

ROBERT KOCH INSTITUT



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Berlin

Göttingen Minipigs and Xenotransplantation

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Ellegaard Göttingen Minipigs Webinar

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Xenotransplantation: Definition

- Transplantation of cells, tissues and solid organs from animals
- *Ex vivo* perfusion of human blood using cells, tissues and organs from animals

Xenotransplantation: The need

- Organs - shortage of human donor organs, 25% die on waiting list
- Diabetes - late complications in diabetes patients despite insulin treatment due to insufficient compliance

Why pigs?

- Unlimited availability
- Short breeding time, large litters
- Similar size: Pig kidneys and hearts are comparable in size and function to human organs
- Physiological similarity: Pig insulin has been used to treat diabetes
- Genetic modification
- Cloning
- Low costs
- Apes are endangered species

The problems

- Severe immune rejection – genetically modified pigs
- Physiological incompatibility – genetically modified pigs
- Transmission of porcine microorganisms
Viruses, bacteria, fungi, parasites, protozoa, prions

The achievements

Pig to non-human primates

Pig transplant	Longest survival time (days)
Islet cells	950
Heart, heterotopic	945
Heart, orthotopic	195
Kidney	499
Neurones	549
Cornea	511
Liver	29
Lung	10

First clinical trials: Islet cells from Auckland Island pigs

- Donor pigs negative for 26 microorganisms
- Islet cells encapsulated
- New Zealand (14 patients)
- Argentina (40 patients)
- No transmission of porcine viruses



Viruses screened for:

PCV2, Porcine Circovirus Type 2; PCV1, Porcine Circovirus Type 1; PLHV, Porcine Lymphotropic Herpesvirus; PCMV, Porcine Cytomegalovirus; RV, Rotavirus; PEV1, Porcine Enterovirus Type 1; PEV3, Porcine Enterovirus Type 3; PHEV, Porcine Hemagglutinating Encephalomyelitis Virus; HEV, Hepatitis E Virus; BVD, Bovine Virus Diarrhea; AujD, Aujeszky's Disease; PPV, Porcine Parvovirus; PRRSV, Porcine Reproductive and Respiratory Syndrome Virus; EMCV, Porcine Encephalomyocarditis Virus

Genetic modifications of pigs

- Deletion of sugar moieties
- Complement regulation
- Coagulation regulation
- Prevention of cell-mediated rejection – T cells, natural killer cells and macrophages
- Expression of anti-inflammatory proteins or knockout of pro-inflammatory proteins

- Reduction/elimination of the risk of PERV transmission

Genetic modifications of pigs

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

α -1,3-galactosyltransferase knockout (GGTA1-KO)

cytidine monophosphate-N-acetylneuraminic acid hydroxylase knockout (CMAH-KO)

β -1,4-N-acetyl-galactosaminyl transferase 2 knockout (B4GALNT2-KO)

Complement regulation by human complement-regulatory gene expression

human membrane cofactor protein transgenic (hCD46-tg)

human decay-accelerating factor transgenic (hCD55-tg)

human protectin or membrane inhibitor of reactive lysis transgenic (hCD59-tg)

human complement-regulatory protein C1 inhibitor transgenic (hC1-INH-tg)

Coagulation regulation by human coagulation-regulatory gene expression

human thrombomodulin transgenic (hTM-tg)

human endothelial protein C receptor transgenic (hEPCR-tg)

human tissue factor pathway inhibitor transgenic (hTFPI-tg)

human ectonucleoside triphosphate diphosphohydrolase-1 transgenic (hCD39-tg)

human ecto-5'-nucleotidase transgenic (hCD73-tg)

Prevention of cell-mediated rejection – T cells

human LEA29Y transgenic (LEA29Y-tg)

human CTLA4-Ig transgenic (hCTLA4-Ig-tg)

porcine CTLA4-Ig transgenic (pCTLA4-Ig-tg)

SLA class I knockout

human dominant-negative mutant class II transactivator transgenic (CIITA-DN-tg)

human TNF-related apoptosis-inducing ligand transgenic (hTRAIL-tg)

human programmed cell death 1 ligand 1 transgenic (PD-L1-tg)

Prevention of cell-mediated rejection – natural killer cells and macrophages

HLA-E/human b2-microglobulin transgenic (HLA-E/b2M-tg)

human signal regulatory protein alpha transgenic (hCD47-tg)

Expression of anti-inflammatory proteins or knockout of pro-inflammatory proteins

human tumor necrosis factor α -induced protein 3 (TNFAIP3) transgenic (A20-tg)

human heme oxygenase 1 transgenic (hHO-1-tg)

soluble human TNFRI-Fc transgenic (shTNFRI-Fc-tg)

Reduction/elimination of the risk of PERV transmission

Knockdown of PERV expression

Genome-wide inactivation of PERV pol gene

Kemter, Denner, Wolf, Curr Diab Rep. 2018;18(11):103.

Microbiological safety

Infection after allotransplantations

- Herpesviruses: CMV, EBV, HSV
- HIV-1
- Rabies virus
- EBV
- HSV
- Hepatitis B
- Jacob Creutzfeld disease after transplantation of dura mater

Sensitive and specific detection methods

Direct detection methods

Viral DNA or provirus	PCR, nested PCR, real time PCR, Southern blot, droplet digital PCR
mRNA, viral RNA	RT-PCR, real time RT-PCR
Viral proteins	Immunofluorescence, immunoperoxidase assay, immunogold, immunohistochemistry
Viral particles	RT activity, electron microscopy
Infectious virus	Infection assay

Indirect detection methods

Detection of an antibody response

Western blot analysis, ELISA

Known zoonotic viruses

Hepatitis E virus (HEV)

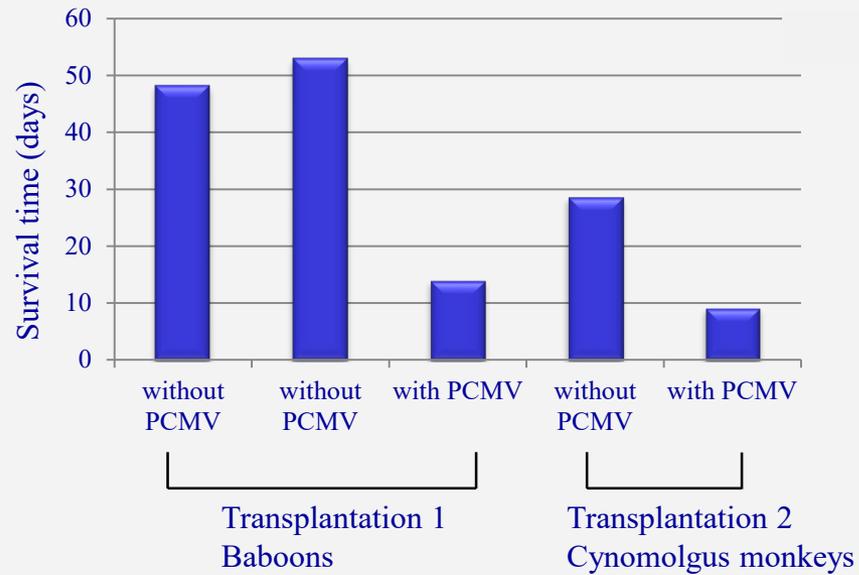
- From pigs to humans: eating pork and contact, from human to human: blood transfusion
- Chronic infection in immunocompromised humans
- Disease in individuals with preexisting liver diseases
- Treatment: Ribavirin (inhibitor of RNA synthesis), no vaccine

