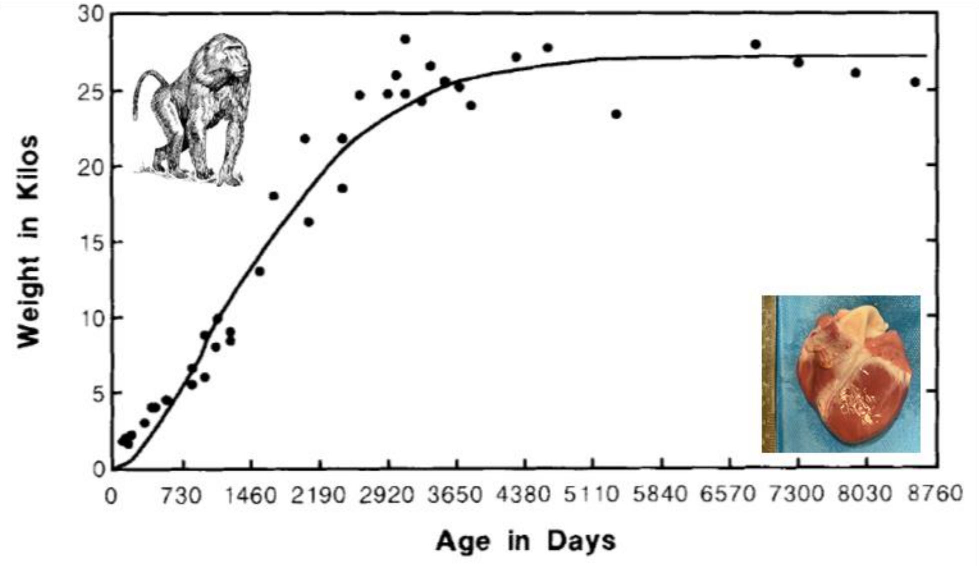
Serum cytokine levels in recipient baboons

