Deleting PERV C infectious potential from donor pigs for xenotransplantation

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Inaugural-Dissertation zur Erlangung der Doktorwürde

(Dr. rer. biol. vet.)

der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

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München 2019

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Lehrstuhl für Molekulare Tierzucht und Biotechnologie

Arbeit angefertigt unter der Leitung von Univ.-Prof. Dr. Eckhard Wolf

Mitbetreuung durch: Priv.-Doz. Dr. Nikolai Klymiuk

Gedruckt mit Genehmigung der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.

Berichterstatter: Univ.-Prof. Dr. Eckhard Wolf

Korreferent/en: Univ.-Prof. Dr. Gerd Sutter

Tag der Promotion: 27.07.2019

An meine liebe Familie!

“It's just when you think you know something, that you have to look at it from another perspective!”

From the movie “Dead poets’ society”

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Index of Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATG	Anti-thymocyte globulin
ATP	Adenosine triphosphate
B4GALNT2	β-1, 4-N-acetyl-galactosaminyl transferase 2
BAC	Bacterial artificial chromosome
bp	Basepairs
cDNA	Complementary DNA
CRISPR	Clustered regularly interspaced palindromic repeats
CRPs	Complement regulatory proteins
crRNA	CRISPR RNA
CTLA4	Cytotoxic T lymphocyte-associated antigen
ddNTPs	Dideoxynucleotides
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
DPF	Designated pathogen-free
DSB	Double strand break
dsDNA	Double strand DNA
DTT	Dithiothreitol
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
EPCR	Endothelial cell protein C receptor

ERV	Endogenous retrovirus
EtOH	Ethanol
FCS	Fetal calf serum
FDA	US Food and Drug Administration
FFF3	Porcine fetal fibroblast cell line
gRNA	guiding RNA
HAR	Hyperacute rejection
hDAF	human Decay-accelerating factor
HLA-E	Human leukocyte antigen
hPBMC	Human peripheral blood mononuclear cells
HR	Homologous recombination
Ig	Immunoglobulin
INDEL	Insertion or deletion
kb	Kilo basepairs
KO	Knockout
LTR	Long terminal repeat
Mb	Mega basepairs
min	Minute
MLV	Murine leukemia virus
mm	Millimeter
mM	Millimolar
Neu5Gc	N-glycolylneuraminic acid
NHEJ	Non-homologous end-joining repair

NK	Natural killer
ORF	Open reading frame
PAEC	Primary aortic endothelial cells
PBMC	Peripheral blood mononuclear cells
PCiA	Phenol-chloroform-isoamylalcohol
PCMV	Porcine cytomegalovirus
PCR	Polymerase chain reaction
PERV	Porcine endogenous retroviruses
PRR	Proline reach region
RNA	Ribonucleic acid
SCNT	Somatic cell nuclear transfer
siRNA	Short interference RNA
SNP	Single nucleotide polymorphism
TAE	Tris acetate buffer
TALEN	Transcription activator-like effector nucleases
TB	Thrombin
TBM	Thrombomodulin
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TLA	Targeted locus amplification
tracrRNA	Trans-activating RNA
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
ZFN	Zinc-finger nucleases
α-Gal	Galactosyl α-(1,3)-galactosyl β-1,4-N-acetyl glucosaminyl

I. Introduction

There is a large number of patients waiting for cells, tissues and organs for transplantation worldwide, but the available human donors cannot fill this need. Xenotransplantation might be a good solution to close this gap. Pigs seem to be the best source as potential donor animals because of their physiological similarity with humans, ethical issues, easy genetic manipulation and high reproduction rate. Recent progress in xenotransplantation preclinical studies suggest that immunological hurdles and species-specific incompatibilities are manageable. At this step it becomes necessary to elucidate safety issue before going to the clinical studies on human recipients. Transmission of pathogens like bacteria, fungi, protozoa and many viruses can be controlled or eliminated with different approaches like designated pathogen-free (DPF) breeding conditions, vaccines, drugs or early weaning. These strategies, however, are not able to eliminate porcine endogenous retroviruses (PERV), which are permanently integrated in the porcine genome and are inherited according to Mendelian rules. There are three relevant subtypes of PERV. Among them A and B are able to infect human cells in vitro, whereas subtype C alone infects only porcine cells. However, when PERV C recombined with PERV A, a highly infectious PERV A/C was capable to infect also human cells. Therefore, it is highly recommended for xenotransplantation to use donor pigs that do not contain PERV C in their genome. The objective of this thesis was to delete the infectious potential of PERV C from donor pigs for xenotransplantation through two different approaches, namely selective breeding and excision of PERV C by CRISPR/Cas technology. Both strategies needed at first the detection, localization and characterization of the provirus loci within the genome. For selective breeding all animals of our herd were tested for PERV C loci to learn more about the PERV C load and to design breeding strategies for provirus eradication. CRISPR/Cas technology was used to excise PERV C from the genome of a multimodified pig line and to generate single cell clones, which can be then used for somatic cell nuclear transfer (SCNT) to generate PERV C free animals.

II. Review of Literature

1. Xenotransplantation

The term transplantation designates the replacement of cells, tissues and organs between individuals of the same species, allotransplantation, or between different species, xenotransplantation. Allotransplantation is the preferred solution for patients who are waiting for an organ graft, but the number of patients is much higher than the number of potential organ donors (PUGA YUNG et al., 2017). For example, more than 100.000 patients only in the United States are currently waiting for kidney transplants and a presumably larger number of patients around the world are on waiting lists (WIJKSTROM et al., 2017). Likewise, there is a big shortfall of cells or tissues like corneal and pancreatic islet for millions of blind or diabetic patients (EKSER et al., 2015). Xenotransplantation might fill this gap. The practice of xenotransplantation started already in the 19th century using skin, cornea, cells, slice of tissue from different animals into humans; after establishing techniques for blood vessel anastomosis whole organ xenotransplantation became an option as well (COOPER et al., 2015). Clinical attempts of renal xenotransplantation were performed from the beginning of the 20th century but due to inadequate knowledge of rejection mechanisms and insufficient immunosuppression the patients died within a very short period of time (SAMSTEIN & PLATT, 2001). 60 years later the survival of a patient receiving a kidney transplant from chimpanzee was prolonged till 9 months when using strong immunosuppressant drugs (REEMTSMA et al., 1964). Although primates are the closest relatives to humans, they are not seen optimal xenograft donors because of the high risk of infectious agent transmission, ethical reasons, size of organs, expensive breeding and long reproduction time (COOPER et al., 2002). Instead, the pig is seen nowadays as the preferred donor species because of the high similarities to humans in physiological, anatomical and immunological terms (SACHS, 1994).

2. Xenograft rejection: Hurdles and approaches to overcome them by genetic modification or systemic drugs

When transplanted into a primate a pig organ becomes a target of different immunological responses that cannot be sufficiently controlled with immunosuppressant therapy alone (COOPER et al., 2016). However, the option of genetic modification offers a way for overcoming immunological rejection of xenografts either by deleting unwanted genes or adding desired genetic functions (SACHS & GALLI, 2009; COOPER, 2015).

2.1. Hyperacute rejection

The first reason of the xenograft loss is the hyperacute rejection (HAR), which normally happens immediately after transplantation due to the interaction of preformed anti-pig-antibodies with targets on the graft cells. The main responsible epitope for HAR is the galactosyl α-(1,3)-galactosyl β-1,4-N-acetyl glucosaminyl (α-Gal), a carbohydrate that is present in high quantity on pig cells (TANEMURA et al., 2000) and other mammals species, while it is lacking in humans, apes and Old World monkeys (GALILI et al., 1988). The production of antibodies against α-Gal epitopes in humans is induced by bacteria in the intestinal flora (GALILI et al., 1984). These antibodies bind α-Gal epitopes on the vascular endothelial cells of the xenograft and trigger the activation of complement system and coagulation (YANG & SYKES, 2007). Eventually this process results in vascular thrombosis, necrosis and finally xenograft rejection (PIERSON, 2009). To overcome or to reduce HAR, different approaches were examined, but the most efficient one proved the disruption of *GGTA1*, the gene which is responsible for the synthesis of α-Gal on the porcine endothelial cells surfaces. After the knockout of *GGTA1* on one allele (DAI et al., 2002; LAI et al., 2002), pigs were produced that did not express α-Gal after homozygous deletion of *GGTA1* (PHELPS et al., 2003). Further *GGTA1* knock out (GTKO) pigs were produced with different methods (WATT et al., 2006; KLYMIUK et al., 2010; HAUSCHILD et al., 2011). Since GTKO effectively prevented hyperacute rejection of porcine xenografts and significantly prolonged their survival in non-

human primates this approach became an absolute prerequisite in xenotransplantation practice (KUWAKI et al., 2005; SHIMIZU et al., 2008). Other glycosylic xenoantigens induce binding of human antibodies on the porcine cell surface in addition to α-Gal (PADLER-KARAVANI & VARKI, 2011). Very important among them is N-glycolylneuraminic acid (Neu5Gc), which is not produced in humans due to the inactive *CMAH* gene (TANGVORANUNTAKUL et al., 2003), and an oligosaccharide synthesized by β-1, 4-*N*-acetyl-galactosaminyl transferase 2 (B4GALNT2), which is homologous to the enzyme that synthesizes the SDa blood group antigen in humans (BYRNE et al., 2018). Tests for antibody-binding with cells from pigs missing both *GGTA1* and *CMAH* genes shown a decreased bound of IgM compared to GTKO pig cells (LUTZ et al., 2013). On the other hand, human embryonic kidney (HEK-B4T) cells expressing B4GALNT2 when sensitized with primate serum after cardiac xenotransplantation demonstrated a 20-fold increased antibody binding and complement-mediated cell lysis (BYRNE et al., 2014). For both genes, however, it is unclear if their deletion is an ultimate necessity for xenotransplantation.

2.2. Vascular rejection

As shown, humoral rejection of xenograft by preformed antibodies can be attenuated, but the release of induced antibodies can still activate xenograft endothelium and result in complement mediated acute vascular rejection (COWAN et al., 2009). The complement system is a set of proteins expressed on the surface of cells and controlled by complement regulatory proteins (CRPs) with the function to protect from complement mediated injury (SAMSTEIN & PLATT, 2001). Porcine cells have similar complement regulatory proteins (CRPs) as humans, but their endogenous expression is not able to inhibit human complement (COOPER et al., 2016). To overcome this obstacle, it is tempting to generate genetically modified pigs that express human CRPs. Different of such attempts on the most relevant CRPs have been documented.

Human decay-accelerating factor (hDAF or CD55) is expressed on the surface of vascular endothelial cells and is able to inhibit complement activation (KIM et al.,

2012). Moreover, this regulatory protein is a ligand of CD97, which is expressed on granulocytes and monocytes and can inhibit natural killer (NK) cells and lymphocyte T cells (DHO et al., 2018). Therefore, different groups generated pig lines that express CD55 (LANGFORD et al., 1994; LAVITRANO et al., 2002) and others established double transgenic pigs with lack of *GGTA1* gene in addition to CD55 expression (MCGREGOR et al., 2012; AZIMZADEH et al., 2015).

Another important CRP is the human membrane cofactor protein called CD46 that is responsible for the inactivation of C3b and C4b (SEYA et al., 1999). CD46 expression prolonged survival of baboons who received CD46 transgenic porcine hearts for up to 23 days, while wild-type grafts were rejected within 90 minutes (DIAMOND et al., 2001). A kidney from a CD46 transgenic pig transplanted without immunosuppression demonstrated xenograft protection from complement mediated lysis (LOVELAND et al., 2004). A transgenic pig line that expresses a combination of CD46 and CD55 plus a third complement regulatory protein, CD59, which inhibits formation of the complement membrane attack complex, demonstrated that the expression of more CRPs results in a better xenograft protection against human complement mediated injury (FODOR et al., 1994; NIEMANN et al., 2001; ZHOU et al., 2005).

To further limit activation of vascular endothelial cells, damage and acute vascular rejection of xenograft, pigs were established that expressed anti apoptotic and anti-inflammatory molecules like heme oxygenase-1 (HO-1) and tumor necrosis factor alpha-induced protein (A20) (OROPEZA et al., 2009; PETERSEN et al., 2011). HO-1 reduces formation of pro-inflammatory factors, NK cells activity and its overexpression diminishes IgM deposition and thrombus formation (YEOM et al., 2012). HO-1 and A20 were also used in addition to CRPs to generate multiple transgenic pigs (AHRENS et al., 2015a; FISCHER et al., 2016).

2.3. Cellular rejection

Even if antibody-mediated rejection is sufficiently controlled, xenografts become object of delayed xenograft cellular rejection mediated by T lymphocytes, macrophages and natural killer cells (cellular rejection) (SCHNEIDER & SEEBACH, 2008). Again, genetic modifications of pigs might overcome these hurdles. T cell

mediated response against the vascular endothelium of the transplanted organ is triggered either directly by pig SLA class I and II proteins or indirectly when porcine peptides are presented by the recipient's major histocompatibility complex (MHC) class II (HIGGINBOTHAM et al., 2015). Transgenic pigs were produced that express a human dominant negative mutant MHC class II trans activator gene (CIITA-DN) in order to down regulate the expression of SLA class II on the porcine cells (IWASE et al., 2015). This transgene decreased significantly the $CD4^+$ T-cell response against aortic endothelial cells from CIITA-DN pigs (pAECs) (HARA et al., 2013). Alternatively, cellular rejection might be also avoided by preventing T-cells activation through the expression of cytotoxic T lymphocyte-associated antigen (CTLA4) fused to the Fc of human IgG (CTLA4-Ig) (PHELPS et al., 2009). CTLA4-Ig inhibits the interaction between T-cells B7 receptor and CD80 and CD86 ligands during antigen presentation (KOSHIKA et al., 2011; ZHANG et al., 2015). As CTLA4-Ig does not fully prevent T-cells activation, a more potent variant of this protein (LEA29Y) was developed by the exchange of two amino acids (LARSEN et al., 2005). Specific expression of LEA29Y in pancreatic β-cells (INSLEA29Y) resulted in prolonged islet xenograft function (KLYMIUK et al., 2012; BUERCK et al., 2017).

Another promising target is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This ligand interacts with different receptors, induces apoptosis in most transformed cells, downregulates immune responses via cell cycle inhibition of lymphocytes (SONG et al., 2000) and of plasma cells after the end of antibodies secretion (URSINI-SIEGEL et al., 2002). In vitro and in vivo studies demonstrated the capacity of TRAIL to induce apoptosis in Jurkat and Hut cells (immortalized T lymphocytes) (KLOSE et al., 2005) and to inhibit human T lymphocytes cell cycle when exposed to dendritic cells obtained from pigs that express TRAIL on a *GGTA1* KO/CD46 background (KEMTER et al., 2012). Moreover, when expressed on NK cells TRAIL eliminates activated $CD4^+$ T lymphocytes and neutrophils (RENSHAW et al., 2003; SCHUSTER et al., 2014).

NK cells distinguish self from non-self by recognizing MHC class I through inhibiting receptors (RAULET et al., 2001; CARRILLO-BUSTAMANTE et al., 2016). One of them is CD94/NKG2, which interacts specifically with the human leukocyte antigen

(HLA-E) (LEE et al., 1998). NK cells were demonstrated to infiltrate and destroy porcine tissues (INVERARDI et al., 1992; KHALFOUN et al., 2000) and to activate vascular endothelial cells (GOODMAN et al., 1996; VON ALBERTINI et al., 1998). Thus, HLA-E was expressed on pig endothelial cells in order to decrease NK cells response (WEISS et al., 2009). In vitro experiments and different ex vivo studies with pigs expressing HLA-E in different constellations with other transgenes confirmed the protective effect against NK cell-mediated cell lysis or xenograft rejection (LAIRD et al., 2017; ABICHT et al., 2018; PUGA YUNG et al., 2018).