Porcine cytomegalovirus/porcine roseolovirus (PCMV/PRV)

- Significant reduction of transplant survival in pig – non-human primate transplantation
- HCMV – risk factor in allotransplantation
- No treatment, no vaccine

Effect of PCMV/PRV

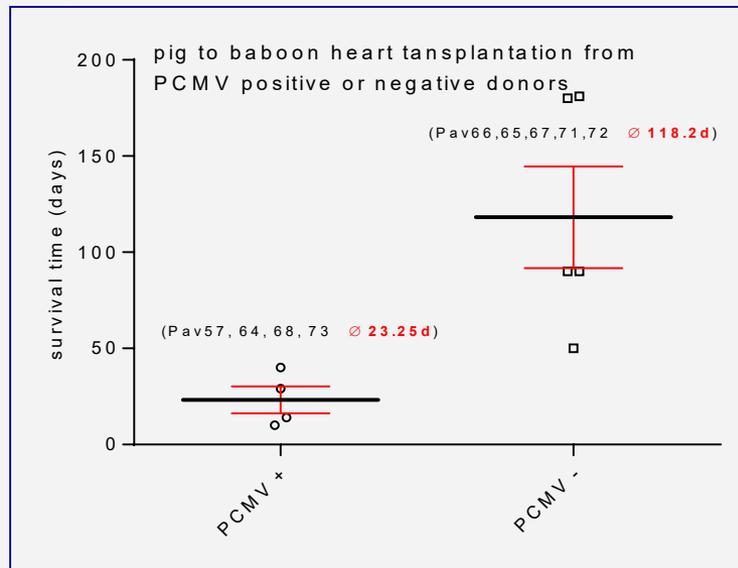
Kidney transplantation



Effect of PCMV/PRV

Orthotopic heart transplantation

Pig to baboon



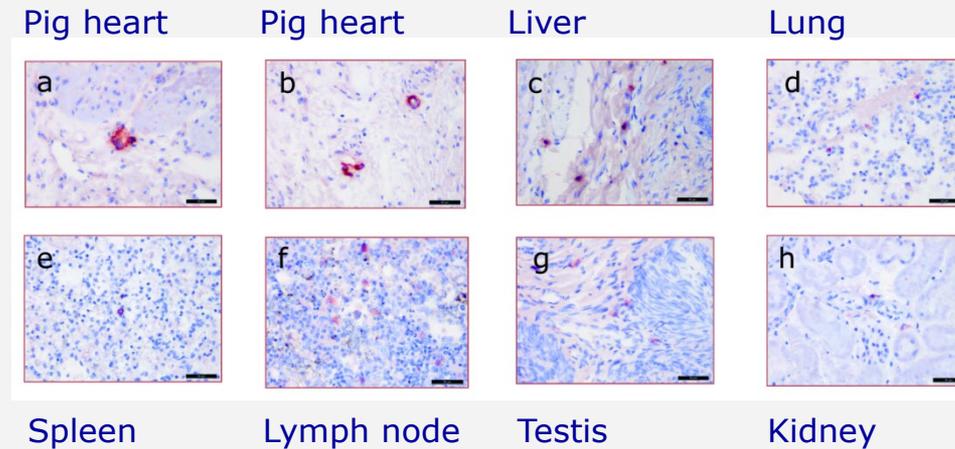
- reduced survival time of the transplant
- increased levels of IL-6 and TNF α
- high levels of tPA-PAI-1 complexes, suggesting a complete loss of the pro-fibrinolytic properties of the endothelial cells

Effect of PCMV/PRV -2-

Orthotopic heart transplantation

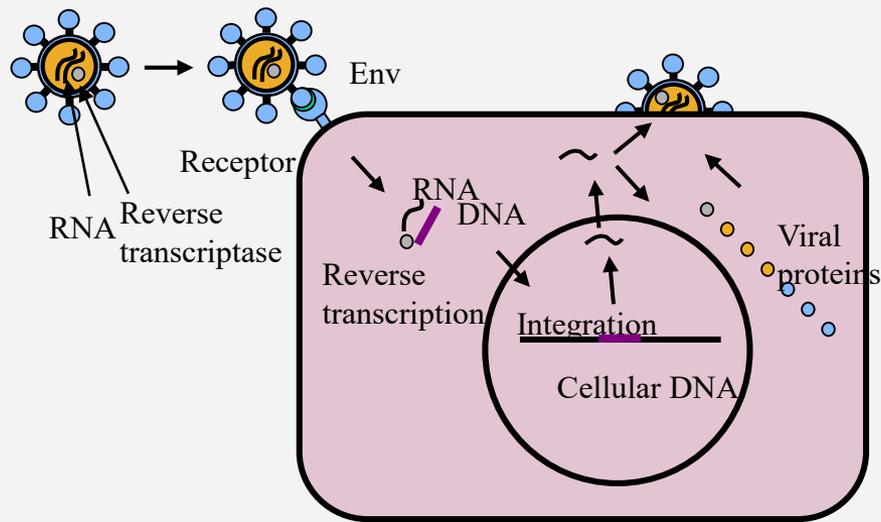
Pig to baboon

➤ unclear, whether PCMV infects baboon cells

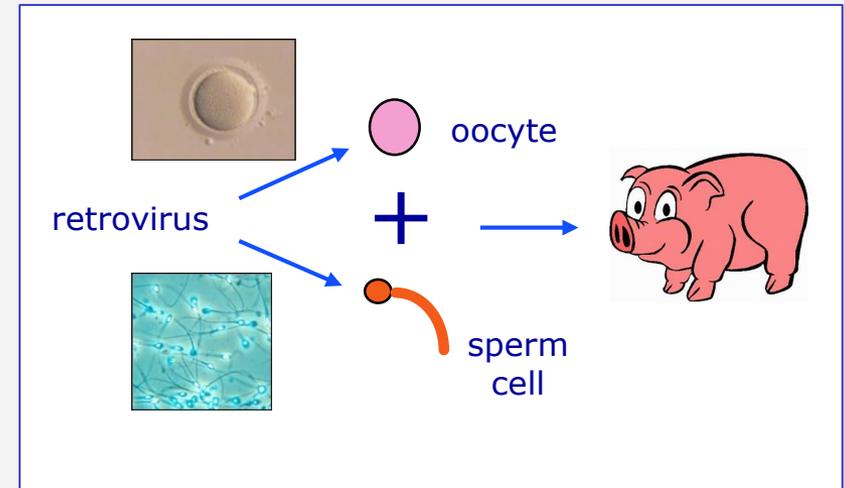


Porcine endogenous retroviruses (PERVs)