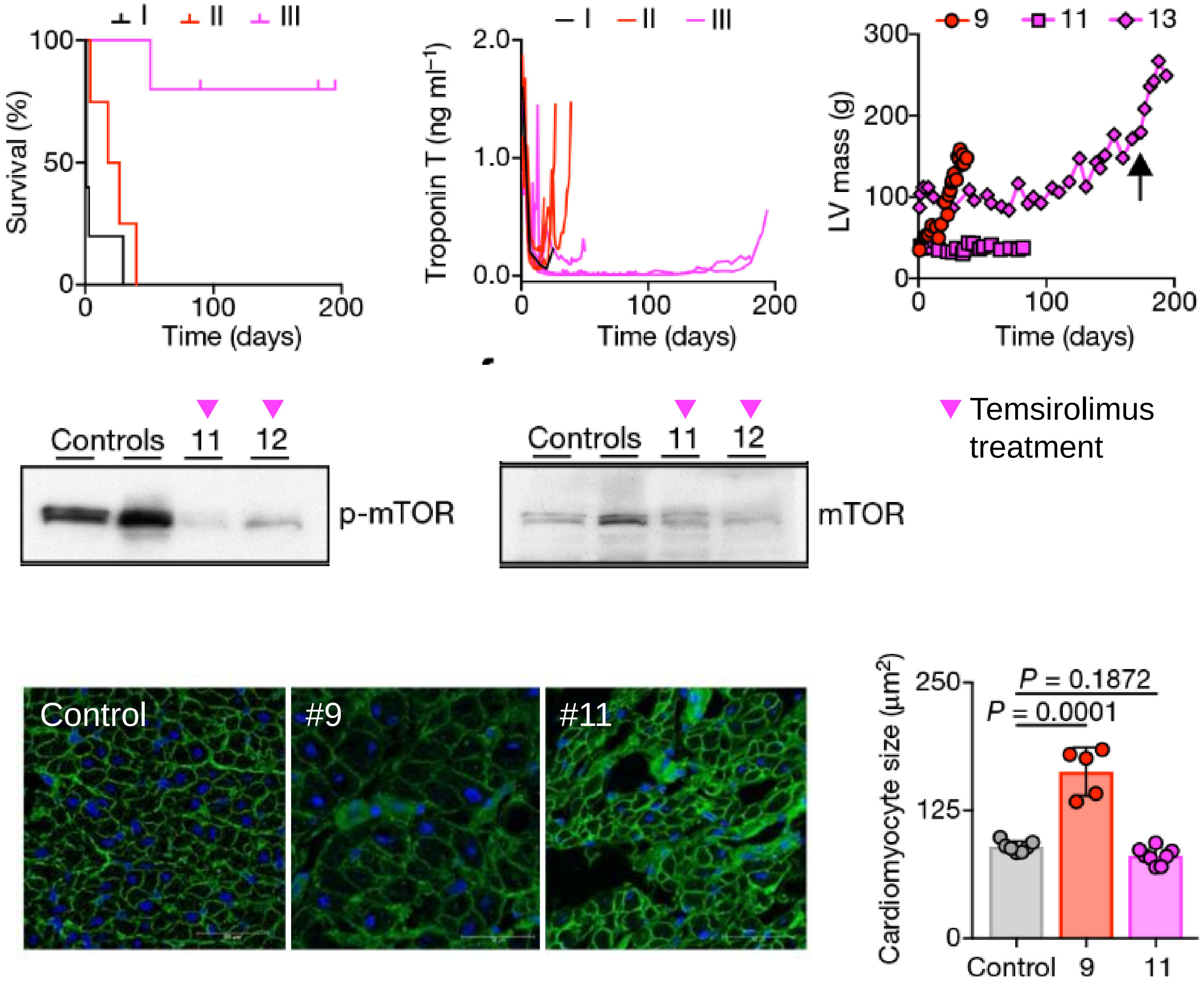


Denner et al., Sci Rep 10, 17531 (2020)

Size matters in pig-to-baboon cardiac xenotransplantation trials







Längin et al., Nature 564, 430-433 (2018)

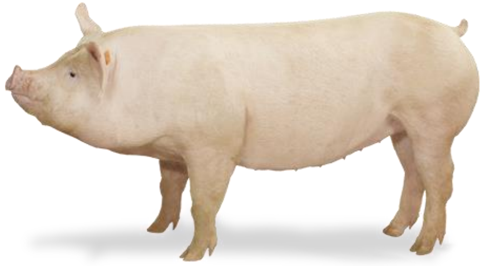
Control of organ growth after (xeno)transplantation

Model: Kidney allotransplantation in pigs

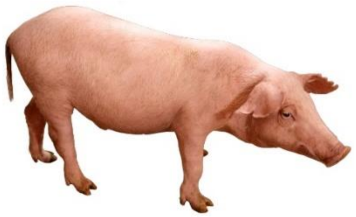
Size-matched Yorkshire pig → Miniature pig (n = 3)

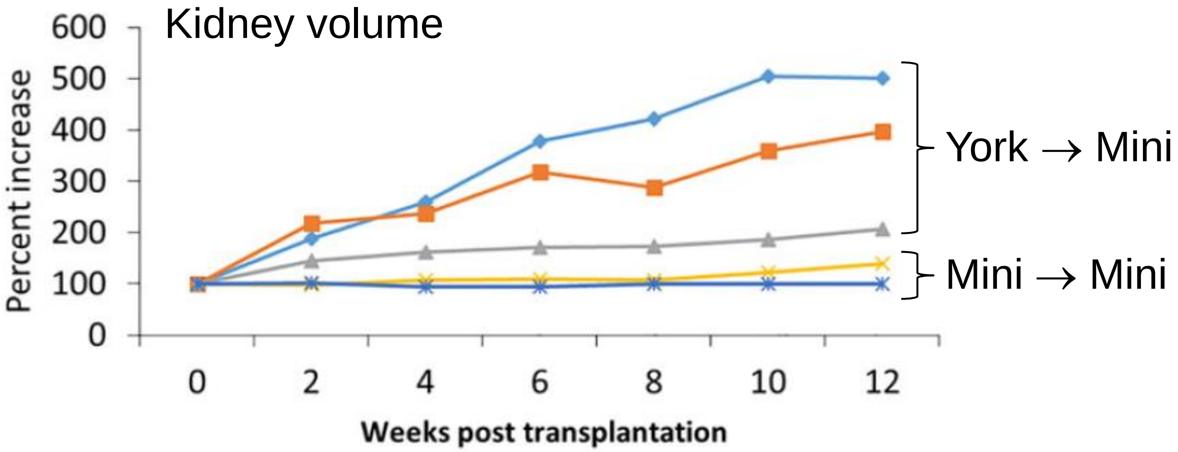
Size-matched Miniature pig → Miniature pig (n = 2)