2.4. Coagulation

In addition to triggering complement-mediated rejection, induced xenoantibodies can also activate endothelial cells to express tissue factor (TF), thus switching the anticoagulant state into a procoagulant one (LIN et al., 2009). TF binds coagulation factor VII, which auto-cleaves (TF-FVII/FVIIa) activating factor IX and factor X of the coagulation cascade. Activation of this pathway can be counteracted by tissue factor pathway inhibitor (TFPI) by inhibiting the TF-FVIIa complex (LWALEED & BASS, 2006). TFPI fused to the membrane anchor of CD4 receptors was thus expressed on the cell surface of mouse fibroblasts and demonstrated that TFPI does not lose activity in the tethered form (RIESBECK et al., 1997). In a murine heart transplantation model tethered TFPI was expressed on donor ECs and showed that platelets and fibrinogen were in normal levels and that coagulation was prevented (CHEN et al., 2004). Lin and colleagues demonstrated the ability of TFPI to inhibit TF activity in vitro (LIN et al., 2010) and knockdown of TF expression showed to reduce coagulation process in transgenic pigs (AHRENS et al., 2015b).

In the procoagulant state, porcine endothelial cells release von Willebrand factor, which induces the aggregation of human platelets and results in deposition of fibrin polymers due to thrombin enzymatic activity (SCHULTE AM ESCH et al., 2005). Normally this coagulation process is blocked by activated protein C, which is produced by a complex of thrombomodulin (TBM) on the vessel wall and thrombin (TB) in the blood stream. In the xeno situation, however, porcine TBM and human TB fail to activate protein C (TAYLOR et al., 2001; ROUSSEL et al., 2008). Expression of

human TBM (hTBM) on the surface of porcine endothelial cells led to high quantity production of protein C (KOPP et al., 1998). Similarly, hTBM transgenic pig fibroblasts were effective in preventing clotting (PETERSEN et al., 2009; MIWA et al., 2010). In addition to thrombomodulin, endothelial cell protein C receptor (EPCR) is important for the activation of protein C. In large vessels EPCR increases the concentration of protein C in the vicinity of the TM/TBM complex but inside capillaries where the initial thrombosis process in xenografts takes place the concentration of EPCR is very low (STEARNS-KUROSAWA et al., 1996; FUKUDOME et al., 1998; TAYLOR et al., 2001). As its function is further decreased by the presence of non-Gal antibodies (BYRNE et al., 2011), EPCR expression became an option to overcome coagulation disorders (LEE et al., 2012; IWASE et al., 2014a; GOCK et al., 2016).

Activated platelets secrete adenosine diphosphate, which recruits and aggregates other platelets and boosts the thrombosis mechanism (IWASE et al., 2014b). CD39, a nucleoside triphosphate diphosphohydrolase (NTPDase), is expressed on inactive endothelial cells and hydrolyzes ATP and ADP into AMP (KACZMAREK et al., 1996). Studies in mouse models have shown that the overexpression of CD39 can prevent renal ischemia reperfusion injury (CRIKIS et al., 2010) and thrombosis although bleeding time in CD39 transgenic mice was prolonged causing hemorrhagic shock (DWYER et al., 2004). The protection through CD39 was tested in pig as well as in an in vivo model of myocardial ischemia reperfusion injury where reduction in infarct size was observed (WHEELER et al., 2012).

2.5. Systemic drugs

Using systemic drugs was the first approach tested to prevent xenograft rejection even before genetically modified pigs became available (REEMTSMA et al., 1964). As these drugs were highly immunosuppressant and highly toxic for the recipient, this regimen was not clinically acceptable. As described in the previous paragraphs, genome engineering of the xenograft donor has the ability to resolve many immunological hurdles in xenotransplantation, but they until now are not sufficient to fully prevent rejection. Therefore, it is still necessary to modulate the adaptive immune

response by immunosuppressive drugs. Typically such drugs are directed to limit T lymphocytes activation by blocking costimulatory signals via anti-CD154 monoclonal antibodies (HIGGINBOTHAM et al., 2015) and by the depletion of B lymphocytes through proliferation inhibition and apoptosis induction via anti-CD20 mAbs (MATHAS et al., 2000; MOHIUDDIN et al., 2012). Because of its pro-thrombotic side effects (KUWAKI et al., 2004), anti-CD154mAb is preferentially replaced by anti-CD40 mAb (BUHLER et al., 2000). T- and B-cells activation is prevented by continuous application of mycophenolate mofetil (MMF) in combination with cyclosporine A and corticosteroids (COZZI et al., 2003; IWASE & KOBAYASHI, 2015).

2.6. The state of the art

Many combinations of genetically modified donor pigs and systemic immunosuppression have been explored, but until recently success was limited. Nowadays, however, new optimism has taken the field. This is best exemplified by heart xenotransplantation. In a heterotopic heart transplantation setting in baboons, grafts survived for more than 2 years using GTKO, hCD46, hTBM pigs and the immunosuppressive regimen described above (MOHIUDDIN et al., 2012; MOHIUDDIN et al., 2016). However, in this case the heart was transplanted in the abdomen and did not have any physiological function. In a life-supporting setting survival time was prolonged till 6 months (LANGIN et al., 2018). Life-supporting kidney transplantation into macaques reached 499 days using a GTKO/CD55 donor pig in association with anti-CD154mAb, anti CD4mAb, MMF and corticosteroids (KIM S, 2017). In contrast, liver and lung xenotransplantation seems to be more difficult. The longest liver survival time is 29 days (SHAH et al., 2017), when livers from GTKO pigs were transplanted into baboons, accompanied by continuous infusion of prothrombin in addition to an immunosuppressive regimen consisting in anti-CD40mAbs, ATG and corticosteroids. For lung, donors with different combinations of genetic modifications were tested and 9 days maximal survival time was achieved for GTKO pigs and hCD47/hCD55 expression in alveolar epithelial and capillary cells and pulmonary arterial endothelium (WATANABE et al., 2018).

Besides whole organ xenotransplantation, progress was made for transplantation of pancreatic Langerhans islet cells, important for the type 1 diabetes treatment. The longest control of blood glucose homeostasis for up to 6 months was achieved through intraportal transplantation of islets isolated from Seoul National University miniature pigs into streptozotocin-induced diabetic rhesus monkeys, using a moderate immunosuppressive protocol with anti-CD154 mAbs, ATG and sirolimus (SHIN et al., 2015). To reduce administration of immunosuppressive drugs, physical separation of the islets from the recipient was investigated (CUI et al., 2009; DUFRANE et al., 2010). Islets from Goettingen minipigs were encapsulated in BetaO_2 devices and transplanted for 6 months into nonhuman primates (LUDWIG et al., 2017). Glucose-regulated insulin secretion and absence of immunoreaction events without requirement of immunosuppressive regimen was demonstrated. Till now only the transplantation of islets was tested in a clinical trial. In 2009 in New Zealand patients with severe unaware hypoglycemia were transplanted with Diabecell or rather neonatal islets encapsulated in alginate microcapsules. The patients showed amelioration in the severity of the hypoglycemic episodes and stable glucose levels (GARKAVENKO et al., 2012).

3. Safety of xenotransplantation

With the recent progress in preclinical studies, the application of xenotransplantation in human patients appears realistic in the next decade. However, for such an undertaking, safety concerns need to be resolved. Most importantly, transmission of pathogens from the donor to the human recipient needs to be prevented. Xenozoonose are assumed to represent an even higher safety risk than infection in allotransplantation (DENNER & TONJES, 2012), although human allotransplantation resulted in infection with human immunodeficiency virus, human cytomegalovirus (HCMV), hepatitis C virus (HCV), human T cell lymphotropic virus (HTLV), Mycobacterium tuberculosis, Candida and other bacteria and fungi at considerable rates (FISHMAN et al., 2012b). The unforeseeable consequence of transmission of infectious agents across species barriers raised severe concerns about the clinical perspectives of xenotransplantation. The use of systemic immunosuppressant regimen can further

facilitate the infection ability of microorganisms. For instance, porcine cytomegalovirus (PCMV) might be dangerous for the human recipient as it was shown to infect a human fibroblasts cell line in vitro (WHITTEKER et al., 2008). Moreover, the infection with PMCV was associated with reduced graft survival in kidney transplantation into baboons (YAMADA et al., 2014) or into Cynomolgus monkeys (SEKIJIMA et al., 2014). Absence of PMCV coincided with prolonged porcine heart survival and prevented consumptive coagulopathy in the recipient (MUELLER et al., 2004).

On the other hand and in contrast to allotransplantation, where microbiological screenings of donors are difficult to apply due to the narrow time window between explantation and implantation (NELLORE & FISHMAN, 2018), there would be enough time to screen xenograft donors before transplantation (DENNER & MUELLER, 2015).

To eliminate the risk of infection in xenotransplantation, the donor has to be free from microorganisms like bacteria, fungi, protozoa and especially viruses. Therefore, it is important to establish adequate and sensitive screening protocols (FISHMAN & GROSSI, 2014). For addressing these issues, the World Health Organization (WHO) recommended to regulate safety aspects of Xenotransplantation before clinical application (DENNER, 2017). Regulations and guidelines considering the "Precautionary principle", as postulated by the Food and Drug Administration (FDA) and the European Medicines Agencies (EMEA) specify surveillance programs and safety standard operation procedures concerning screening for the source pigs and materials derived from them (FISHMAN et al., 2012a; SPIZZO et al., 2016; NOORDERGRAAF et al., 2018; TONJES, 2018). Evidently, designated pathogen-free (DPF) breeding conditions are required for the production of donor pigs. Most microorganisms like bacteria and fungi as well as many viruses can be eradicated with antibiotics, anti-fungal treatments, vaccines, antiviral drugs and DPF conditions can be established by Cesarean delivery and early weaning (EGERER et al., 2018). Unfortunately, some pathogens such as the porcine gammalymphotropic herpesvirus (PLHV) cannot be eradicated by these means and other methods have not been suggested so far (MUELLER et al., 2005; DENNER & MUELLER, 2015).

Maintenance of DPF status has to be confirmed routinely by regular screening of the production herd for infectious microorganisms and viruses (SPIZZO et al., 2016). For example, the donor herd used in New Zealand for the first clinical trial of porcine islet cells transplantation were quarterly or annually tested for 10 bacteria, 15 viruses and 1 protozoon (WYNYARD et al., 2014). In addition, to assure a clean porcine material for xenotransplantation, sensitive detection assays and new screening methods are important for the identification of new virus infections as they could be present in the xenograft in low quantity or could be in a latent phase of their life cycle (DENNER,2017; HARTLINE et al., 2018).

3.1. Retroviruses

Common eradication strategies fail to remove viruses that are permanently integrated into genome, specifically porcine endogenous retroviruses (PERV) (ONIONS et al., 2000). Among RNA viruses, retroviruses have the special feature of being able to reversely transcribe their RNA genome into double stranded DNA and to integrate this DNA as provirus into the host genome. Retroviruses comprise viruses with relatively complex genomes like lentiviruses, deltaviruses and spumaviruses as well as viruses with very simple genomes like alpha, beta, gamma and epsilon retroviruses (WEISS, 2006). Viruses that contain RNA genomes were discovered in 1961 when the Rous sarcoma virus was isolated (CRAWFORD & CRAWFORD, 1961). The name retrovirus was chosen after that reverse transcriptase enzyme was discovered (TEMIN & MIZUTANI, 1992). All retroviruses consist of phospholipid envelope which embeds viral envelope (env) proteins. env constitutes two parts, the transmembrane (TM) component which is anchored in the membrane as well as the surface (SU) protein which facilitates uptake into host cells and, thus, defines the tropism of the virus (COFFIN, 1997; VOGT, 1997). Within the phospholipid envelope, the virion consists of a capsid formed by the nucleocapsid protein. This capsid protects the core of the virus comprising the reverse transcriptase, protease and integrase as well as two copies of the RNA genome. In addition to protecting the virus core, nucleocapsid proteins play an important role during reverse transcription due to their chaperone function (RENE et al., 2018). The virions have in general a diameter of 80-100 nm,

but the protein content of the core is different in the distinct retrovirus genera. Simple retroviruses do only comprise three different genes, *gag*, *pro/pol* and *env* encoding the nucleocapsid, reverse transcription components and the envelope proteins. In contrast, more complex retroviruses encode additional genes which, for example, facilitate the integration into non-dividing cells (COFFIN, 1997).

The life cycle of a virion starts with the infection of a host cell. In the early phase the virion envelope fuses with the host cell membrane through binding of receptors and attachment factors on the host cell surface. Upon internalization into the cytoplasmic space, the virus starts its reverse transcription to a double strand cDNA which integrates as provirus in the host chromosomal DNA by the viral integrase (NISOLE & SAIB, 2004; JERN & COFFIN, 2008). The dsDNA of simple retroviruses like Murine Leukemia Virus (MuLV) needs the breakdown of nucleus membrane for entering the nucleus (GOFF, 2001). Once integrated, the provirus starts to transcribe the entire viral genome taking advantage of the host RNA polymerase II as well as non-coding small RNAs which have modulation activity on viral and cellular gene expression (JERN & COFFIN, 2008; ZHANG et al., 2018). From the viral RNA, the cellular translation machinery translates the viral proteins. The virion's elements are transported to the cytoplasmic space nearby the phospholipid bilayer where the assembly process of the viral core is initiated. The capsid is then enveloped by the host cell membrane, comprising the TM and SU, releasing the virion via membrane fission or budding as a new viral particle (COFFIN, 1997; WELSCH et al., 2007).

Mostly, retroviruses infect somatic cells and, thus, are transmitted horizontally from host cell to host cell. Therefore, they are called exogenous retroviruses. When retroviruses infect germline cells, the integrated provirus becomes an endogenous retrovirus (ERV). Upon infection, germline cells can evidently produce intact virus particles, similar to somatic cells. In addition, however, proviruses can be transmitted to the offspring as integral components of the germ cell genome. Therefore, ERV are said to be vertically transmitted (KATZOURAKIS & GIFFORD, 2010).

3.2. Endogenous retroviruses

The first ERV have been identified about 10 years after the discovering of retroviruses when vertical transmission of murine leukemia virus (MLV), murine mammary tumor virus (MMTV) in the laboratory mouse (*Mus musculus*) and avian leukosis virus (ALV) in the domestic fowl (*Gallus gallus*) was documented (WEISS, 2006). With the establishment of molecular genetic techniques, like DNA hybridization, PCR and DNA sequencing, ERV were found in any vertebrate examined so far. In humans, ERV represent up to 8% of the genome, illustrating that the integration of retroviruses is a frequent process and relevant in evolution (LANDER et al., 2001; GIFFORD & TRISTEM, 2003; MAGER & STOYE, 2015). ERV have a common genetic composition comprising an LTR-*gag*-*pro/pol*-*env*-LTR structure. Once integrated into the germline, ERV behave like other transposable elements, which might result in the increase of copy numbers (GRIFFITHS, 2001). On the other hand, the faith of ERV after integration into the germ line is dominated by the effect of ERV on host evolution. In the case of a detrimental effect, negative selection causes the extinction of the host and, thereby, also of the provirus. In the case of a neutral selection, ERV remain in the genome, are transmitted vertically and accumulates mutations like insertions, deletions, rearrangements and epigenetic silencing, leading to inactivation of ERV and the loss of the ability to produce infectious virions (BOEKE & STOYE, 1997). Importantly, parts of ERV might also have beneficial function on host and, therefore, underlie positive selection (VARELA et al., 2009). One of the best evidence of beneficial function is the placental expression of HERV-W and HERV-FRD *env* genes (the only ORF conserved) which generate the fusion of cytotrophoblast in human (MI et al., 2000; BLAISE et al., 2003). Similar fusogenic proteins derived from ERV are found in murine species as well (DUPRESSOIR et al., 2005). Another example for beneficial ERV function is the specific expression of the salivary amylase gene (AMY1C) in the parotid gland due to the presence of the endogenous retroviral derived element ERVA1C in the proximity of the *AMY1C* gene promoter (TING et al., 1992). Proteins expressed from ERV genes can also induce resistance against exogenous retrovirus infection by behaving as restriction factor. The binding of *env* product to a cellular receptor interferes and inhibits the surface attachment of new virus through

downregulated expression and saturation of cell receptors (MCDOUGALL et al., 1994; WU et al., 2005). The murine Friend virus susceptibility -1 (Fv1) gene for example has 40% sequence similarity with the gag gene of murine ERV-L (MERV-L) and inhibits MLV and non-MLV infection (SANZ-RAMOS & STOYE, 2013; YAP et al., 2014).