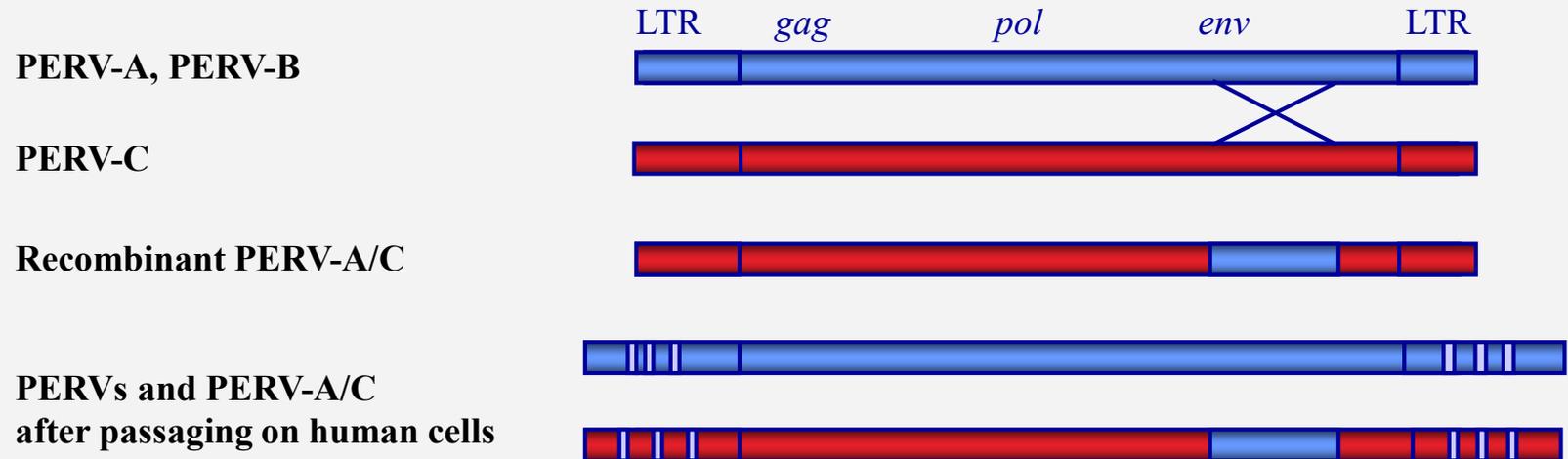
Life cycle



Endogenization



PERVs: Recombination and adaptation



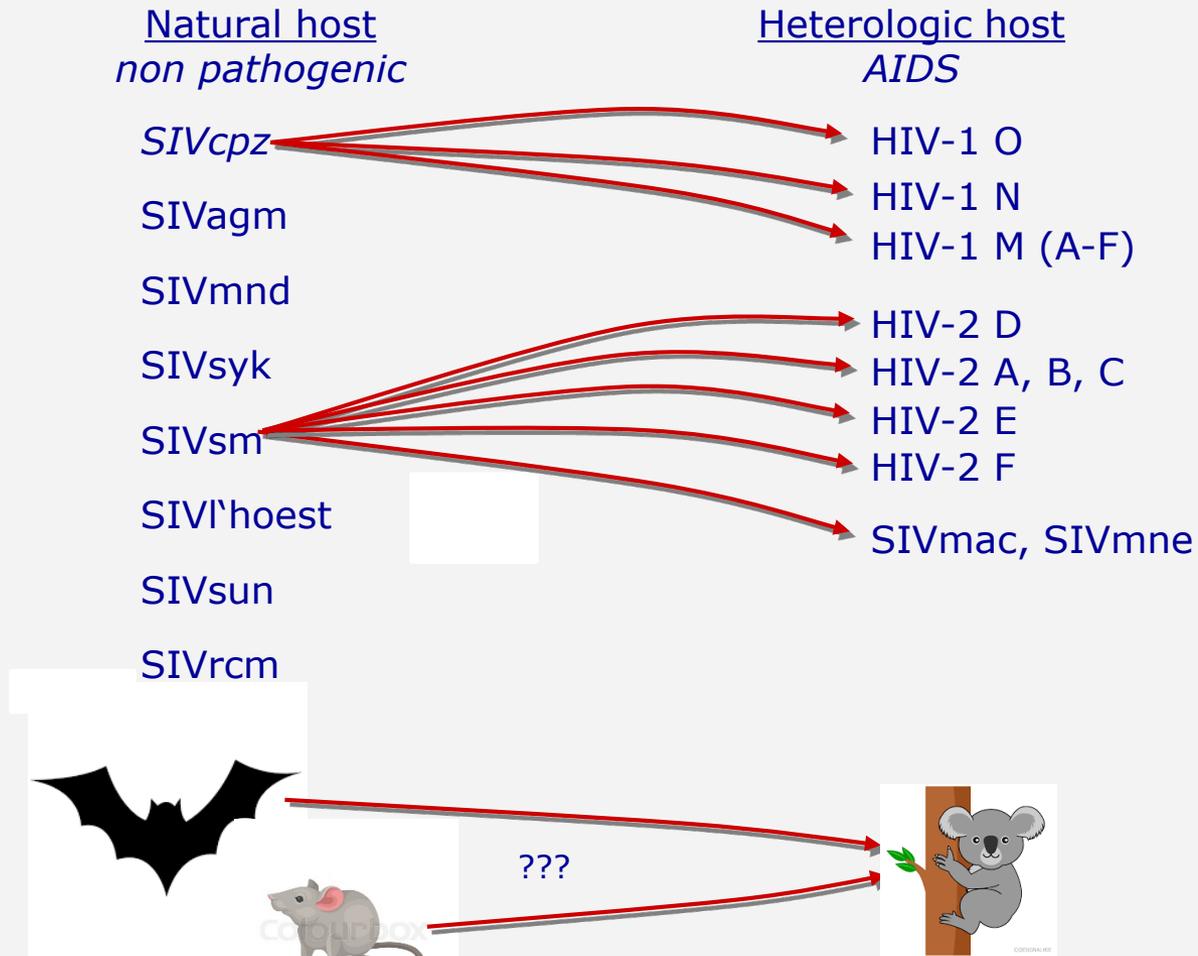
PERV-A/C

- increased titres
- multimerisation of NF-Y binding sites in the LTR
- not integrated in germ line