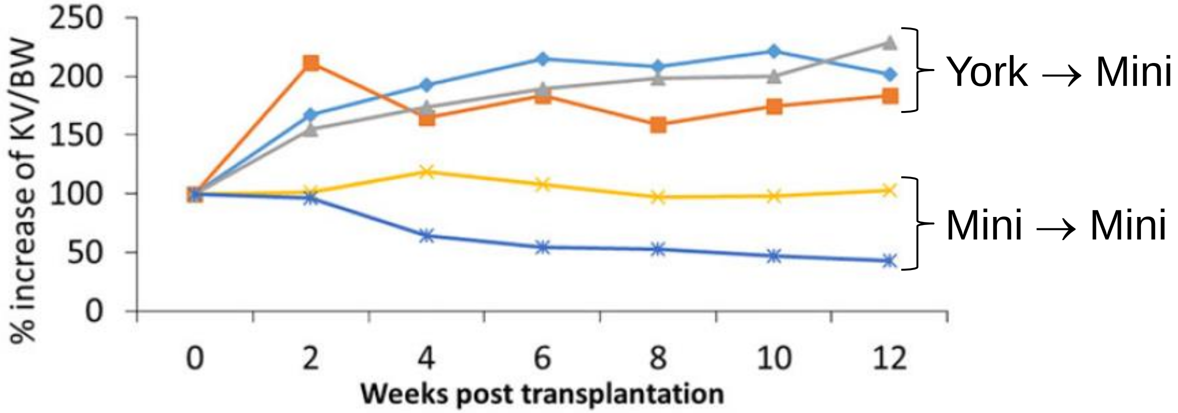
Yorkshire pig
>100 kg BW at 6 mo



MGH minipig
<40 kg BW at 6 mo

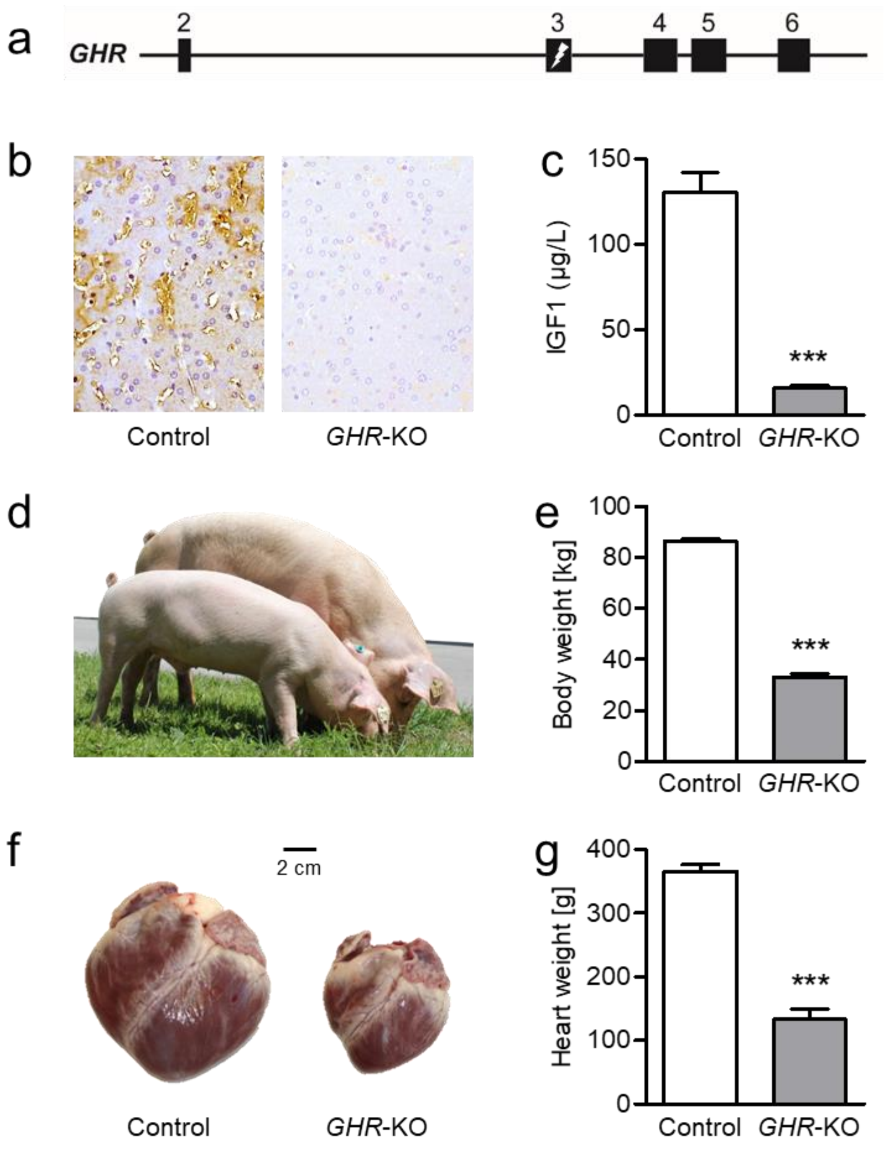