Like in other mammals, ERV were found in the pig (PERV), the preferred species for xenotransplantation. Different types of PERV from α, β and γ classes have been identified, but only γ type viruses have been shown to be infectious (PATIENCE et al., 1997; PATIENCE et al., 2001; GROENEN et al., 2012). Replication competent PERV were observed for the first time in a porcine kidney cell line (PK15) (ARMSTRONG et al., 1971). The determination of PERV copy number by genomic mapping, cDNA screening or droplet digital polymerase chain reaction (ddPCR) in different pigs indicate that there is a different number of PERV among pig breeds and that the number of PERV ranges from 10 to 134 (AKIYOSHI et al., 1998; HERRING et al., 2001; FIEBIG et al., 2018). As in all ERV, the general genomic organization comprises 3 ORF containing genes enclosed by non-coding sequences on the 5' and 3' end. Reverse transcription is induced by the binding of a specific tRNA in the so-called primer binding site near the 5´ end of the viral RNA genome. For PERV A and PERV B this tRNA is a $tRNA_{Gly}$ and for PERV C is $tRNA_{Pro}$ (VOGT, 1997; BARTOSCH et al., 2002). Synthesis of the primary DNA strand continues to the 5´ end, including the R and U5 region. Then the primary strand is transferred to the 3´end of the viral genome, where an identical R sequence is found. First strand synthesis is again continued to the 5´end, resulting in identical U3-R-U5 elements (LTR) at both ends of the provirus. Therefore, the PERV comprise the common LTR-*gag*-*pro/pol*-*env*-LTR structure (STOYE, 2012; KIMSA et al., 2014). Importantly, the initially identical LTR of a provirus facilitates estimating the time point of integration, because mutations in the LTR accumulate over time, when neutral selection is assumed. Based on the molecular clock hypothesis this event found state approximately 7,6 million years ago (TONJES & NIEBERT, 2003). Between the 5´LTR and the coding region of gag, non-translated segments of regulatory function are located. These components are the primer binding site (PBS), the splice donor (SD) site as well as the packaging

signal psi Ψ which is essential for the packaging of the viral RNA genome into the virion capsid (CHOI et al., 2015; LOPATA et al., 2018). The presence of all 3 intact viral genes, *gag, pro/pol* and *env*, in the coding region is prerequisite for the formation of replication competent ERV. The *gag* gene encodes for a precursor protein that forms the viral core. The *pro/pol* gene encodes protease, reverse transcriptase and integrase enzymes, responsible for the generation of dsDNA and its integration into host chromosomes (DENNER & TONJES, 2012). The *env* gene encodes the surface and transmembrane envelope protein and is expressed as a polyprotein that is glycosylated in the host cell on multiple sites (AKIYOSHI et al., 1998; LEE et al., 2006). Importantly, the *gag* and *pro/pol* genes are highly conserved among active PERV whereas significant differences have been found in the *env* gene, resulting in different tropism and the consequent definition of the subfamilies A, B and C (LE TISSIER et al., 1997; AKIYOSHI et al., 1998; TAKEUCHI et al., 1998; PATIENCE et al., 2001).

3.3. Pathogenic potential of ERV

Replication competent ERV can lead to pathological conditions and similarly to most retroviruses, most ERV have been identified upon their pathogenic effect on the host. Like already described for exogenous retroviruses, ERV can cause disease and cancer via different mechanisms (STOYE, 2012; KASSIOTIS, 2014). Active ERV integrate in the host genome in a random manner which can result in an insertional activation of protooncogenes, downregulation of tumor suppressor genes, mutation or destruction of genes with consequent loss or gain function (MAGER & STOYE, 2015). It is of note that endogenized forms of PERV-related murine leukemia virus (MuLV), feline leukemia virus (FuLV) and gibbon ape leukemia virus (GaLV) have tumorigenic and immunodeficiency potential. These retroviruses possess a region of transmembrane envelope protein (TM), called immunosuppressive domain, which was shown to inhibit in vitro a few of different immune responses like mitogen stimulated lymphocyte proliferation and mitogen-induced proliferation of human PBMC (HARAGUCHI et al., 1997; DENNER, 1998; TACKE et al., 2000). For PERV itself however, tumorigenic and immunodeficiency potential has not been described so far (ROSENBERG & JOLICOEUR, 1997; DENNER, 1998; TACKE et al., 2000).

Although, in Munich miniature swine Troll, a model for hereditary cutaneous melanoma, high expression of recombinant PERV-A/C has been detected in the diseased pigs compared to healthy controls (DIECKHOFF et al., 2007). Even defective ERV can be involved in disease when they recombine with an exogenous virus (ROSENBERG & JOLICOEUR, 1997). Another possibility of pathogenic effects of ERV is when they express viral proteins that act as a superantigen and, thus, cause autoimmune disease (SICAT et al., 2005; KATOH & KURATA, 2013). Even solo LTR can influence genome activity by acting as an enhancer to adjacent genes (JERN & COFFIN, 2008; WEISS, 2016).

Considering all these potential pathological effects and the potential transmission of retroviruses from one species to another as well as the fact that PERV are replication competent, it is important to consider pathogenicity risks of PERV in xenotransplantation (ARMSTRONG et al., 1971; DENNER, 2007). The finding that different human cell lines (PATIENCE et al., 1997) as well as primary human endothelial cells (MARTIN et al., 2000) were infected by PERV released from the PK15 porcine kidney cell lines was of outstanding importance. In the field of xenotransplantation in addition, human cell lines were permissive to PERV produced from primary porcine peripheral blood mononuclear cells (PBMC) (WILSON et al., 1998) as well as from porcine primary aortic endothelial cells (PAEC) (MARTIN et al., 1998). Specifically, PERV-A and PERV-B are able to infect human cells in vitro as well as pig cells (LE TISSIER et al., 1997). In contrast, PERV C alone is an ecotropic ERV that infects only pig cells, (AKIYOSHI et al., 1998; TAKEUCHI et al., 1998; PATIENCE et al., 2001). When recombining with PERV A, however, PERV C can contribute to a super-infectious PERV A/C, that highly effectively infects human cells (OLDMIXON et al., 2002; BARTOSCH et al., 2004). The enhanced infectivity of PERV-A/C (titer 500 fold higher than PERV A) is associated with mutation of isoleucine into valine in the 140 position in the PERV A derived receptor binding site (RBD) and the PERV C derived proline reach region (PRR) (HARRISON et al., 2004). Moreover, 4 specific residues in the PERV C derived terminus of SU promote infection of human cells through the interaction with receptors HuPAR 1 and HuPAR 2. Therefore, it can be postulated that spontaneous mutations can induce switching of

ecotropic PERV C in to a human-tropic PERV (ERICSSON et al., 2003; GEMENIANO et al., 2006; ARGAW et al., 2008). In addition, increasing the viral titer through serial passaging in human cells facilitates productive infection on human primary cells like pulmonary artery endothelial cells (HPAEC), aortic endothelial cells (HAEC), human peripheral blood mononuclear cells (PBMC) and human lymphocytic cell lines C8166 by PERV A/C (SPECKE et al., 2001b; SPECKE et al., 2001a). This increase in viral titer of PERV is related to the extension of the LTR length as a consequence of multiplication of direct repeats, which act as transcription factor binding sites and therefore enhance the activity of retroviral promoters (DENNER et al., 2003; DENNER, 2015). The incapability of PERV produced freshly from porcine cells to infect hPBMCs is due to the inability of PERV to overcome protective factors like APOBEC and Tetherin (DORRSCHUCK et al., 2011; BAE & JUNG, 2014; DENNER, 2015). This finding might explain why till now there are no evidences for PERV infection in different patients who have received porcine materials. It is of note that any of these studies have shown the transmission of the porcine-human species barrier only in vitro. In contrast, retrospective studies in over 200 human recipients of porcine skin, fetal neuronal cells, encapsulated and neonatal islet, extracorporeal kidney and liver perfusion, aortic valves etc. did not reveal PERV RNA, DNA or antibodies against PERV (PARADIS et al., 1999).

Studies of PERV infection and transmission were also done in other species, including non-human primates in which xenotransplantation preclinical studies are performed. Again, neither antibodies production and virus reproduction nor transmission of PERV has been demonstrated so far (PLOTZKI et al., 2015; DENNER, 2018). The lack of PERV infection seems to result from non-functional cell receptors for the first PERV-cell interaction (MARTINA et al., 2006; MATTIUZZO et al., 2007; MATTIUZZO & TAKEUCHI, 2010; PLOTZKI et al., 2015). Therefore, it remains important to study the pathogenicity and infection mechanisms of PERV and to minimize or delete the infection risks from PERV. In particular, it will be important to provide donor pigs that are free of PERV C to avoid the generation of high titer of PERV A/C.

4. PERV deletion approaches

To minimize the infectious risk from PERV for pig xenograft recipients the first thing to do is to select donor pig that have the lowest possible copy number of PERV C in their genome. By using screening methods like PCR, nested PCR, real time PCR (RT-PCR), reverse transcriptase RT-PCR or co-culture pig-human cell assays it was found that the copy number of PERV C in pigs is generally low and even PERV C free pigs have been identified (DENNER et al., 2009; KAULITZ et al., 2011b; FIEBIG et al., 2018). Most existing genetically modified donor pigs for xenotransplantation, however, contain PERV C proviruses. Different PERV inactivating approaches might reduce the risk of transmission. As for other gamma retroviruses, the establishment of vaccines against PERV can protect the human recipient. First PERV vaccines have been developed on the basis of neutralizing antibodies against membrane proximal external region in the N-terminal of the TM envelope protein (FIEBIG et al., 2003; KAULITZ et al., 2011a; WAECHTER & DENNER, 2014). Simultaneous immunization with TM and SU proteins revealed a higher neutralizing activity as with the respective proteins alone (DENNER et al., 2012). Other strategies target PERV at the nucleic acid level. For example, the inhibition of PERV expression might be mediated through short interfering RNA (siRNA), a mechanism which is known to be common in eukaryotic cells to suppress protein synthesis and to act as anti-viral responses (CAPLEN et al., 2001). siRNAs are double strand RNA components with a length of about 22 nucleotides. They are recognized by RNA induced silencing complex (RISC) which induces the degradation of RNA targets that are homologous to the siRNA (YANG et al., 2000). Different studies were conducted by introducing siRNA against conserved regions of PERV into PERV-infected human cell lines (KARLAS et al., 2004), porcine cells (MIYAGAWA et al., 2005) and in vivo in transgenic pigs (DIECKHOFF et al., 2008; RAMSOONDAR et al., 2009). All these attempts worked efficiently and demonstrated that pol siRNA expressed in transgenic pigs reduced PERV expression over 3 years without adverse effects (SEMAAN et al., 2012).

Alternatively to the RNA level, PERV can be inactivated also at the genomic level by the novel technique of genome editing. Such an attempt provides the most sustainable

effect by introducing deleterious mutations into the provirus. This inactivation can be induced by zinc-finger nucleases (ZFNs) which induce double strand breaks (DSB). After this break, the affected cell activates DNA repair processes like the error prone non-homologous end joining (NHEJ) (KIM et al., 1996b; RAMALINGAM et al., 2013) or the faithful homologous recombination (HR) (RICHTER et al., 2013). NHEJ often results in small deletions or insertions in the cut location which often cause a frameshift and the consequent generation of stop codons while the HR mechanism can be advantageous to introduce new DNA sequence having flanking regions homologous to the sequences present upstream and downstream of the DSB break (RAMALINGAM et al., 2013; MAHFOUZ et al., 2014). Considering the successful generations of knock out pigs using gene editing with ZFNs (KIM et al., 1996b; HAUSCHILD et al., 2011; BAO et al., 2014) this technology was used to delete PERV proviruses integrated in the genome. The only attempt for ZFN reported so far, however, failed because the multiple DSB destabilized the cell genome bringing the cell to die (SEMAAN et al., 2015). Meanwhile, ZFN and the related TALEN technologies have been outdated by the much more flexible CRISPR/Cas endonuclease.

4.1. Inactivation by CRISPR/Cas9

Described for the first time in 1987 in *Escherichia coli*, this system is a natural adaptive immune response that bacteria and archaea have developed to protect themselves from viral and plasmid sequences (ISHINO et al., 1987). First step in this adaptive process is the integration of exogenous viral or plasmid DNA at a length of 20-30 bp into the so-called clustered regularly interspaced short palindromic repeats (CRISPR) locus in the bacterial genome. After integration of the exogenous DNA, the RNAs (crRNA) are expressed and activate CRISPR associated endonucleases(Cas) RNA dependent by annealing to the trans-activating crRNA (tracrRNA) (HORVATH & BARRANGOU, 2010; RAMALINGAM et al., 2013; AHMAD et al., 2018). The Cas protein is then directed to its target site by the specific binding of crRNA to its complementary strand in newly invading viruses. Eventually the virus is inactivated through cleavage by the Cas protein (MOJICA et al., 2000; JANSEN et al., 2002). A useful adaptation of this

system is the possibility to produce a guide RNA (gRNA) which acts as crRNA and tracrRNA at once (CHO et al., 2013; CONG et al., 2013; MALI et al., 2013). Thus, for biotechnological purposes mostly a two-component system is used: the Cas9 protein and a synthetic gRNA. In addition to the desired DSB of the target sequence, cleavage can also occur unspecifically at so-called off target sites because CRISPR/Cas9 system tolerates up to 5 mismatches within the 20 bp recognition site (FU et al., 2013). However, the genome editing specificity by this system can be improved by double nicking strategy which combines a pair of gRNAs guiding two mutated Cas9 nickases to cleave the two strands of the target site separately (RAN et al., 2013). Alternatively, mutated Cas proteins with higher specificity have been developed (KLEINSTIVER et al., 2016; IDOKO-AKOH et al., 2018). Since its discovery, CRISPR/Cas9 genome editing system has been used for many different purposes in different fields and after the first application in targeting mammalian genes (CONG et al., 2013) this approach became an important tool in xenotransplantation to generate knockout pigs (PETERSEN et al., 2016; CHO et al., 2018) or knock-in pigs (WANG et al., 2015), to delete multiple genes simultaneously (WANG et al., 2016), and to exchange porcine genes with its human ortholog (NUNES DOS SANTOS et al., 2018). In addition, it would be interesting to use the CRISPR/Cas system also to insert xenoprotective genes that simultaneously inactivate unwanted genes like already performed with other gene editing approaches (KWON et al., 2017; NOTTLE et al., 2017). The multiple gene editing ability of CRISPR/Cas has been used to reduce PERV infectious potential by inactivating all PERV copies present in the pig genome. Two studies inactivated PERV, first in a porcine kidney cell line PK15 (YANG et al., 2015) and then in a primary porcine fetal fibroblast cell line (FFF3) (NIU et al., 2017). In both cases, certain cell clones were identified in which all PERV were successfully inactivated by insertion and deletion events. In the PK15 cells the PERV copy number was 62 while in the FFF3 it was 25. In cells with all PERV inactivated no reverse transcriptase (RT) activity and no off target effect were shown. The FFF3 cells were then used to clone embryos and to transfer them into surrogate sows which generated PERV inactivated healthy piglets (NIU et al., 2017). It is of note that in both studies, neither porcine cells nor the foster mother did contain any PERV C proviruses. Considering the pathogenicity risk of recombinant PERV A/C it would be, however,

important to perform such CRISPR/Cas9 inactivation experiments in cells containing also PERV C (SCOBIE et al., 2017). The inactivation of all PERV integrated in a genome presents the risk of chromosomal aberrations and requests high effort and may be not necessary since only few PERV might be active. Therefore, the excision of PERV C is a good strategy to prevent the recombination with PERV A.