Risk posed by retroviruses

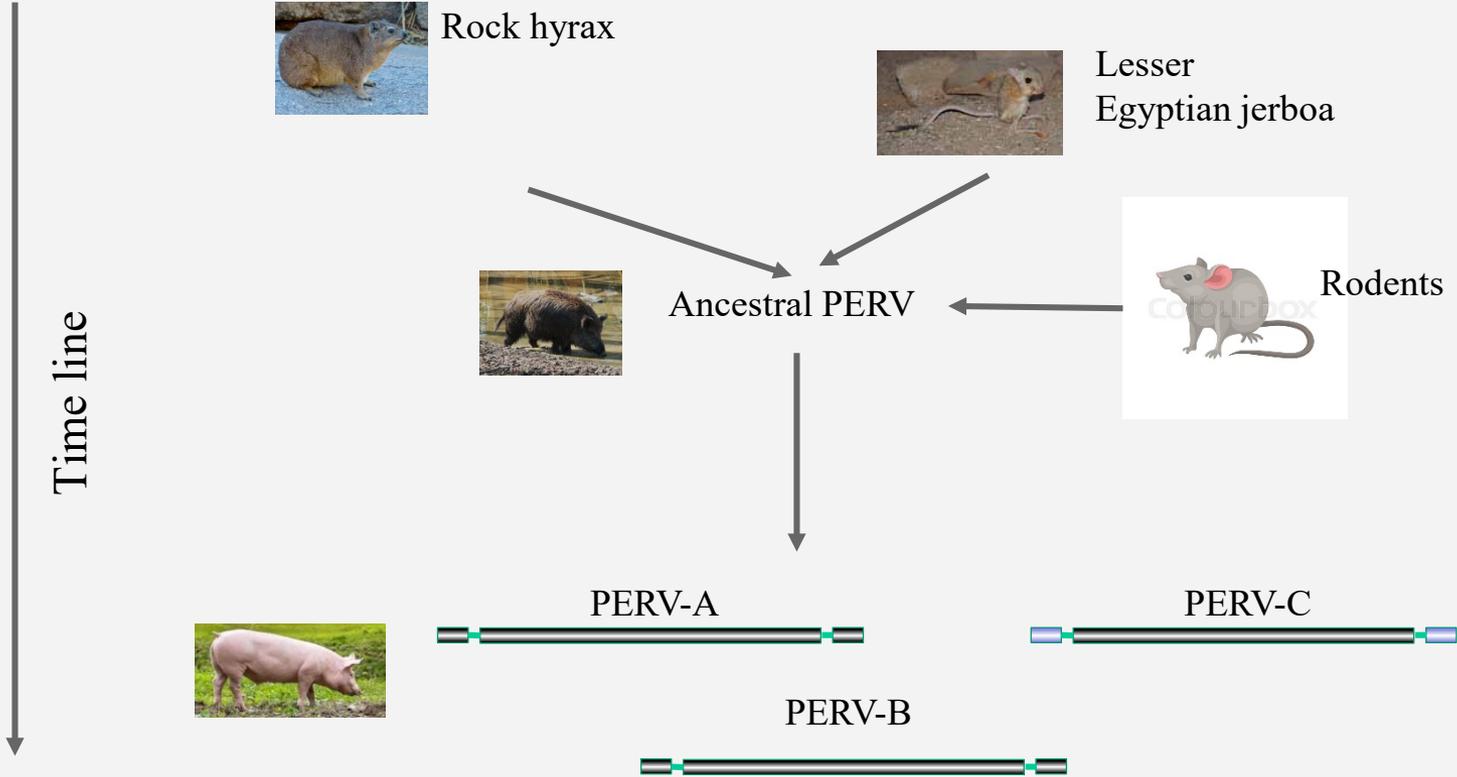
- Tumors, leukemia (FeLV, MuLV, KoRV, HTLV)
- Immunodeficiency (AIDS) (HIV)

Transspecies transmission of retroviruses



Denner et al., *Virology*. 2003;314(1):125-33
 Karlas et al. *Ann Transplant*. 2010;15(2):45-54.

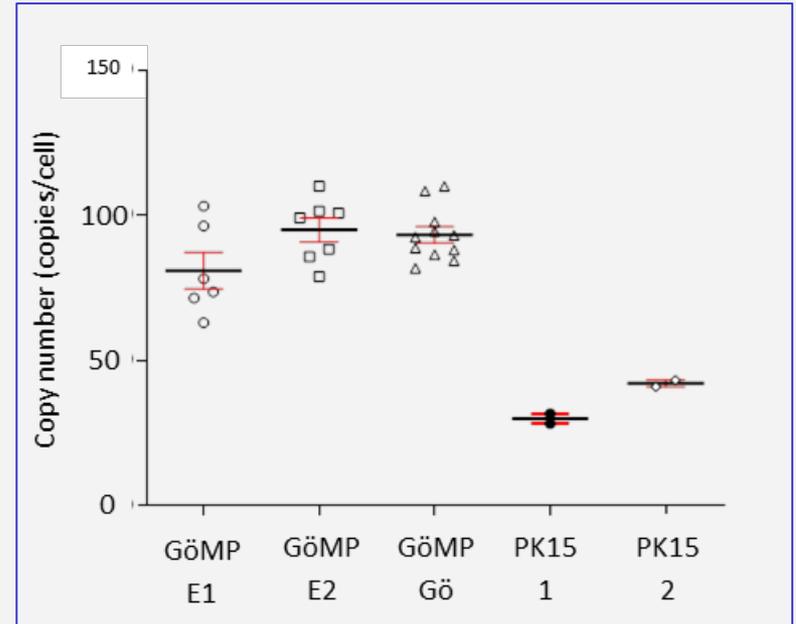
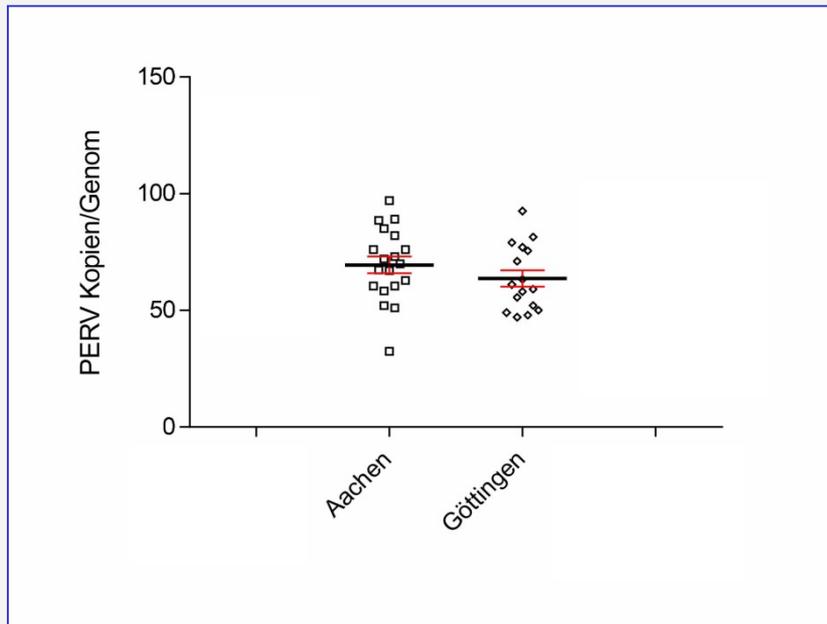
Origin of PERV



PERVs in Göttingen Minipigs

In all pigs PERV-A, PERV-B, in most PERV-C.

Number of integrated proviruses (droplet digital PCR)

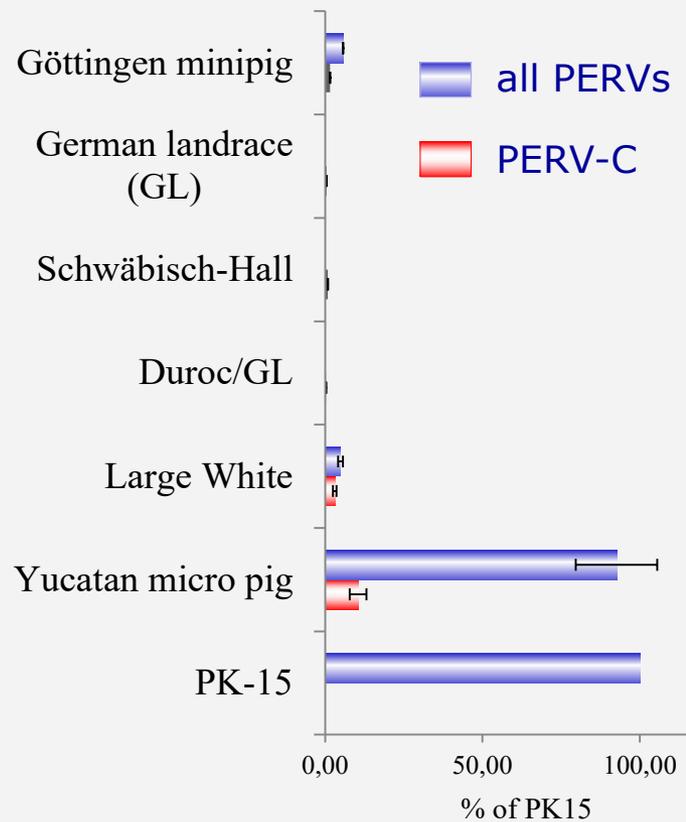


Semaan et al., Xenotransplantation, 2013
Fiebig et al., Xenotransplantation, 2018