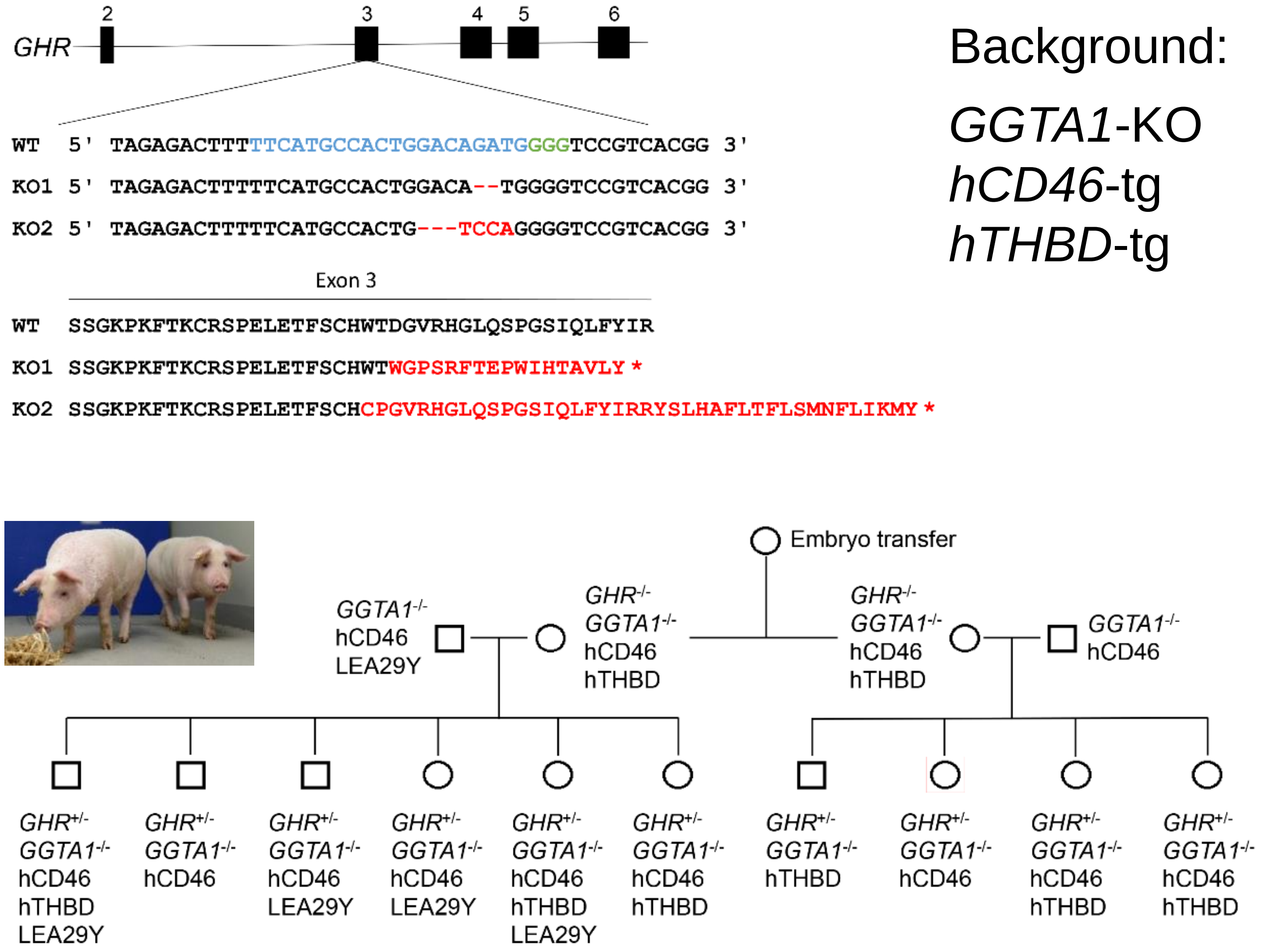


Tanabe et al., Am J Transplant 17, 1778-1790 (2017)

Size reduction of donor pigs by KO of the *GHR* gene