4.2. Mapping PERVs integration sites

To inactivate or to delete PERVs function there is no ultimate need to identify their exact location in the porcine genome, but excision of the entire provirus requires at first the identification of their integration site. PERV integration sites can be explored by mapping fragments of genomic DNA of a certain pig through publicly accessible reference genomes (LUFINO et al., 2016). Different approaches were used to generate pig libraries on the basis of BAC, lamda phages or cosmids (ROUQUIER et al., 1993; WANG et al., 2003). In fact, BAC libraries were used for the first pig genome sequencing project (GROENEN et al., 2012). The bacterial artificial chromosome (BAC) is a highly stable circular self-replicating vector conserved in *E.coli.* BAC facilitates cloning of large DNA fragment from 100 to 300 kb and thus allows identification of the adjacent genomic region of a provirus (SHIZUYA et al., 1992; KIM et al., 1996a). For identifying PERV containing BAC, clones are screened for the sequence of interest (the provirus) by using labelled probes. BAC-end sequencing can be performed to localize the position of the BAC on the reference genome (YU et al., 2012). Mapping and characterization of PERV in the porcine genome using different vector for the construction of DNA libraries have already been done (C. ROGEL-GAILLARD, 1999; HERRING et al., 2001; NIEBERT et al., 2002). In these studies, DNA libraries from a Large White pig were screened to identify intact PERV by Southern blotting, inverse PCR and sequencing. The integration sites or proviral flanking sequences were characterized through linker-mediated PCR methods with a primer in the PERV LTR and a primer within blunt-end-cut of DNA vector in order to enable the identification of their chromosomal locations (SIEBERT et al., 1995). The LTR flanking region were than used to design primers useful to explore the PERV prevalence in different hDAF transgenic Large White pig (HERRING et al., 2001).

The number of potentially functional PERV found in the Large White pig is low and there is no evidence for the presence of PERV C. However, PERV have been further characterized using DNA libraries from different type of pigs (LEE et al., 2002; JUNG et al., 2010) like in National Institutes of Health miniature pigs which appeared to contain also PERV-C (YU et al., 2012). Nevertheless, BAC library construction and all subsequent work need considerable amounts of time and resources to identify possible integration site of a specific PERV.

A new strategy seems to be more efficient in localizing of a given genomic structure using only genomic DNA (DE VREE et al., 2014). This strategy is called targeted locus amplification (TLA) which in principle combines inverse PCR with next generation sequencing and permits analysis of genomic regions of around two hundred kilobases. TLA is based on the principle that genomic vicinity on the DNA strand sequences does also result in spatial 3D vicinity in the nucleus, and therefore can be stabilized by cross-linking. After fragmentation and religation circular DNA is obtained. Following the inverse PCR principle, primers are then used which bind in the known sequence (i.e the provirus) but are directed a part from each other. PCR thus amplifies the adjacent region of the provirus. The amplified fragments are then sequenced to reconstruct the locus of interest. Different studies have shown the efficiency of TLA technology to map transgenes integration sites, breakpoints induced by transgenes integration and local structural changes (CAIN-HOM et al., 2017; TOSH et al., 2017) as well as to make prenatal diagnosis (VERMEULEN et al., 2017) and genetic screening in acute leukemias (KUIPER et al., 2015; ALIMOHAMED et al., 2018). Considering the characteristics of this approach, TLA can be used to map and to detect the integration sites of a sequence of interest like for example a provirus at base-pair resolution.

Considering the known information about structural PERV sequences, the pathogenic potential of PERV and the preference of PERV C absence in donor pigs for xenotransplantation (https://www.who.int/transplantation/xeno/en/), the targeted locus amplification can be used together with CRISPR/Cas9 genome editing tool to identify and characterize the integration sites of PERV C in our herd and to design guide RNAs used from CRISPR/Cas9 to excise PERV C definitively from the genome

of somatic cells obtained from a pig carrying the lowest PERV C copy number. This and the screening of all our bred animals for the identified PERV C integration sites in order to support their removal as well as by breading was the aim of this research work.

III. Materials and Methods

1. Materials

1.1. Devices

Name	Manufacturer
Accu-jet pro pipette controller	Brand, Wertheim
Agarose gel electrophoresis chamber	WG - Biotech, OWL Inc., USA
Benchtop 96 tube rack	Stratagene, USA
Biochrom™ SimpliNano Spektrometer	Biochrom GmbH, Berlin
Biofuge pico centrifuge	Heraeus, Osterode
Centrifuge 5415 D	Eppendorf, Hamburg
Centrifuge 5810 R	Eppendorf, Hamburg
Centrifuge 5417 R	Eppendorf, Hamburg
Centrifuge Labofuge M	Heraeus, Osterode
Cellavista	SynenTec, Münster
Centrifuge Rotina 380R	Hettich, Tuttlingen
Cell CO2 incubator	MMM Group, Munich
CoolCell	BioCision, USA
Centrifuge Spectrafuge 24D	Labnet International, USA
EASYpure® II	pure aqua, Schnaitsee
Gel documentation system	BioRad, Munich
Geneamp PCR System 9700 thermocycler	Applied Biosystems, USA
GeneQuant Pro spectrophotometer	Amersham, Uk
Glass pipets	Brand, Wertheim
Incubator 37°C and 60°C	Memmert, Schwabach
inoLab® pH meter 7110	WTW, Weilheim
Laminar flow HB 2448K	Heraeus, Hanau
Laminar flow HeraSafe HS12	Kendro, Hanau
Mastercycler® gradient	Eppendorf, Hamburg

MS 5 Stereomicroscope	Leica, Wetzlar
Microwave	DAEWOO, Korea
Pipettes (1000 µL, 200 µL, 20 µL, 10 µL, 2 µL)	Gilson Inc, USA
Power Pac 300 gel electrophoresis unit	BioRad, Munich
Power Station 300 gel electrophoresis unit	Labnet International, USA
RH Basic heating plate with magnetic stirrer	IKA, Staufen
Shaking Incubator	GFL, Burgwede, DE
Select vortexer	Select BioProducts, USA
SilberCycler, 96er mit Gradient	Süd-Laborbedarf, Gauting
Thermomixer 5436	Eppendorf, Hamburg
Varioklav 400 autoclave	H+P Labortechnik, Oberschleißheim
Thermostat Plus	Eppendorf, Hamburg
Water bath JB Nova	Grant Instruments, UK
Water bath AQUAline AL 12	Lauda-Brinkmann

1.2. Consumables

Name	**Manufacturer**
96-well culture plate	Nunclon delta surface, USA
96-well half area culture plate	Corning Costar, USA
Centrifuge tubes (15 ml, 50 ml)	Falcon, Becton Dickinson, Heidelberg
Cryotubes 1 and 2 ml	NuncTM, Denmark
Cryo vial (0.5 ml, 1.5 ml)	TPP, Switzerland
Disposable sterile culture tubes	Simport, Canada
Glass pasteur pipettes	Brand, Wertheim
Parafilm® M	American Can Company, USA
PCR reaction tubes (0.2 ml)	Brand, Wertheim
Petri culture dishes	Sarstedt, Nümbrecht
Pipet tips	Eppendorf, Hamburg
Pipet tips with filter	Thermo Scientific, Schwerten
SafeGrip® latex and nitril gloves	SLG, Munich
Safe-Lock reaction tubes (1.5 mL, 2 mL)	Eppendorf, Hamburg

1.3. Software and Bioinformatic tools

Bio Edit Sequence Alignment Editor	Tom Hall, USA
Finch TV Version 1.3.1	Geospiza Inc., USA
Microsoft Office Suite	Microsoft, USA
Cellavista Application software	SynenTec, Münster
Basic Local Alignment Search Tool	https://blast.ncbi.nlm.nih.gov/Blast.cgi
e!Ensembl	https://www.ensembl.org
Primer designing tool	https://www.ncbi.nlm.nih.gov/tools/primer-blast/
PrimerQuest Tool, IDT	https://eu.idtdna.com
chopchop gRNA	http://chopchop.cbu.uib.no/
Tm Calculator	https://www.thermofisher.com
Repeat masker	http://www.repeatmasker.org/

1.4. Kits

Name	Manufacturer
Double Pure Kombi Kit	Bio&SELL, Nürnberg
NexttecTM Genomic DNA Isolation Kit	Nexttec Biotechnologie GmbH
Easy-DNA™Kit	Invitrogen, Karlsruhe
NucleoSpin®Gel and PCR Clean-up	Macherey-Nagel, Düren

1.5. Oligonucleotides

All designed primers and guide RNAs were purchased from Biomers.net GmbH, Ulm.

Name	forward 5'-3'	reverse 5'-3'
PERVC set1	TGGCCTGATCTATACGTTTG	CCTATGCGCATACCATTAGT
PERVC set2	CAACCCAAGGACCAGGAC	TCATTAGAGGTTACACAGTTCC
gRNA 141C51	GCATTATTTTCAGGTCACTG	
gRNA 141C52	GAAGCTTTGAACATGATTCC	
gRNA 141C31	GAATCTTGGTTCCTGGCCCT	

Name	forward 5'-3'	reverse 5'-3'
EnvC1	CAGTTATTCCTAGTCTGACCTCAC	AGCCATTGGAGGCTCCAGCTG
141int1	AGAGCAGCATTGAGGCAAGG	AGTACCAAAGCCCGTCTAGC
141up2	ATCTCTGCTTTCCTCCCTCTA	CGAATAAGTGCAGTCCAGATCA
141int3	TTTACCTCCAAGTCGGTTCTC	CAGCAGGTGAGAGTATGCTATG
141int4	GATCCTCCTAGCACGTCTGC	CCCAGCAGGTGAGAGTATGC
141exc1	ATTGAGGCAAGGCTGGTTCC	TTTCTGTGTTGAGGAACCCCT
141exc2	ATCTCTGCTTTCCTCCCTCTA	CAGCAGGTGAGAGTATGCTATG
141s1	AGTTTGATGAAGCAAGTATCC	ACTCACACTTTGGTTATGG
141s2	ACTCATTTAGCTTTTTGGTCC	AAGGGAGTTTCTAACTGC
141s3	GGAGACATGTTGTCACC	GAATTATTCTTCTACTGG
141s4f	GTCTGCTCTTCTACAGG	
141s5f	GATGGATGGAGGATGC	
141s6r		GTAGAAGAGCAGACCCAACAAT
141s7r		AACCATAGTTAGTACCTCTGGAAC
141s8r		GGATCCGAGCCGCGTCTGCAA
141s9r		TGGTTCCTAGTCGGGTTCGTTAAC
141s10r		CGACAGAGTATCTGAACATAA
141s11r		CAGAAATAGTCGCAATGTGCC
141s12r		TAGCACAGAAGATAATCTCAAGA
141s13f	ATGTCTTAGTGTGGGCACATT	
13int1	CTTTCCGCATCCGATAGCCT	GTACCAAAGCCCGTCTAGCA
13int2	GTAGGGCGGTGACCTTGAAA	GTGGATCATTCAGGGGCCAT
13int3	CAGCCTGTGATCCTCCTAGC	CATCCCTTTTGTGTCCGCCA
13int4	ACACTCAGAACAGAGACGCC	GTGCACGACGGGTTATCCTA
n523int1	GTTCAGACCATCAGGGCTCC	GTTCTCGGGTGTTAGGGGTG
n523int2	TTGCTAACCAGGCGTCATCA	GCGCACCACTGAAAACAGAC
523int3	GTACCAAAGCCCGTCTAGCA	CACATGCAGCAGTCCTACCA

Name	forward 5'-3'	reverse 5'-3'
523int4	AAAGCCCGTCTAGCAGGGAA	CCAGATGCCCCGACTATTCA
641int1	AGTTCAGATGGTCTGTTGGGTTCT	GCGCACCACTGAAAACAGAC
641int2	AACCTTCAGAGGCGAGGTCT	GCCGGTACATGCATACACGT
641int3	GTACCAAAGCCCGTCTAGCA	ATCGGGACCTCAGGTGAGAA
641int4	GCCGCTAGCTCTACCAGTTC	TGAGCTGCCCAAGATACAGC
n641int2	CTTCAGAGGCGAGGTCTCG	
718int1	TCTTCCTCCTTCCTTCCACCA	CCTTACGGCCTGAGAGGACA
718int2	GACCTCCTTCCTTGTGTCCTC	GTGATCCTCCTAGCACGTCTG
718int3	TAACCTGGAGGAATCCCTAACC	GAGGCAGGGACAGTTTGGAG
718int4	CTGGAGGAATCCCTAACCTC	GAGGCAGGGACAGTTTGGAG
718int5	GGTGGGGCGTTGAAATTGAT	TGAGAGGACAGCTGCAAACC
718int6	TGTGGAGAGAGGAAGGGATTTA	AAACAGACACTCAGAACAGAGA C
718int7	AGACAACAGTACCAAAGCCCG	TTGAGAGGCAGGGACAGTTTG
718int8	GCAGTCCAGATCATGGTACTTAG	GGGACAGTTTGGAGAACACA
1679int1	GTGCTTTTGCGAACAAGGCT	TGACCGACAGTAGGAGACCA
1679int2	AGTGAACAAGGTGCTTTTGCG	TGAGAGGACAGCTGCAAACC
1679int3	AGTACCAAAGCCCGTCTAGC	AGCACCTATGCAATGGCTCC
1679int4	TCCTGTTACTCACAGTTGGGC	TGCAATGGCTCCAGATGCTAA
13104int1	AGTTTCTTGTGTGTCGGTTGC	GCCTGTGATCCTCCTAGCAC
13104int2	TGCATACCAGATTCAGGCAAGT	AGCAGCCTGTGATCCTCCTA
13104int3	AAGGGTGGTTTGAGGGATGG	AGGGGCAAGGTAAGCTTTTCA
13104int4	GTACCAAAGCCCGTCTAGCA	CTTTGGCAAGGGGCAAGGTA
n13104int2	TCTTGTGTGTCGGTTGCCAT	
13104s5f	GCCGCTAGCTCTACCAGTTC	
13104s6f	ACTCACAGTTGGGCCATGTA	
13104s7r		GCTTCTGACCAACTGACTCTAA
13104s8r		CCAGGGTTGGTTATTTGTGAGA
210int1	TCTCATGCCCAATGGTTTGC	CAACAGTACCAAAGCCCGTC

Name	forward 5'-3'	reverse 5'-3'
210int2	CCTCTGTGGGGACAATGGAT	TCCTGTTACTCACAGTTGGGC
210int3	GGACAGCTGCAAACCGAAAG	ACCCTCCCTTTAGGAAAAACCC
210int4	TAGCACGTCTGCTGATCACAGTC	CCATTCCCTCCAGAAGCCA
210int5	CCAGCCTAATTCCCATCCCTC	AGACAACAGTACCAAAGCCCG
210int6	CAGGTATCAGCATCTCCTCCTA	AGGAGGACTATTAAGGGT
210int7	GTGATCCTCCTAGCACGTCTG	GTCTTGGGCTTCCATGTGGATT
210int8	AACAGACACTCAGAACAGAGAC	TGGGCTTCCATGTGGATTT
X14int1	CATGTCTGTCCTGCCTCTGG	GTACCAAAGCCCGTCTAGCA
X14int2	ACCAGTAAGAGAAGGTCGATGT	AACAGTACCAAAGCCCGTCT
X14int3	TGAGAGGACAGCTGCAAACC	GGGCGTGTTCAAAAGAGTGC
X14int4f	GCCTGTGATCCTCCTAGCAC	CCTCGCTGGTCAATTGCTTG
4135int1	ACTGGCTCTGAACCGTTTGG	CACTCAGAACAGAGACGCCG
4135int2	TTTGGGGCTGAGGCTGTTAGA	CGGCCGGTACATGCATACAC
4135int3	AACAGTACCAAAGCCCGTCT	CGTATCTGTCTGTGTCCGGG
4135int4	CAACAGGTCTCTTTGGTTGGC	TCCCATTTCTGGCAAGAGCC
120int1	GTACTGGGCTTTTGCATCGC	GCCTGTGATCCTCCTAGCAC
120int2	CTCTTGCAGGTAGCCTTCGC	CGGTCCTCTGACCGACAGTA
120int3	TACTCACAGTTGGGCCATGT	GGGCTGGTAAGGAAAGCAAAC
120int4	AGACAACAGTACCAAAGCCCG	TTAAGCAGGGCTGGTAAGGAA