Krüger et al., Archives of Virology 2021;166(2):1-7

PERVs in Göttingen Minipigs

Expression (PBMCs) (RT-PCR)



Göttingen Minipigs: source of islet cells

Virus	PCR methods* Positive/negative (%)	Western blot Positive/negative (%)	Reference
HEV	Retired breeders 0/6 (0%)	0/6 (0%)	Morozov et al., PLOS, 2015
	Adults 0/6 (0%)	n.t.	
	Fattener 3/10 (30%)	0/10 (0%)	
	Sow-piglet 6/12 (50%)	2/6 (33%)	
	Summary 9/40 (22.5%)	2/22 (9%)	
PCMV	12/39 (30%)	8/67 (12%)	Plotzki et al., J. Immunol. Methods, 2016; Morozov et al., Xenotransplantation, 2015
PLHV-1, -2, -3	0/14 (0%)	1/10 (10%)	
PCV2	3/21 (14%)	n.t.	Heinze et al., Ann. Virol. Res., 2016

* PCR, nested-PCR, real-time PCR
n.t., not tested

Testing Göttingen Minipigs

Göttingen minipigs were tested negative for 88 individual microorganisms

Viruses, bacteria, fungi, parasites, protozoa

Pathogens	Methods
Bacteria (20)	
<i>Brucella abortus</i> , <i>B. microti</i> , <i>B. melitensis</i> , <i>B. pinnipedalis</i> , <i>B. suis</i> , <i>B. canis</i> , <i>B. ovis</i> and <i>B. neotomae</i>	real-time PCR
<i>Burkholderia mallei</i> , <i>pseudomallei</i>	PCR
<i>Chlamydomydia felis</i> / <i>Chlamydomydia psittaci</i>	real-time PCR
<i>E. coli</i>	real-time PCR
<i>Fusobacterium</i>	real-time PCR
<i>Bacillus anthracis</i>	real-time PCR
<i>Listeria monocytogenes</i>	real-time PCR
<i>M. tuberculosis</i> , <i>M. bovis</i> , <i>M. microti</i> , <i>M. intracellulare</i> , <i>M. avium</i> , <i>M. gastri</i> , <i>M. africanum</i> , <i>M. scrofulaceum</i> , <i>M. ulcerans</i> , <i>M. simiae</i> , <i>M. kansasii</i> , <i>M. chelonae</i> , <i>M. fortuitum</i> , <i>M. marinum</i> , <i>M. genavense</i> , etc.	real-time PCR
* <i>Actinobacillus pleuropneumoniae</i>	real-time PCR
* <i>Bordetella bronchiseptica</i>	real-time PCR
* <i>Brachyspira</i> (<i>Serpulina</i>) <i>pilosicoli</i>	real-time PCR
* <i>Campylobacter</i> , <i>Campylobacter jejuni</i> , <i>C. coli</i> and <i>C. lari</i>	real-time PCR
* <i>Leptospira</i>	real-time PCR
* <i>Pasteurella multocida</i>	real-time PCR
* <i>Salmonella</i>	real-time PCR
* <i>Yersinia enterocolitica</i>	real-time PCR
* <i>Clostridium perfringens</i>	real-time PCR
* <i>Erysipelothrix rhusiopathiae</i>	real-time PCR
* <i>Staphylococcus</i>	real-time PCR
* <i>Streptococcus</i>	real-time PCR
Viruses (20)	
Nipah virus	reverse transcription / real-time-PCR
Porcine cytomegalovirus	real-time PCR
Porcine lymphotropic herpesvirus 1 and 2, PLHV-1 and -2	real-time PCR
Rabies virus	reverse transcription / real-time PCR
Hepatitis E virus	reverse transcription / real-time PCR
PERV	reverse transcription / real-time PCR
*Encephalomyocarditis	reverse transcription / real-time PCR
*Rotavirus	reverse transcription / real-time PCR
Influenza virus, H5N1, H5N2, *H1N1, *H2N2, H3N8, H4N6, H7N7, H8N4, H9N2	reverse transcription / real-time PCR
BVDV	reverse transcription / real-time PCR
Swine fever virus	reverse transcription / real-time PCR
PHEV	reverse transcription / real-time PCR
Pseudorabies	real-time PCR
Porcine adenovirus	real-time PCR
Porcine circovirus type 1	real-time PCR
*Porcine circovirus type 2	real-time PCR
Porcine enterovirus	reverse transcription / real-time PCR
PRRSV	reverse transcription / real-time PCR
Swine pox virus	real-time PCR
Swine vesicular disease	reverse transcription / real-time PCR
Vesicular stomatitis virus	reverse transcription / real-time PCR
Nematode/worm (2)	
<i>Taenia solium</i>	real-time PCR
<i>Trichinella spiralis</i>	real-time PCR
Protozoa (3)	
<i>Cryptosporidium</i>	PCR
<i>Trypanosoma cruzi</i>	real-time PCR
* <i>Toxoplasma gondii</i>	real-time PCR
Fungi (4)	
<i>Aspergillus</i>	real-time PCR
<i>Cryptococcus neoformans</i>	real-time PCR
<i>Microsporium</i>	real-time PCR
* <i>Candida albicans</i>	real-time PCR
Total: 49 groups of pathogens (>88 individual microorganisms)	