Hinrichs et al., Mol Metab 11:113-128 (2018)

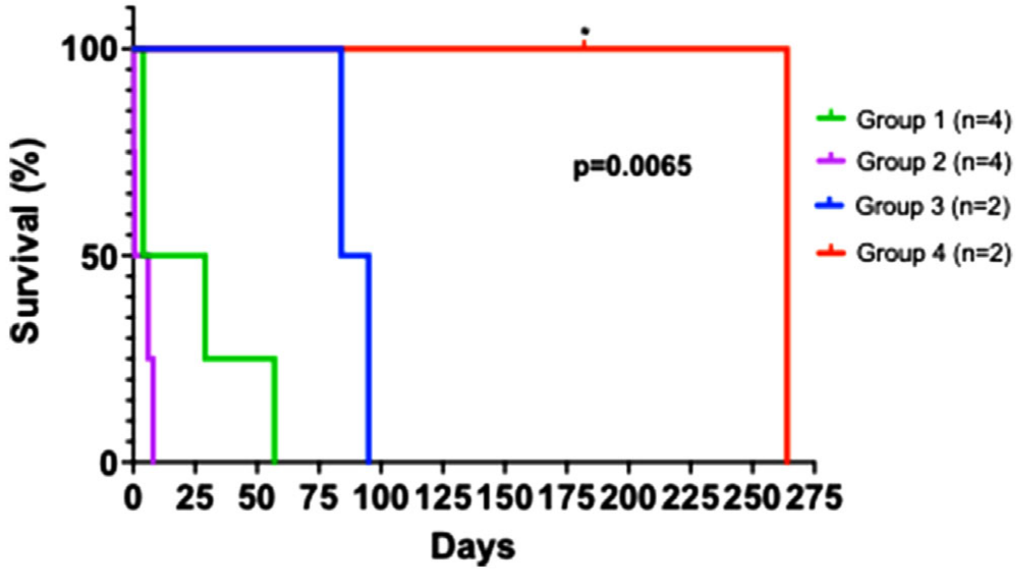


Hinrichs et al., Xenotransplantation 28, e12664 (2021)

Background:

GGTA1-KO
hCD46-tg
hTHBD-tg

KO of the *GHR* gene prolongs cardiac xenograft survival

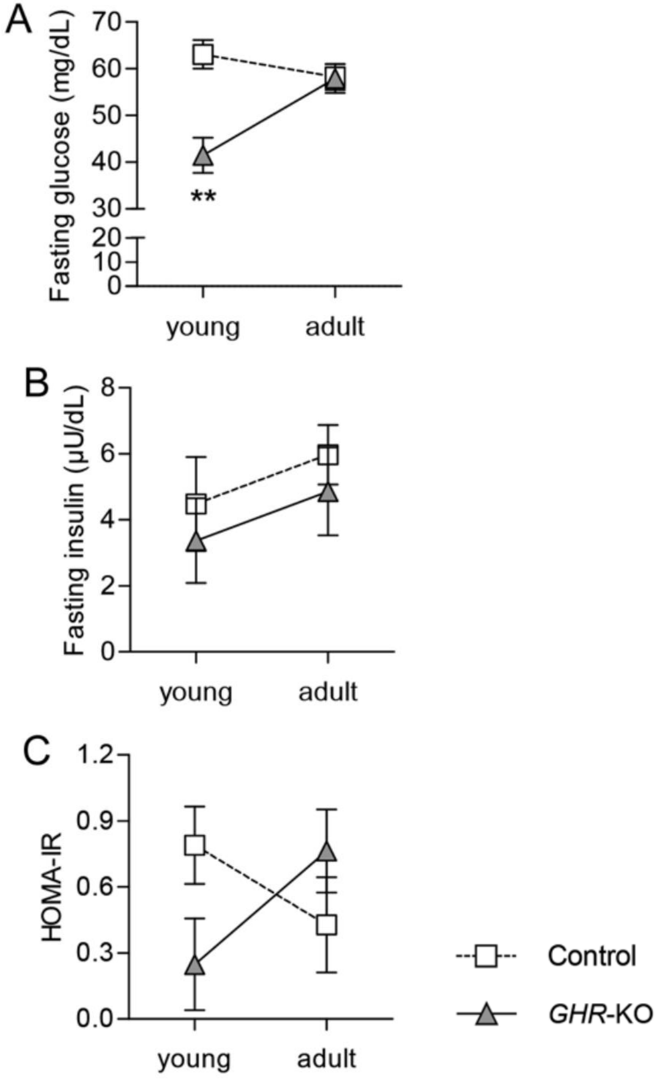


Group	Knockouts				Transgenes					
	GGTA1	*CMAH*	*B4GALNT2*	*GHR*	*hCD46*	*hCD55*	*hTHBD*	*hEPCR*	*hCD47*	*hHMOX1*
1	X				X		X			
2	X	(X)	(X)		(X)	(X)				
3	X		X		X	X	X	X	X	X
4	X		X	X	X		X	X	X	

Mohiuddin et al., Xenotransplantation. 2022 Mar 31:e12744. doi: 10.1111/xen.12744. Online ahead of print.