1.6. Primary cells, bacterial strains and plasmid vectors

		Description/ Manufacturer
Pig primary kidney cell line	3990	GalKO+/-,CD46, INS-LEA
	1476	INS-LEA
	4504	GalKO+/-, CD46, hTM
	4686	GalKO-/-, CD46
	PKCm	Wild type Landrace pig
Bacterial strain	Top 10	Invitrogen, Karlsruhe
Plasmid	gRNA vectors	Thermo Scientific, Schwerte
	Cas9 vector	Addgene, USA

1.7. Chemicals, enzymes and other reagents

Acetic Acid (glacial) (HOAc)	Roth, Karlsruhe
Agarose Universal	Bio&Sell, Nürnberg
Ampicillin	Roth, Karlsruhe
BigDye® Terminator v3.1	Applied Biosystems, Weiterstadt
β-mercaptoethanol	Sigma, Steinheim
Bromophenolblue	Roth, Karlsruhe
Chloroform (Tricholomethane)	Roth, Karlsruhe
CollagenR	Serva, Heidelberg
Difco™ Trypsin 250	BD,USA
Dimethyl sulfoxide (DMSO)	Sigma, Steinheim
Dithiothreitol (DTT)	Sigma, Steinheim
DNA loading dye (6x)	Thermo Scientific, Schwerte
Dulbecco modified Eagle medium (DMEM)	Gibco, Darmstadt
dNTPs (dATP, dCTP, dGTP, dTTP)	Thermo Scientific, Schwerte
EDTA (Ethylenediaminetetraacetic acid)	Roth, Karlsruhe

Ethanol (EtOH)	Roth, Karlsruhe
GelRed^{TM} Nucleic Acid Gel Stain	Linaris, Dossenheim
Gene Ruler™ 1 kb	Thermo Scientific, Schwerte
Glutamax Gibco	Thermo Scientific, Schwerte
Glycerin (Glycerol)	Roth, Karlsruhe
Fetal calf serum (FCS)	Invitrogen, Karlsruhe
Herculase II Fusion DNA Polymerase	Agilant technologies, USA
Herculase II 5x reaction buffer	Agilant technologies, USA
Hydrochloric acid, 37 % (HCL)	Roth, Karlsruhe
Isoamylalcohol	Roth, Karlsruhe
Isopropanol	Roth, Karlsruhe
LB-Agar	Roth, Karlsruhe
LB-Medium	Roth, Karlsruhe
Magnesium chloride ($MgCl_2$)	Fluka Chemie, Switzerland
Non-essential amino acids (100x)	Gibco, Darmstadt
Polyethylenglycol (PEG) 8000	Roth, Karlsruhe
Phenol	Roth, Karlsruhe
Proteinase K	Roth, Karlsruhe
Potassium acetate (KOAc)	Roth, Karlsruhe
Potassium chloride (KCl)	Sigma, Steinheim
di-Potassiumhydrogenphosphate (KH_2PO_4)	Roth, Karlsruhe
Ribonuclease A	Thermo Scientific, Schwerte
SOB-Medium	Roth, Karlsruhe
Sodium acetate	Sigma, Steinheim
Sodium dodecyl sulfate (SDS)	Serva Electrophoresis, Heidelberg
Sodium chloride (NaCl)	Sigma, Steinheim
Sodiumdihydrogenphosphate (Na_2HPO_4)	Sigma, Steinheim
Sodium hydroxide (NaOH)	Roth, Karlsruhe
Sodium Pyruvate	Invitrogen, Karlsruhe
T4 DNA ligase and buffer (10x)	Thermo Scientific, Schwerte
Tris-(hydroxymethyl)-aminomethane (TRIS)	Roth, Karlsruhe

1.8. Buffers and solutions for molecular genetic protocols

All buffers and solution were prepared with water deionised in a Millipore machine (EASYpure® II, pure aqua) called aqua bidest.

Name	Components
DNA molecular weight standard	100 µL Gene RulerTM 1 kb 100 µL 6x DNA loading dye 400 µL aqua bidest. Aliquoted and stored at -20°C
DNA loading buffer (10×)	10 % glycerol in aqua bidest. 1 spatula tip of Bromophenol Blue 0.5 M NaOH added till color turns blue Aliquoted and stored at 4°C
dNTPs mixture	2 mM dATP, dCTP, dGTP, dTTP Dissolved in aqua bidest Aliquoted and stored at -20°C
TAE buffer (50×) (2l)	2 M Tris (484 g) 200 mL 0.5 M EDTA (pH 8.0) 114 mL glacial acetic acid Brought to 2 L volume with aqua bidest. Filtrated and autovlaved Before use diluted to 1X concentration
Sequencing buffer (5×)	17.5 mL 1 M Tris/HCl (pH 9.0) 125 µL 1 M MgCl2 50 mL aqua bidest. Aliquoted and stored at -20°C
PK buffer (10x)	200 mM Tris 1 M NaCl 40 mM EDTA Stored at room temperature

Name	Components
PEG-$MgCl_2$	40 % (w/v) PEG 8000 30 mM $MgCl_2$ Stored at room temperature
Phenol-Chloroform-isoamylalcohol (PCiA)	25 mL Phenol 24 mL Chloroform 1 mL Isoamylalcohol
T-buffer	10 mM Tris HCl added till pH 8.0
Plasmid A solution	50 mM glucose 25 mM Tris/HCl pH 8.0 10 mM EDTA/NaOH pH 8.0
Plasmid B solution	0.1 M NaOH 0.5 % (w/v) SDS Freshly prepared
Plasmid C solution	3 M KOAc 9 M HOAc till pH 4.8 Autoclaved
STE	10 mM Tris/HCl pH 8.0 100 mM NaCl 1 mM EDTA/NaOH pH 8.0

1.9. Media and solutions for cell culture protocols

Name	Components
Cell culture medium	DMEM with Glutamax 1 % Non-essential amino acids (100 %) 15 % Fetal calf serum (FCS) 0.1 mM β-mercaptoethanol
Stop medium	DMEM with Glutamax 10 % FCS

Name	Components
Cryo medium	10 % DMSO 90 % FCS Freshly prepared
PBS without Ca^{2+}/Mg^{2+}	8 g NaCl 0.2 g KCl 0.2 g KH_2PO_4 2.14 g $NaH_2PO_4 \cdot H2O$ 1000 mL aqua bidest.
Trypsin/EDTA	PBS without Ca^{2+}/Mg^{2+} 0.5 % Trypsin 0.04 % EDTA

2. Methods

Molecular genetics and cell culture methods as well as bioinformatics approaches were used for detection of PERV C loci in the genome of 4 chosen animals, characterization of PERV C integration sites in these cells, PERV C screening in our xeno herd, for the excision of PERV C from the genome of the selected animal through engineered CRISPR/Cas9 system and for following analysis of the produced single cell clones.

2.1. PERV diversity by Targeted Locus Amplification

PERV C loci detection was performed by Cergentis Ltd, Utrecht, Netherlands (https://www.cergentis.com/) through TLA sequencing described in de Vree et al. Nat. Biotechnol. 2014. The primary kidney cells used for the analysis were isolated from the animals 3990 (GalKO+/-, CD46, INS-LEA), 1476 (INS-LEA), 4504 (GalKO+/-, CD46, hTM) and 4686 (GalKO-/-, CD46). For the screening 4 probes were designed on a non-homologous region of PERV C *env* gene by Dr. Nikolai Klymiuk.

2.2. Chromosomal integration sites characterization of PERV C loci and primer designing for their screening

The chromosomal integration site localization of all PERV C loci was performed using the bioinformatics tool Ensemble starting from the known flanking regions of every PERV C found. Once the localization sites were found, the relative sequences were used in the repeat masker tool to find in this regions repetitive segments in the entire pig's genome in order to design primer pairs with specificity for the region of interest. To design specific primer pairs, tools like Primer Blast, PrimerQuest and Tm calculator were used.

2.3. Genomic DNA isolation from primary kidney cells

In order to set up optimal conditions for new screening PCRs and to characterize PERV C integration sites, genomic DNA was isolated from the primary kidney cells of the animals 3990, 4686, 4504, 1476 and as control from a primary porcine cell line "Kidney M" isolated from the kidney of a 3 months old male Landrace pig. The isolation, cultivation, freezing and thawing protocols for these cells have been established and performed from Dr. Annegret Wünsch and Eva Maria Jemiller according to RICHTER et al. (2012). The DNA isolation was performed using the Easy-DNA™ Kit purchased from Invitrogen, Karlsruhe according to manufacturer's instructions and precisely the protocol nr. 3. The obtained DNA was dissolved in 55 µL of T-buffer and 1 µL of it was used to determine the concentration and purity by Biochrom™ SimpliNano Spektrometer. From each DNA than was made an aliquot of 100 ng/ µL which was used as template for the following analysis.

2.4. Genomic DNA isolation from pig tails

To screen the animals of our xeno herd for all PERV C integration sites found by TLA analysis genomic DNA from each pig was isolated with two methods. In the first method DNA was isolated from 2 tail's slices (1 mm each) using the Nexttec™ Genomic DNA Isolation Kit purchased from nexttec GmbH, Leverkusen according to manufacturer's instructions. This DNA was used directly as template for PCR. In the

second method, which demonstrated to be a better option, the DNA was isolated from pig's tail piece of 1 cm^3 using the protocol nr. 8 of the Easy-DNA™ Kit mentioned above. After that, the DNA concentration was measured and diluted to 100 ng/µL as reported in 2.3.

2.5. Polymerase chain reaction (PCR)

PCR protocols used for multiple purposes in this work, have in common the amplification of a determined genomic part using primer pairs specific and complementary to the extremities of the sequence of interest. To identify the best primer pairs and the optimal working conditions useful for the screening of every PERV C locus in the different chromosomes all the designed primer pairs were tested with different working conditions like for example changing the annealing temperature and/or the elongation time and/or primer concentration. All the master mix components were mixed on ice to have a final concentration of 25 µL and the reaction was performed in PCR cycler within 0.2 mL PCR tubes. For every PCR run was used as template the DNA previously isolated and aqua bidest. as contamination control. To sequence the PCR products if further required the PCR experiments for every sample were performed in double. The master mix components as well as the PCR conditions as showed below at test starting point were the same for every designed primer pair and changed depending on the performance of the PCR to find the optimal working requisite.

Table 1: PCR components

Herculase II 5x reaction buffer	5.0 µL
dNTPs (2 mM)	2.5 µL
Primer forward (10 µM)	0.4 µL
Primer reverse (10 µM)	0.4 µL
Herculase II DNA Polymerase	0.2 µL
aqua bidest.	15.5 µL
Template	1.0 µL
Total volume	25 µL

Table 2: ***PCR cycler program***

First denaturation	95 °C	5 min	
Denaturation	95 °C	30 s	x 35
Annealing	58 °C	30 s	
Elongation	72 °C	30 s	
Final elongation	72 °C	10 min	
Termination	4 °C	15 min	

2.6. Agarose gel electrophoresis

In order to detect the amplified DNA, the PCR samples were used to run on an 1% agarose gel electrophoresis. Universal Agarose at concentration 1 g/100 mL was dissolved in 1x TAE buffer by heating in the microwave and after cooling down till 60°C was added GelRed™ Nucleic Acid Gel Stain (2 µL/100 mL). The solution was then poured in an electrophoresis chamber and after its polymerization the chamber was filled with 1x TAE buffer. Each PCR sample as well as 6 µL of 1 kb Gene Ruler™ DNA molecular weight standard useful for the DNA size estimation was mixed with 2.5 µL 10x DNA loading buffer and loaded into single gel slots. In order to separate the DNA fragments depending on the size a 130 Volt electric field was applied to the chamber. At the running end the gel was put in a Gel documentation system and the DNA bands were visualized by UV light. In this case too, in order to sequence the PCR products if further required the DNA bands were excised and used to obtain the DNA elution.

2.7. DNA elution

The DNA was eluted directly from the PCR samples or from the gel containing the band of our interest. In the first case, one reaction was used for agarose gel electrophoresis and the reaction copy was used as sample for the NucleoSpin®Gel and PCR Clean-up kit following manufacturer's PCR clean-up protocol in order to obtain the eluted DNA. In the second case, the excised band was used to obtain the DNA using the protocol Isolation of DNA from agarose gels of Double Pure Kombi Kit

according to manufacturer's instructions. To verify if DNA was really eluted 2 µL of the obtained solution was mixed with 2 µL of 10x DNA loading buffer and with 15 µL of aqua bidest. and was loaded in a 1 % agarose gel together with 6 µL of 1 kb Gene Ruler™ DNA molecular weight standard. The eluted DNA was further used as sample for the following sequencing method.

2.8. Sanger sequencing

The Sanger approach was used to characterize at nucleotide base level all the eleven PERV C integration sites found in the 4 before mentioned primary cells. This sequencing method (SANGER et al., 1977) permits to know the exact nucleotide sequence of an amplified DNA fragment based on the incorporation of labelled dideoxynucleotides (ddNTPs) which induce termination of the DNA chain extension. In each 0.2 mL reaction tube a target DNA fragment is sequenced with one primer so that a DNA fragment can be sequenced with different primer pairs in different reactions. The PCR products were sequenced with the own forward and reverse primers as well as when necessary with primers pairing internal sequences of the target DNA. The master mix components and the sequencing reaction cycler protocol are shown below (Table 3,4). At the end of the running program the sequencing products were purified through ethanol precipitation. To the sequencing reaction was added first 2.5 µL of 125 mM EDTA and then 30 µL of precooled 100 % EtOH. All the volume was then transferred into a 1.5 mL tube and incubated on ice for 15 min. After 13.000 rpm centrifugation at 4°C for 30 min the supernatant was removed and the pellet was washed overnight with 50 µL of 70 % EtOH. The next day after 2.5 min centrifugation at 13.000 rpm and after supernatant removing the pellet was air dried and dissolved in 30 µL of aqua bidest. The purified samples were transferred to a sequencing plate provided from Genome Analysis Center, Helmholtzzentrum Munich which performed the reading of the Sanger sequencing reaction results. The analysis of the nucleotide sequences was made using bioinformatics tools like FinchTV Version 1.3.1 and Bio Edit Sequence Alignment Editor.

Table 3: Sequencing reaction components

5x sequencing buffer	4 µL
BigDye	1 µL
Primer (10 µM)	1 µL
aqua bidest.	2 µL
DNA template	2 µL

Table 4: Sequencing cycler program

First denaturation	95 °C	1 min	
Denaturation	95 °C	5 sec	x 40
Annealing	52 °C	10 sec	
Elongation	60 °C	4 min	
Termination	4 °C	15 min	

2.9. gRNA designing and sequence proofing

After the integration site characterization, gRNAs enclosing PERV C locus were designed using the chop chop software. The construct expressing the designed gRNA fused with tracrRNA and the construct expressing Cas9 was purchased from Thermo Scientific, Schwerte and delivered in MALI vector according to (MALI et al., 2013). In order to proof the commissioned gRNA sequences and to prepare stock, the gRNA vectors were transformed in TOP 10 *E.coli* cells and sequenced after plasmid preparation of the bacterial cultures.

2.10. Heat Shock Transformation

In order to perform the transformation of TOP 10 cells every 5 µg lyophilized gRNA vector was dissolved in 7 µL in endofree T- buffer. From this solution 1 µL was diluted in 999 µL of T-buffer and 1 µL of it was used for the transformation. The -80° C conserved competent cells were thawed carefully on ice and 1 µL of gRNA vector solution was added and mixed gently avoiding re-pipeting. After incubation on ice for

30 min the cells were put in a 42° C water bath for 45 seconds and then again on ice for 2 min. To every reaction tube 1 mL of SOB medium was added under sterile laminar flow and afterwards they were incubated for 45 min at 37° C. During incubation time the LB agar plates added with Ampicillin (50 µg/mL) were signed up and put in the same incubator to equilibrate. After recovering the cells were centrifuged at 4.4 rpm for 4 min and 800 µL of the supernatant were thrown away. The pellet was resuspended in the remaining 200 µL, plated in the LB agar-Ampicillin Petri dishes and incubated overnight at 37° C. Only the cells that have integrated the plasmid will be resistant to Ampicillin, grow in colony and be ready for further analysis.