doi:10.1371/journal.pone.0139893.t001

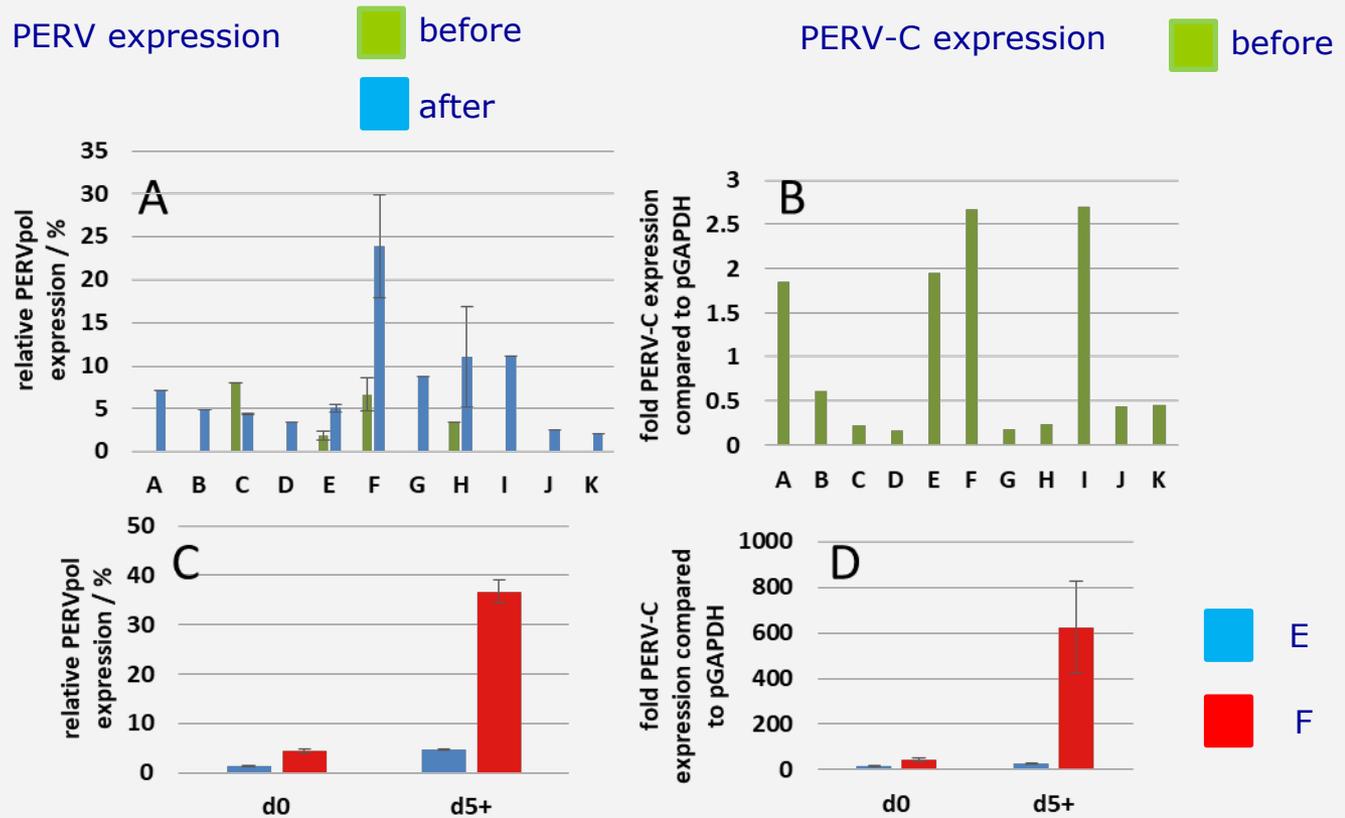
Morozov et al., PLOS, 2015

Comparison: Göttingen Minipigs

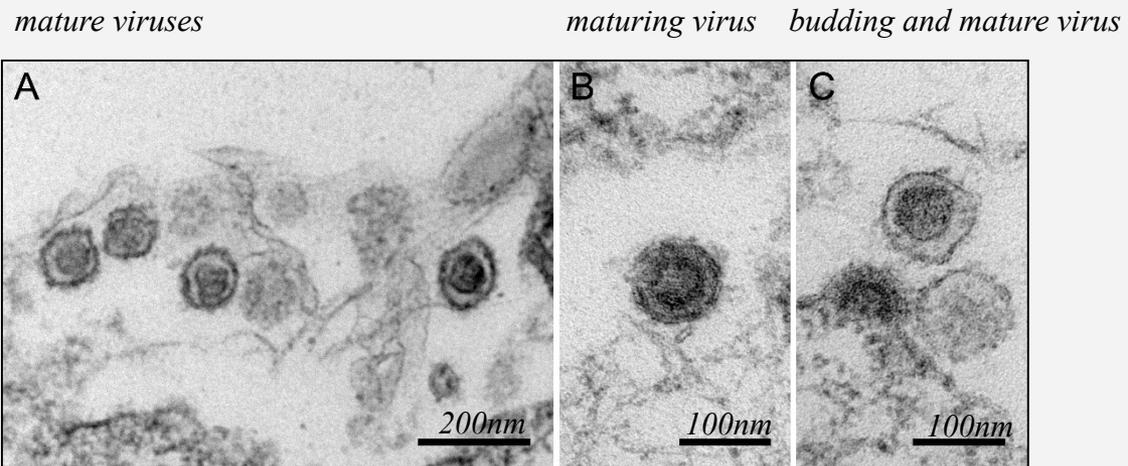
Virus	Ellegaard	University Göttingen
PERV-A, PERV-B	40/40 (100%)	10/10 (100%)
PERV-C	28/28 (100%)	10/10 (100%)
HEV	9/40 (22.5%)	0/10 (0%)
PCMV	10/22 (45%)	0/10 (0%)
PLHV-1	1/10 (10%)	2/11 (18%)
PLHV-2	n.t.	2/11 (18%)
PLHV-3	n.t.	2/11 (18%)
PCV2	3/21 (14%)	2/10 (20%)
PCV3	0/10 (0%)	0/10 (0%)

n.t., not tested

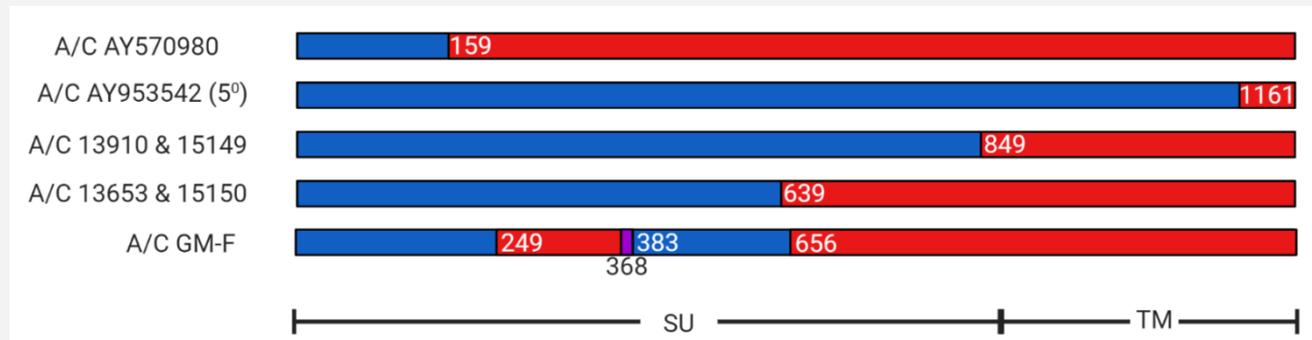
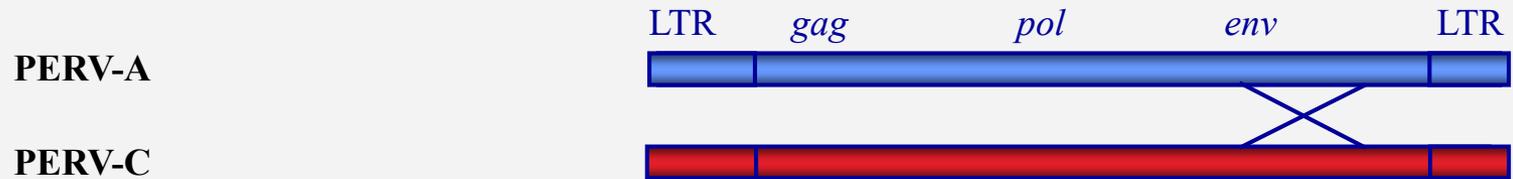
Mitogen stimulation of PBMCs increases PERV expression