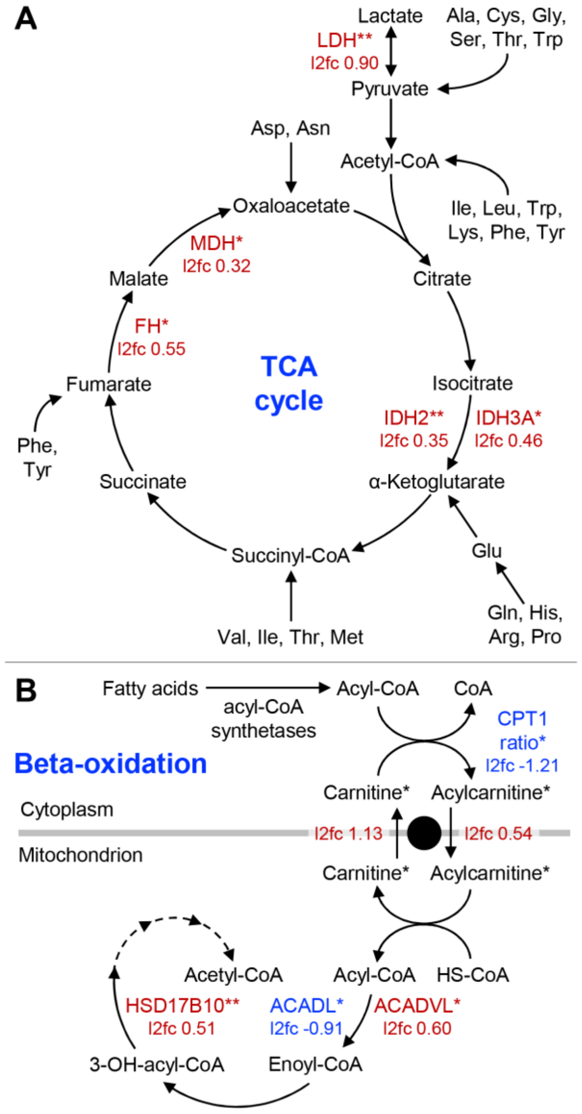
Possible side effects in *GHR*-KO pigs

Juvenile hypoglycemia



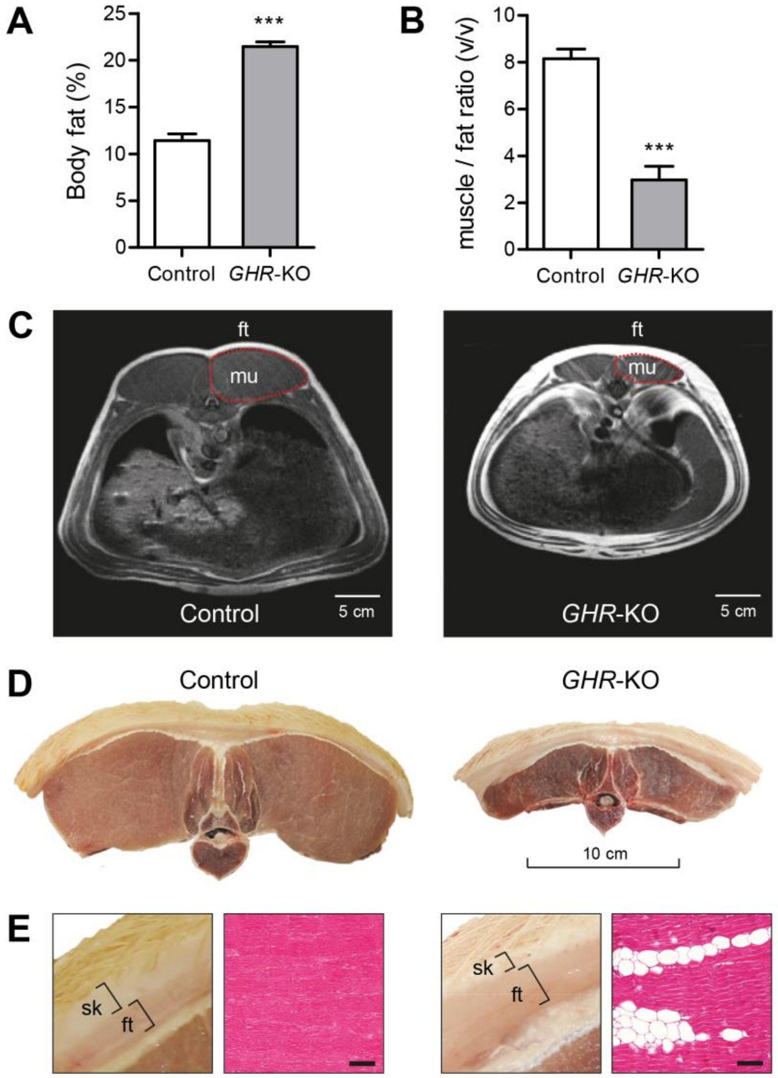
Hinrichs et al., Eur J Endocrinol 185, R35-R47 (2021)

Altered liver metabolism



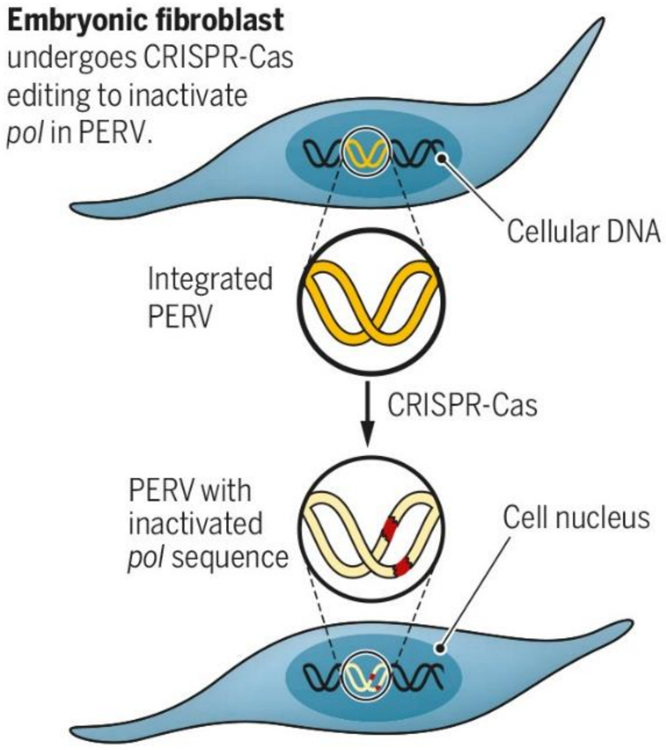
Riedel et al., Mol Metab 36, 100978 (2020)