2.11. Glycerol stock and plasmid preparation

The next day single colonies from agar plates were inoculated into sterile culture tubes containing LB medium-Ampicillin and incubated overnight at 37° C in a shaking incubator. Some of the overnight grown cultures were used for the glycerol stocks preparation consisting in a mixture of 300 µL of glycerol 60 % and 900 µL of bacterial culture which were then stored at -80° C. The other tubes were centrifuged for 10 min at 3000 rpm and the supernatant was discarded. The pellets were resuspended with 750 µL STE buffer and transferred into 1.5 mL tubes before to be centrifuged for 5 min at 700 rpm. After removing the supernatant, the obtained pellets were frozen for 15 min at -20° C and were resuspended by pipetting in 200 µL Plasmid A buffer. Consecutively 400 µL of Plasmid B buffer was added and the samples were mixed by hand, inverting the tubes till a precipitate was observed. After incubation on ice for 5 min 300 µL Plasmid C buffer was added, gently mixed and then again incubated on ice for 5 min. After centrifugation at 13.200 rpm for 10 min, the supernatants were transferred in new signed 1.5 mL tubes containing each 4 µL of RNase A and incubated at 37° C for at least 45 min. In order to extract the plasmid DNA from the solution containing proteins and other cellular residues, 300 µL of PCiA was added in all the tubes which were then shaken for 1 min and centrifuged at 13.200 rpm form 2.5 min in order to separate the two phases. The aqueous phase was transferred into a new tube and after adding 650 µL isopropanol the samples were inverted more time, centrifuged at 13.200 rpm for 10 min and the supernatant was discarded. The DNA pellet was

washed with 700 µL of 70 % EtOH, centrifuged again at 13.200 rpm for 2.5 min and after discarding the supernatant was air dried for 6 min and resolved in 55 µL of T-buffer. After resolution the DNA concentration was measured.

2.12. PEG precipitation

In order to be sequenced the DNA need to became further purified by PEG precipitation. Therefore, 20 µL of the above plasmid DNA was mixed with 20 µL of aqua bidest. and 20 µL of PEG- $MgCl_2$ and the whole was incubated for 10 min at room temperature and then centrifuged at 13.200 rpm for 20 min. After discarding the supernatant, the DNA pellet was washed overnight with 300 µL of 70 % EtOH and centrifuged for 2.5 min at 13.200 rpm the next day. The supernatant was removed, the pellet was air dried for 6 min and then resolved in 20 µL of T-buffer. The final concentration was measured again using Biochrom™ SimpliNano Spektrometer and the samples were diluted to a concentration of 30 ng/µL with T-buffer and directly used as template for the sequencing reaction. In order to verify the exact sequence of gRNAs the samples were sequenced like described in 2.8 with the control gRNA primer (CRP) forward and reverse.

3. Targeted excision of PERV C loci and generation and screening of single cell clones

3.1. Transfection

In order to excise PERV C locus, transfection on 3990 cells was performed with 2 gRNAs and Cas9. One transfection was performed with 2.5 µL of gRNA 141C51, 2.5 µL of gRNAC31 and 1 µL of Cas9 each with a concentration of 300 ng/µL. The other transfection combination consisted in 2.5 µL of gRNA 141C52, 2.5 µL of gRNAC31 and 1 µL of Cas9. Transfection on the 3990 cells and their freezing were performed by Dr. Annegret Wünsch and Eva Maria Jemiller according to (RICHTER et al., 2013).

3.2. Single cell clones generation

To verify the occurred excision of PERV C locus by CRISPR/Cas9 single cells clones were produced from the cells previously transfected. At first, the cells were thawed and diluted in 10 mL DMEM added of fetal calf serum (FCS) in a quantity of 10 % to obtain an amount of 150 cells pro 96 well half area plate. The calculated cells amount then was added to the cell culture medium and in order to have 1 or 2 cells per well 50 µL of this solution was seeded on each well of the cell culture plate which was previously coated with collagen and put in cell CO_2 incubator at 37°C. The cell culture medium was exchanged every 72 hours and every time plates were scanned for single cell clones using Cellavista (Synentec) which take images of each well useful to document the growth of the single cell clones. The obtained single cell clones once being at 80 % of confluence were washed with 100 µL of PBS and detached using 30 µL of Trypsin/EDTA solution and incubated at 37° C for 5 min. After stopping the reaction with 170 µL of cell culture medium added with 15 % of FCS, the cell clones of one well were split on two different wells and cultivated changing medium every 72 hour till the full confluence was achieved. Cells of one well were then cryo preserved as backup adding 170 µL of previously cooled FCS with 10 % DMSO and transferring all in cryo tubes wich were immediately put in –80° C. The cells of the other well were used for DNA isolation useful to screen if the excision took place and therefore 170 µL of stop medium was added and the whole amount was transferred in 1.5 mL tube. The tubes of more single cell clones were then centrifuged for 5 min at 400 x g, the supernatant thrown away and the tubes containing the cell pellet were frozen at -80° C ready for the DNA isolation.

3.3. Single cell clones DNA isolation by high salt precipitation

At first the cells were resuspended in 100 µL of PK buffer and then 10 µL of SDS (10 %) and 4.4 µL of 1M DTT was added before mixing by pipetting. After incubation at 60°C for 1 hour were added 2 µL of proteinase K (20 mg/mL) and again the samples were further incubated like above. At the end of incubation time 30 µL of NaCl (4.5 M) were added and after pipetting and incubation for 10 min on ice the tubes were centrifuged for 20 min at 13.200 rpm. The supernatant was transferred to a new

reaction tube, 110 µL of isopropanol were added and the samples were shaken carefully to precipitate the DNA. The tubes were then centrifuged again for 20 min at 13.200 rpm, the supernatant was discarded carefully and 500 µL of 70 % EtOH were added before incubating at 4°C for the whole night. The next day the samples were centrifuged for 2.5 min at 13.200 rpm, the supernatant thrown away and the pellets were air dried for 6 min. In every tube then were added 45 µL of T buffer and to dissolve the DNA the reaction tubes were incubated for 1 hour at 60°C. The isolated DNA was then stored at 4°C.

3.4. Screening of single cell clones

To verify in which clones the provirus was successfully excised the isolated DNA of the clones was used as template for PCR using different primer pairs such locating on the flanking regions outside the targeted locus. The clones that lack the provirus were further tested either by sequencing as described on paragraph 2.8. or by PCR with primer pair specific for *env* C and with primer pairs specific for the LTRs. The PCR reactions were performed as described in paragraph 2.5.

IV. Results

1. PERV integration sites

To have an overview of the distribution of PERV in porcine species, at first all PERV integration sites were identified in the reference genome and in the 4 chosen animals of our herd. The flanking regions of every PERV C locus were then used to characterize the chromosomal location of proviruses.

1.1. PERV in Tabasco reference genome

The Tabasco reference genome (11.1 version) was examined for PERV A, B and C proviruses using sequences from the most divergent part of the *env* gene. In addition, a sequence from the highly conserved *gag* gene was used for screening the reference genome. Sequences from the *env* of AJ293656, AJ293657 and AF038600 were used as probes for PERV A, B and C, respectively. The sequence of *gag* from AJ293656 was used as a probe for the highly conserved *gag* gene. All positions of PERV and the percentage of homology to the reference PERV are reported in the Figure 1. In total 56 proviruses were identified in the pig reference genome; 15 of them were only identified by the *gag* gene and, thus, likely lack an intact *env.* 7 of the proviruses appeared to have an intact ORF in all 3 retroviral genes. The proposed age of the proviruses is diverse, indicated by difference in the LTRs and ranging from 19 mutations per 590 nucleotides in a PERV at in chromosome Y:20.19Mb to 0 per 631 nucleotides in a PERV at chromosome 15:110.96Mb. Interestingly, no PERV C was identified in the pig reference genome.

Further, all identified PERV sequences then were examined for their internal structure, regarding LTR, *gag*, *pol* and *env.* Not all PERV conserve their intact sequence and a significant proportion has larger deletions in the *pol* and *env* gene or on their terminal ends (Fig. 2). Very interestingly a group of 12 proviruses contain an identical gap in the *env* gene, and some of them have consistent additional gaps in the *pol* and *gag* genes. The gap in the *env* gene prevent speculation about the tropism of this group of

PERV. How this group of proviruses has expanded in the pig genome without a functional *env* remains unclear.

envB		Susscrofa 11.1				ORF				gagA		envA		envC	
length	homology				position	gag	pol	env	LTR	length	homology	length	homology	length	homology
		11	Reverse	29.09Mb	29073991..29091991				3/600	1290	97.21				
457	98.69	11	Forward	38.20Mb	38198436..38210374		++	++	1/629	1271	95.59				
191	90.58	12	Reverse	22.98Mb	22986114..22991923				16/606	1267	95.66				
		12	Forward	28.22Mb	28220526..282304099	++	++	++	1/702	1575	99.43	428	99.77	149	86.58
		13	Reverse	142.04Mb	142029614..142041036			++	8/701	1575	98.92	428	99.53	149	86.58
		13	Reverse	146.75Mb_a	146734411..146856139				0/599	655+613	96.18+94.29	428	96.96	145	83.45
		13	Reverse	146.75Mb_b	146734411..146856139				1/599	1374	96.80				
		13	Reverse	146.75Mb_c	146734411..146856139			++	chim	1374	96.80				
		13	Forward	21.30Mb	21298939..21310939				nd					245	96.33
457	98.69	14	Forward	119.67Mb	119665648:119677596			++	2/590	1275	95.84				
		14	Reverse	62.55Mb	62545046..62565046	++			0/611	1540	98.05				
457	99.34	15	Reverse	110.96Mb	110956688:110968399				0/631	1271	96.07				
104	89.42	15	Forward	116.32Mb	116320090..116336939				7/657	476	93.28	137+166	89.05+93.98	150	84.00
		15	Reverse	66.86Mb	66861922..66873320	++			1/702	1575	99.24	428	99.30	149	85.91
457	99.56	16	Forward	59.57Mb	59569960..59581904	++	++	++	0/629	1575	95.43				
		17	Forward	33.06Mb	33061035..33072922	++	++	++	3/702	1575	98.54	428	98.13	106	89.62
		17	Reverse	3.79Mb	3791734..3803452		++	++	4/619,579	904	95.13	428	96.96	145	83.45
458	96.29	17	Reverse	41.47Mb	41465019..41477727				nd	651	95.08				
104	89.42	17	Reverse	9.53Mb	9534449..9542477				12/592	698+435	96.13+96.09	189+134	91.53+88.81	150	84.00
		1	Forward	132.02Mb	132018432..132029483	++		++	3/701	1575	99.62	428	99.77	149	86.58
		1	Forward	262.17Mb	262164499..262177384	++	++	++	1/701	1575	99.87	428	99.77	149	86.58
457	99.56	1	Forward	38.46Mb	38457085..38467303	++		++	1/590,629	1575	95.49				
		2	Reverse	76.65Mb	76645745..76656125				8/693,676	664+670	96.69+96.87	166+110	94.58+90.00	235	80.43
		3	Reverse	10.66Mb	10658385..10669807	++			3/702	1575	99.37	428	98.83	148	85.14
		3	Forward	17.78Mb	17776901..17789025				17/595	1247	96.31	133+193	87.22+91.71	98	88.78
457	99.34	3	Reverse	51.11Mb	51106305..51118017	++	++	++	2/628	1271	96.22				
457	99.34	4	Reverse	45.60Mb	45597198..45608908		++		0/629	1271	96.14				
		5	Forward	29.35Mb	29351398..29366398					594+977	98.48+95.80				
		5	Reverse	92.18Mb	92182902..92194324	++	++		1/702	1575	99.30	428	99.30	149	85.91
		6	Forward	10.40Mb	10400854..10408780				2/702	837	99.88	428	99.53	151	86.75
		7	Forward	105.71Mb	105708035..105720319	++		++	1/702	1575	99.81	428	99.53	149	86.58
456	95.83	7	Forward	21.23Mb	21234290..21247292				nd	590+660	94.41+96.36				
457	99.56	8	Forward	15.32Mb	15317503:15329445	++		++	0/629	1576	95.30				
		8	Forward	51.57Mb	51568697..51580583	++		++	3/701	1575	99.17	428	99.77	149	86.58
457	98.47	9	Reverse	138.89Mb	138893288..138904994	++	++		5/630	1271	96.22				
		AEM	Reverse	scf133	561590..572996				7/723	1557	97.56	428	96.96	149	87.25
457	99.34	AEM	Forward	scf137	1.. 11892			++	2/631	1575	95.17				
		AEM	Forward	scf141	17669..30669				3/573	653	95.10				
457	99.78	AEM	Forward	scf197	11911..23855	++	++	++	0/629	1575	95.37				
457	94.97	AEM	Forward	scf393	818282..830898				nd	1254	93.86				
457	99.56	AEM	Reverse	scf476	39861..51574				4/590,628	1271	95.83				
		AEM	Forward	scf536	12667..24561				chim	1289	98.22	200+171	97.50+94.15	89	93.26
		AEM	Forward	scf643	1..12000					983	95.02				
457	99.12	X	Reverse	112.84Mb	112840463..112848649			++	2,5/629	934	97.00				
		X	Forward	55.08Mb	55077290..55090290					1575	99.11				
376	96.01	X	Forward	71.40Mb	71400364..71411288				2/593	1110	94.86			89	88.76
		X	Forward	72.24Mb	72242053..72250829				16/592	1576	94.48				
		X	Reverse	73.75Mb	73750754..73762179				4/701	1575	98.22	428	99.30	149	86.58
		X	Reverse	81.54Mb	81535272..81548272					591	95.09				
		Y	Reverse	20.19Mb	20192451..20204270				19/590	1271	94.81	428	97.90	149	84.56
		Y	Reverse	21.87Mb	21865010..21876730				15/589,634	1271	94.49	428	97.90	149	83.89
		Y	Forward	24.41Mb	24405869..24418869					931	92.91				
		Y	Forward	25.24Mb_a	25243758:25274926	++	++	++	nd	1575	99.81	428	99.77	149	86.58
		Y	Forward	25.24Mb_b	25243758:25274926				nd	899	95.11				
		Y	Reverse	25.24Mb_c	25243758:25274926				nd	932	95.49				
		Y	Reverse	25.29Mb	25291276..25304276					783	94.89				

Figure 1: Proviruses from Tabasco reference genome.

Matches identified by BLAST in the Ensemble pig reference genome are characterized by the length of the match and the homology in the matching region. Proviruses are defined by the chromosomal position and their relative orientation. For each provirus,

the open reading frame of the gag, pol and env genes was examined and proviruses with intact ORF in all 3 genes are indicated in bold. If available, the sequences of the 5` LTR and the 3' LTR were compared and the number of mismatches is shown. If one or both LTR were not completely abundant, differences were not determined (nd). Proviruses with more than 95% env A homology are indicated in green; proviruses with more than 95% env B homology are indicated in magenta and the proviruses that have a homology with env of PERV A, B or C less than 95 % are indicated in gray.

1.2. PERV in our herd –TLA sequencing analysis

TLA sequencing was performed by Cergentis Ltd (Utrecht, Netherlands) in primary cells obtained from 4 genetically modified animals from our xenotransplantation breeding herd. Specifically, the animal 3990 was GalKO+/-, CD46, INS-LEA; pig 1476 was INS-LEA; pig 4504 was GalKO+/-, CD46, hTM and the animal 4686 was GalKO-/-, CD46. For the screening 2 sets of primers for *env* and 1 primer pair for each *gag* and *pro/pol* were used to differentiate PERV C subtype from A and B. Flanking sequences were then identified in the pig reference genome version 10.2. available in December 2016. Totally, 11 PERV C loci were found (Fig. 3). Importantly, none of the animals were found to be PERV C free, with PERV load ranging from 1 to 8. The animals 3990 and 4686 comprise only one PERV C at chr1:41Mb and at chr7:18Mb, respectively. Pig 1476 has 5 integration sites at chr1:20Mb, chr1:41Mb, chr4:135Mb, chr5:23Mb and at chr13:104Mb while animal 4504 is the animal with most PERV C proviruses integrated in its genome located at chr1:20Mb, chr1:3Mb, chr1:41Mb, chr2:110Mb, chr6:41Mb, chr7:18Mb, chr16:79Mb and at chrX:14Mb.