Detection of a PERV-A/C recombinant

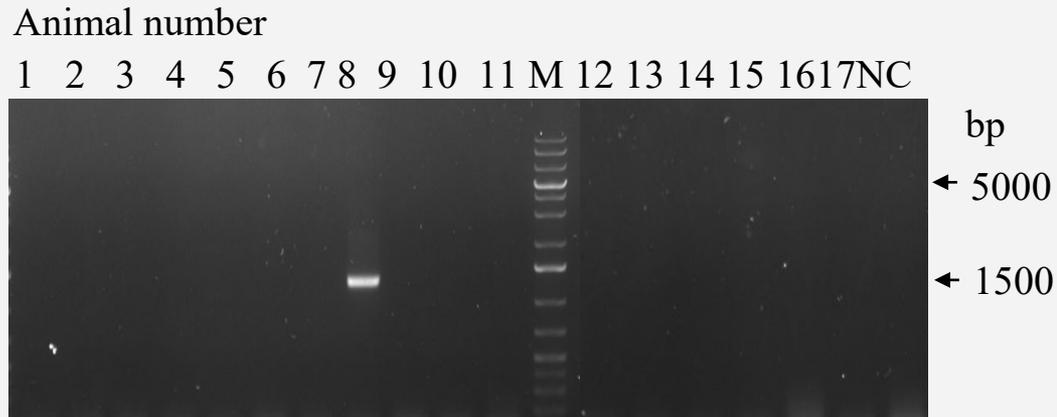


PERV-A/C recombinants



Virus isolation is a rare event

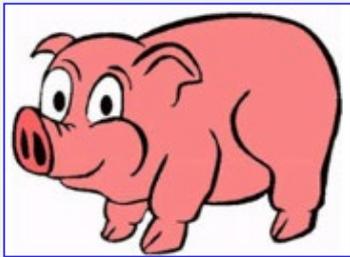
6 Aachen Minipigs		2 proviruses
5 Black forest Minipigs		1 provirus
19 German landrace pigs		No PERV-A/C
11 Göttingen Minipigs		4 proviruses, 1 infectious virus
17 Göttingen Minipigs		1 provirus



Halecker et al., in preparation

Islet cell transplantation

Göttingen minipigs
(Ellegaard)



PERV 3/3
PCMV 0/3
HEV 0/3

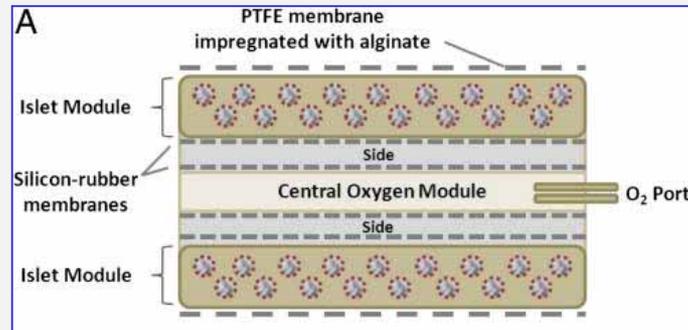
Macroencapsulated
islet cells*
12 months