Obesity



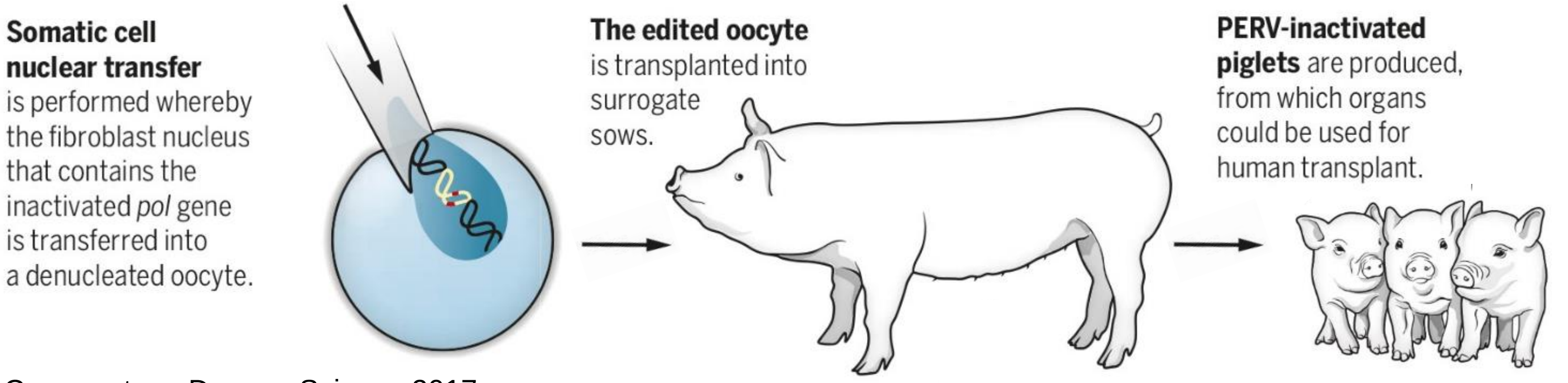
Hinrichs et al., Mol Metab 11, 113-128 (2018)

Pigs with inactivated PERV integrants



- A primary porcine fetal fibroblast cell line (FFF3) with about 25 copies of functional PERVs was used
- Use of p53 inhibitor during genetic modification was necessary to grow up 100% PERV-inactivated FFF3 cell clones
- Five out of eight PERV-inactivated FFF3 cell clones carried chromosomal abnormalities
- No difference in SCNT efficiency between PERV-inactivated (0.9%) and WT cells (0.8%)
- The physiological functions of endogenous retroviruses, which exists in all mammalian species, remain largely unknown

Niu et al., *Science* **357**, 1303-1307 (2017)



Commentary: Denner, Science 2017

Summary of the current state

- The use of pigs as source of cells, tissues and organs for xenotransplantation offers the unique opportunity of genetic engineering the donor animals
- More than 40 different genetic modifications have been introduced into pigs to prevent immune rejection of xenografts, overcome physiological incompatibilities, and reduce the risk of transmitting zoonotic pathogens
- Gen(om)e editing is speeding progress in this field
- The combination of genetic modifications required depends on the type of organ/tissue and – especially for cellular xenografts – the transplantation site
- Cellular localization and level of transgene expression are critical for the functionality and potential side effects of specific modifications
- Remarkable long-term survival and function of pig organs with relatively few genetic modifications has been achieved in stringent NHP models
- Xenotransplantation can thus be considered as realistic future therapeutic option

Genetic modification of xeno-organ source pigs – the more the better?

Donor pigs with a minimum number of essential genetic modifications may be preferred:

- Demonstration of efficacy and safety of individual modifications
- Demonstration of long-term stability and expression of each modification
- Cellular localisation and level of transgene expression are critical
- Transgene expression is difficult to modulate after xenotransplantation, whereas drug treatments can be dose-adjusted of discontinued
- With an increasing number of genetically modified loci the breeding strategy becomes more complex, especially if inbreeding is to be avoided (to maintain a reasonable litter size)
- Cloning is not a reliable procedure for routine production of organ source pigs
- There may be unpredicted interactions between the various modifications
- Certain modifications may have unforeseen negative effects (e.g. increased antigenicity of porcine *CMAH*-KO tissues in non-human primates)

Thank you for your attention!