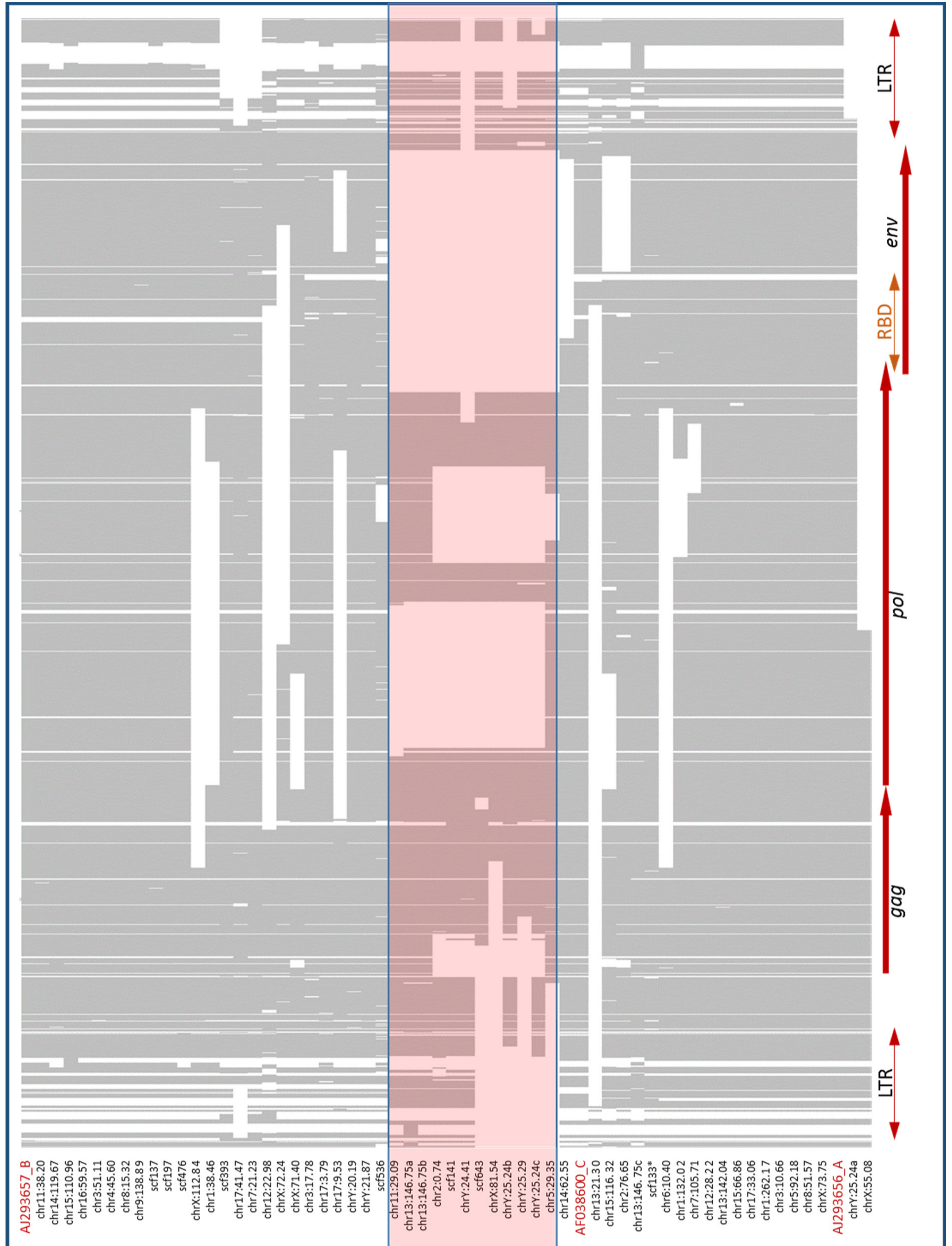


Figure 2: Internal structure of PERVs in Tabasco reference genome.

CERGENTIS
COMPLETE GENE SEQUENCING

SUMARIZING RESULTS PERVC

Chr:Mb	1476				3990				4504				4686				remarks	Exact integration site
peak	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4		Integration or genome position
Chr1:3	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	Good peak, no PERV in genome	Chr1:3014790-3014787
Chr1:20	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	Good peak, no PERV in genome	Chr1: ~20 (JH118480-1)
Chr1:41	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	Good peak, no PERV in genome	Chr1:41140525-41140524
Chr2:77	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	Good peak, PERV in genome	
Chr2:100	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Odd shaped peak	
Chr2:110	-	-	-	-	-	-	-	-	+	+	?	?	-	-	-	-	Good but low coverage peak	Chr2:109094929-109094926
Chr3:40	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Non-specific	
Chr3:142	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Repeats	
Chr4:135	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	Good peak, no PERV in genome	Chr4:135116119-135116116
Chr5:23	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	Good peak, no PERV in genome	Chr5:22743215-22743212
Chr6:4.7	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	Low coverage peak	
Chr6:41	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	Good peak, no PERV in genome, odd shaped due to incorrect genome assembly?	Chr6:40976751-40976748
Chr7:18	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	Good peak, no PERV in genome	Chr7:18356720-18356717
Chr7:44	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Low coverage peak	
Chr7:58	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Low coverage peak	
Chr8:2	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Low coverage peak	
Chr13:104	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	Good peak, no PERV in genome	Chr13:104501280-104501278
Chr14:31	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Low coverage peak	
Chr16:79	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	Good peak, no PERV in genome	Chr16:78812732-78812729
ChrX:14	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	Good peak, no PERV in genome	chrX:14049157-14049160

PERV C - TLA

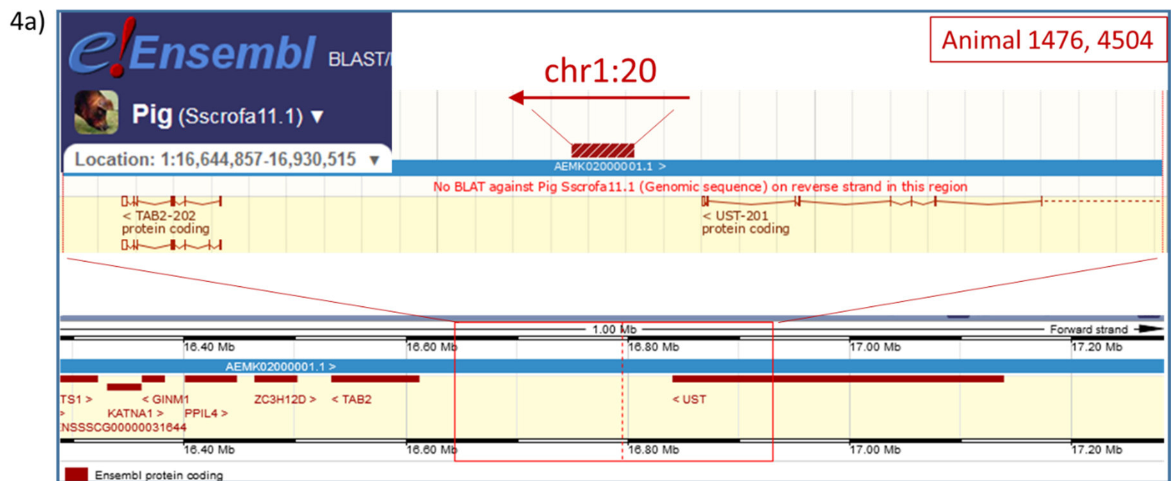
Set1	TGGCCTGATCTATACGTTTG	Set2	CAACCCAAGGACCAGGAC
	CCTATGCGCATACCATTAGT		TCATTAGAGGTTACACAGTTCC

Figure 3: PERV C in our breeding herd. *Genomic matches of provirus-flanking region have been identified with 4 independent primer pairs by TLA sequencing. Only proviruses with consistent matching of all 4 primer pairs were designated as PERV C.*

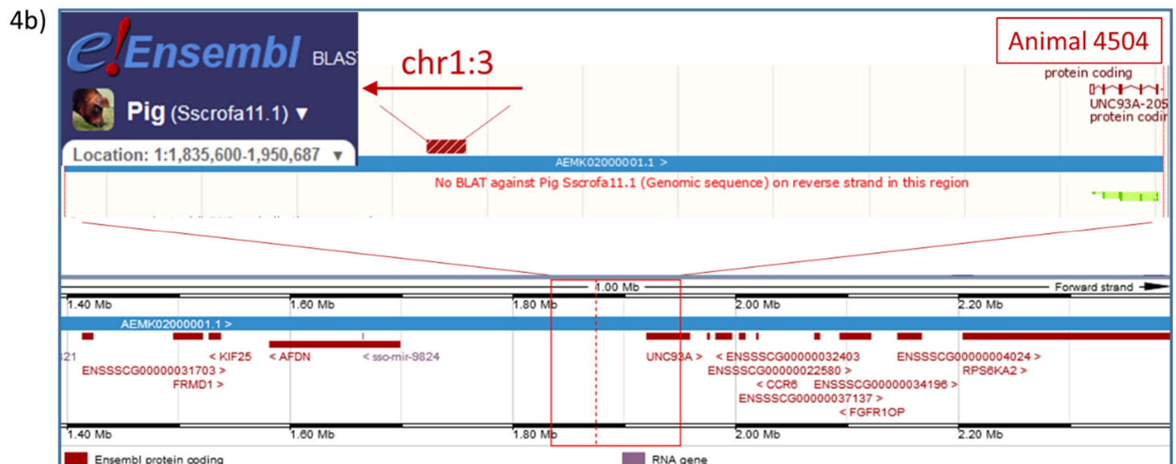
1.3. Chromosomal integration sites characterization of PERV C loci

Next goal was the precise localization of PERV C proviruses with respect to porcine genes. The flanking region sequences from TLA sequencing analysis of all PERV C sites were used to localize the integration sites with respect to annotated genes and to establish specific PCR for each provirus. The screening of provirus integration sites was repeated once the significantly improved reference genome 11.1 was available (Fig. 4 and 5), but the nomenclature of integration sites was maintained as initially defined upon 10.2 reference genome. In 7 of the 11 PERV C loci detected, the integration events have been occurred in intergenic regions of the porcine genome although (Fig. 4).

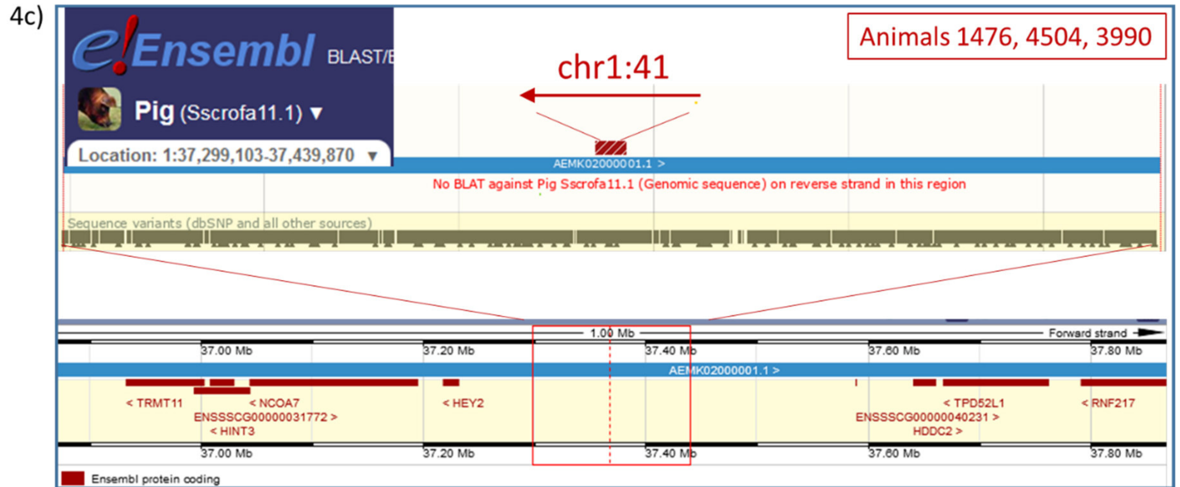
4a)



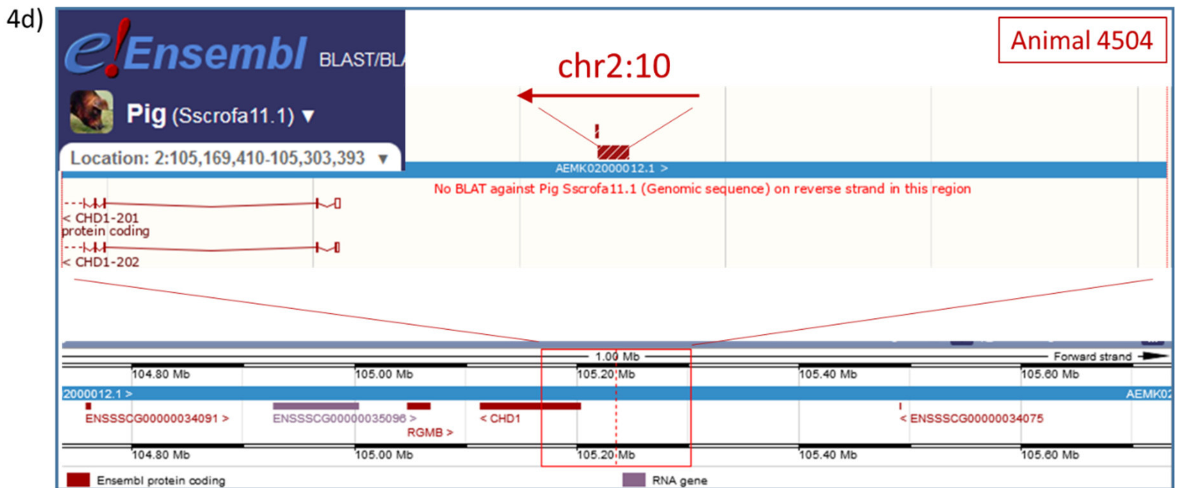
4b)



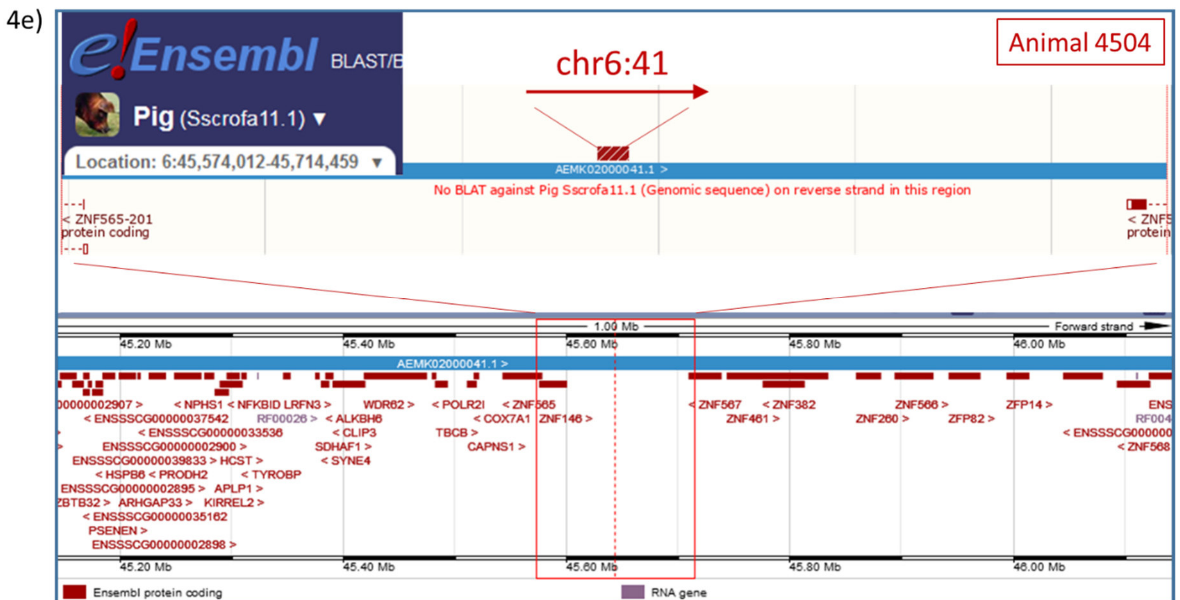
4c)