Cynomolgus
monkeys



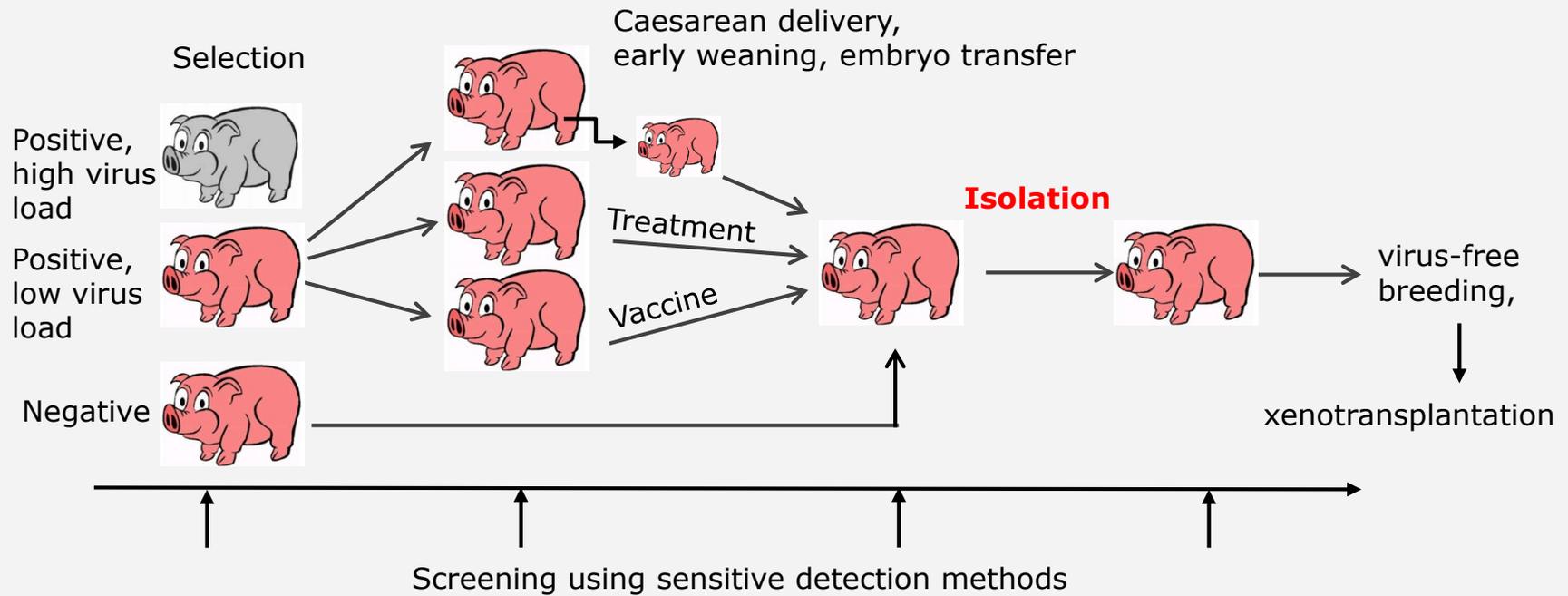
PERV 0/8
PCMV 0/8
HEV 0/8



* BetaAir, Beta-O₂ Technologies, Rosh-Haain, Israel

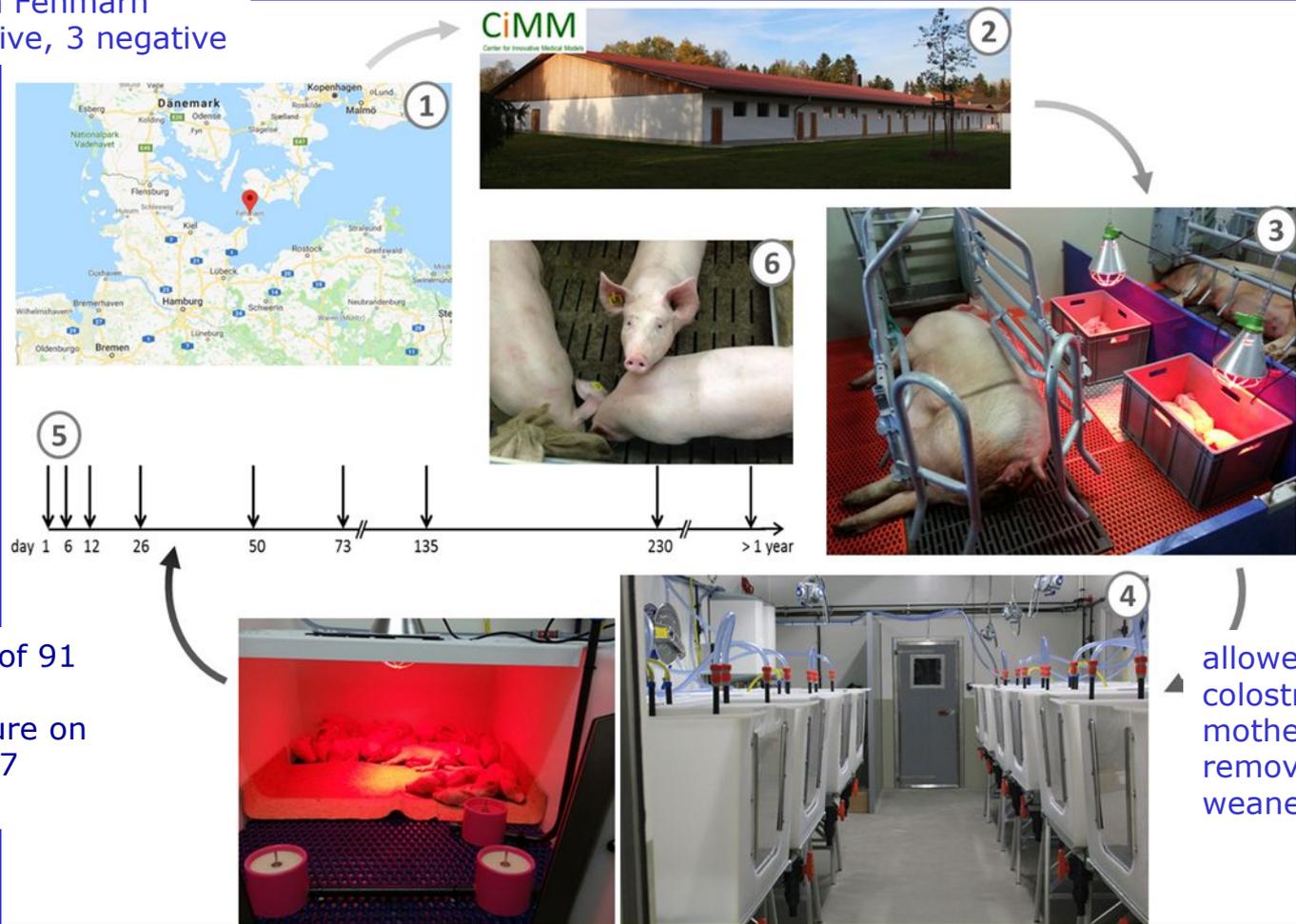
Ludwig et al., PNAS, 2013; Morozov et al., Xenotransplantation, 2016

Elimination programs

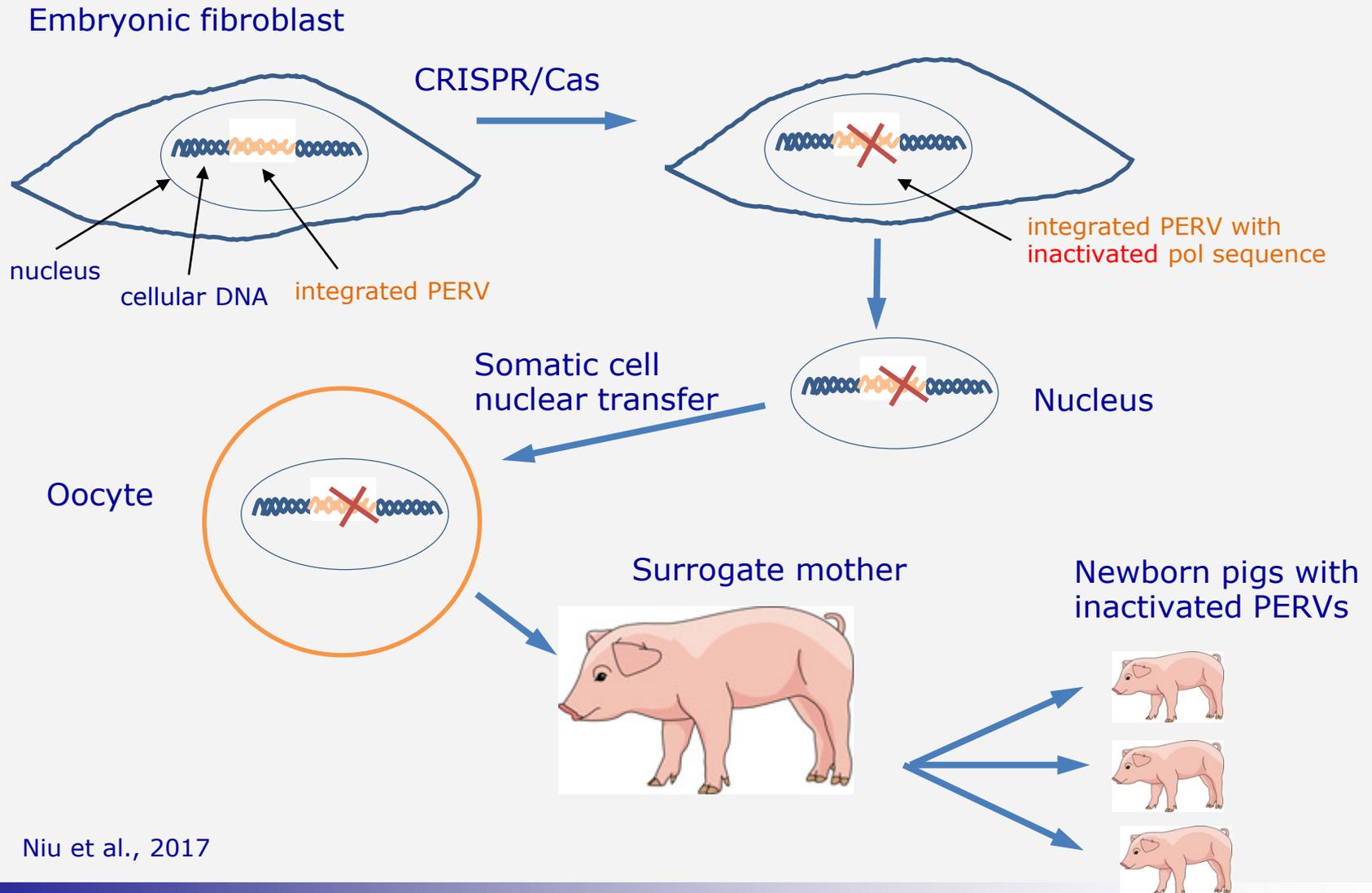


Elimination of PCMV by early weaning

10 sows from Fehmarn
7 PCMV-positive, 3 negative



Inactivation of PERV using CRISPR/Cas



Do we need CRISPR/Cas treated pigs?

- All clinical trials (transplantation of pig islet cells) – no transmission of PERV
- All preclinical trials in non-human primates – no transmission of PERVs
- All infection experiments in small animals and non-human primates with or without pharmaceutical immunosuppression – no infections with PERV
- Off-target effects of CRISPR/Cas
- Risk of inbreeding of CRISPR/Cas-inactivated pigs when generating high numbers of donor pigs

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