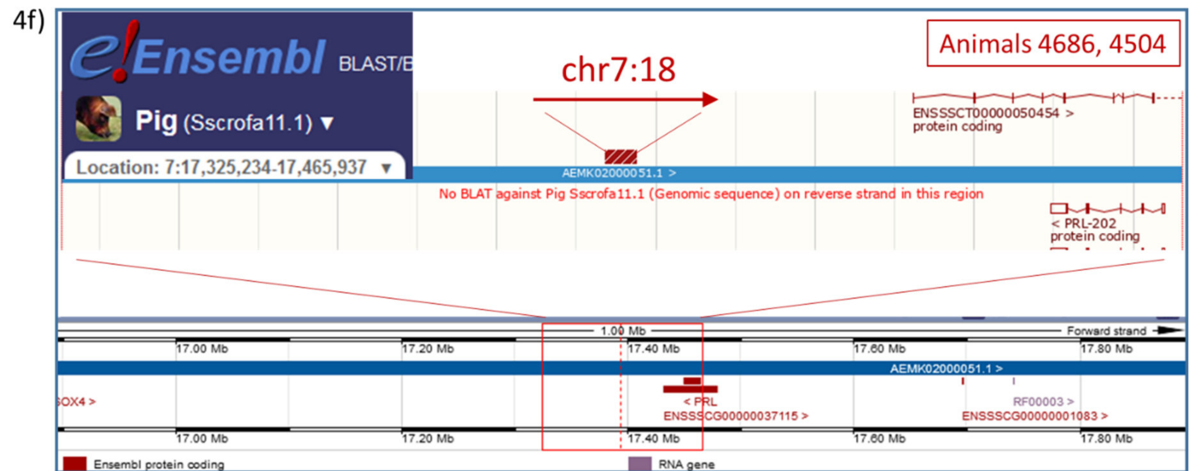


4d)



4e)





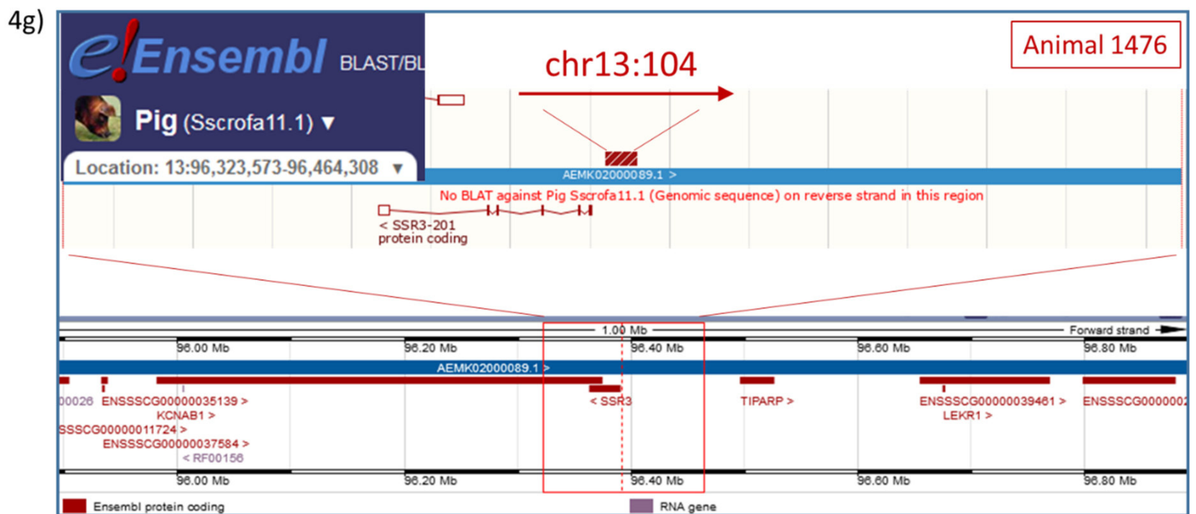
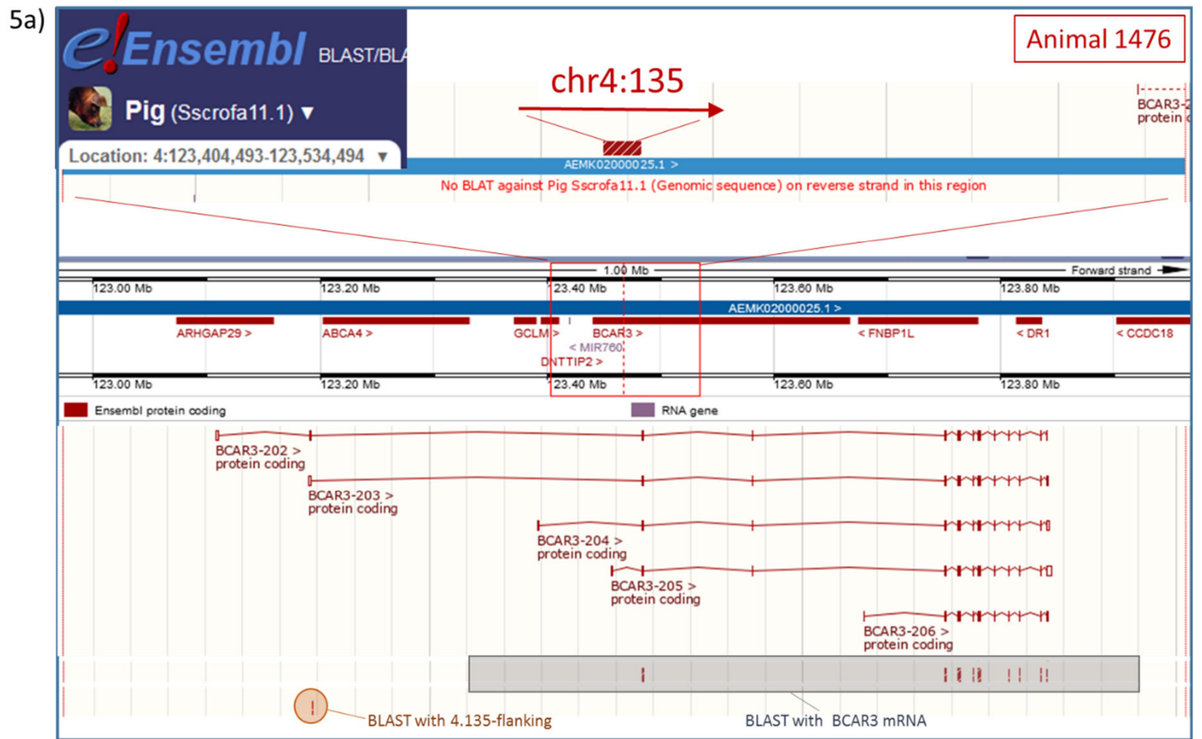


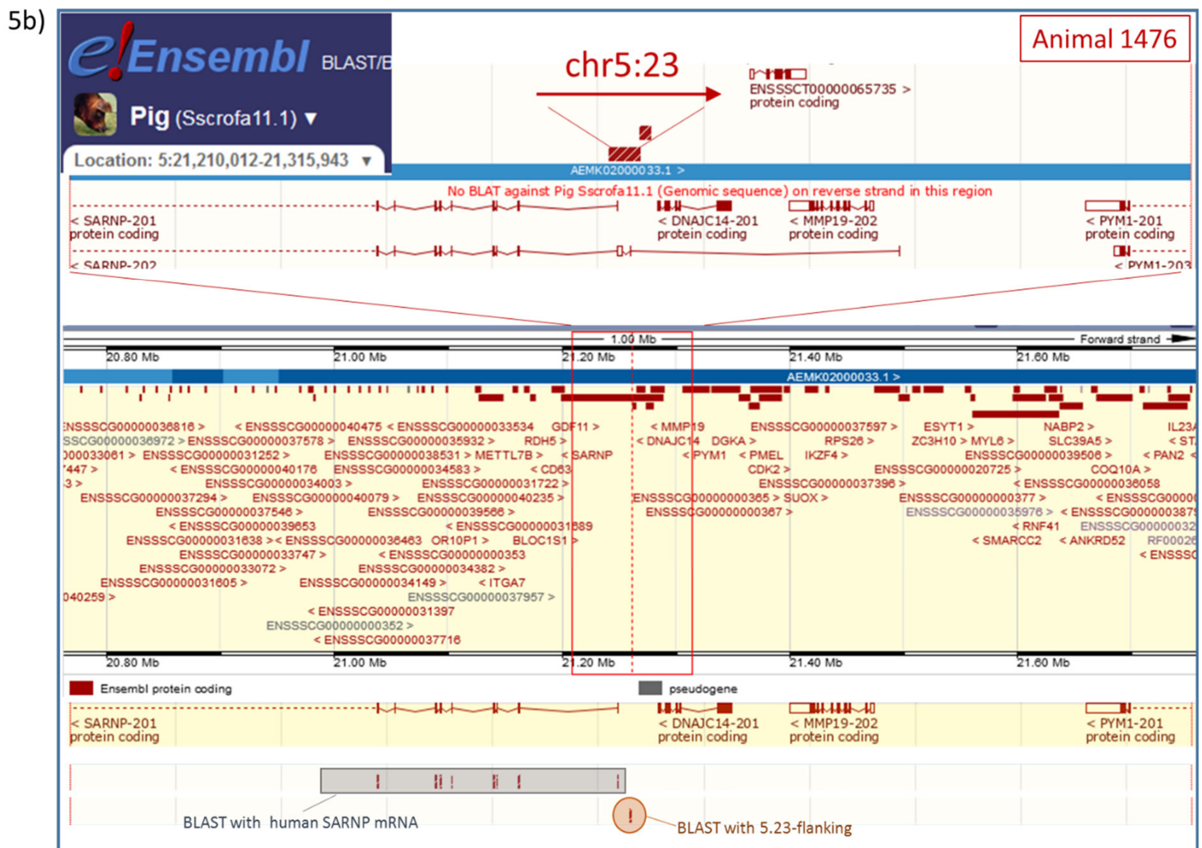
Figure 4: Localization and direction of PERV C loci in intergenic regions. *The flanking regions of the proviruses identified by TLA-sequencing were used to identify the integration site. The direction of the provirus is indicated by an arrow. An overview of the proviral integration site is shown in the lower panel whereas the zoom-in in the upper panel indicates the position of the integration site and the position of the nearby genes in more detail. Gene annotations are indicated by the Ensemble style.*

In the 4 remaining PERV C loci the provirus is integrated within intronic regions of annotated genes (Fig. 5).

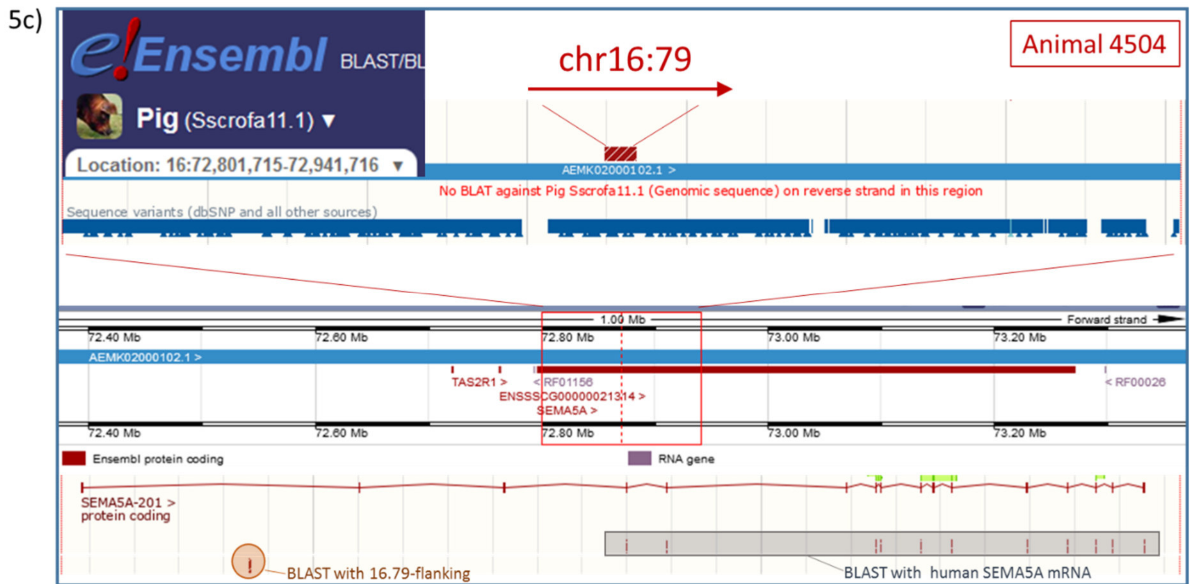
5a)



5b)



5c)



5d)

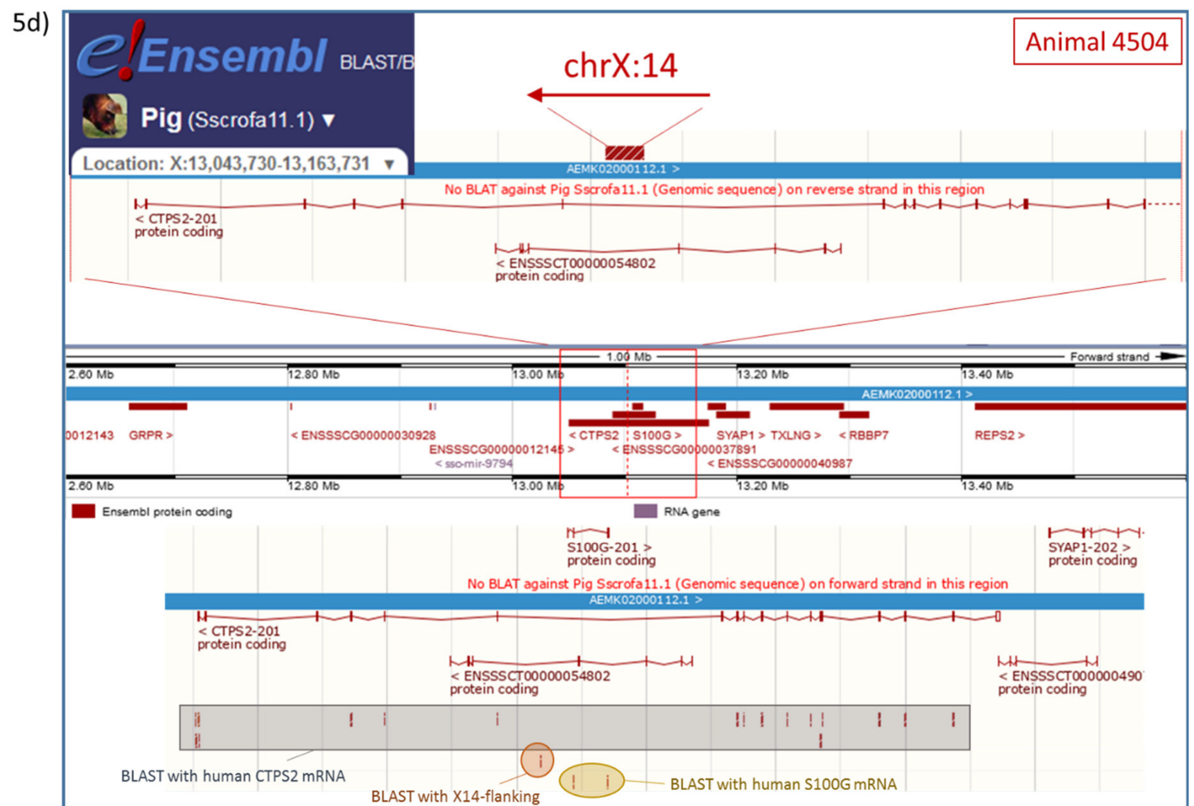


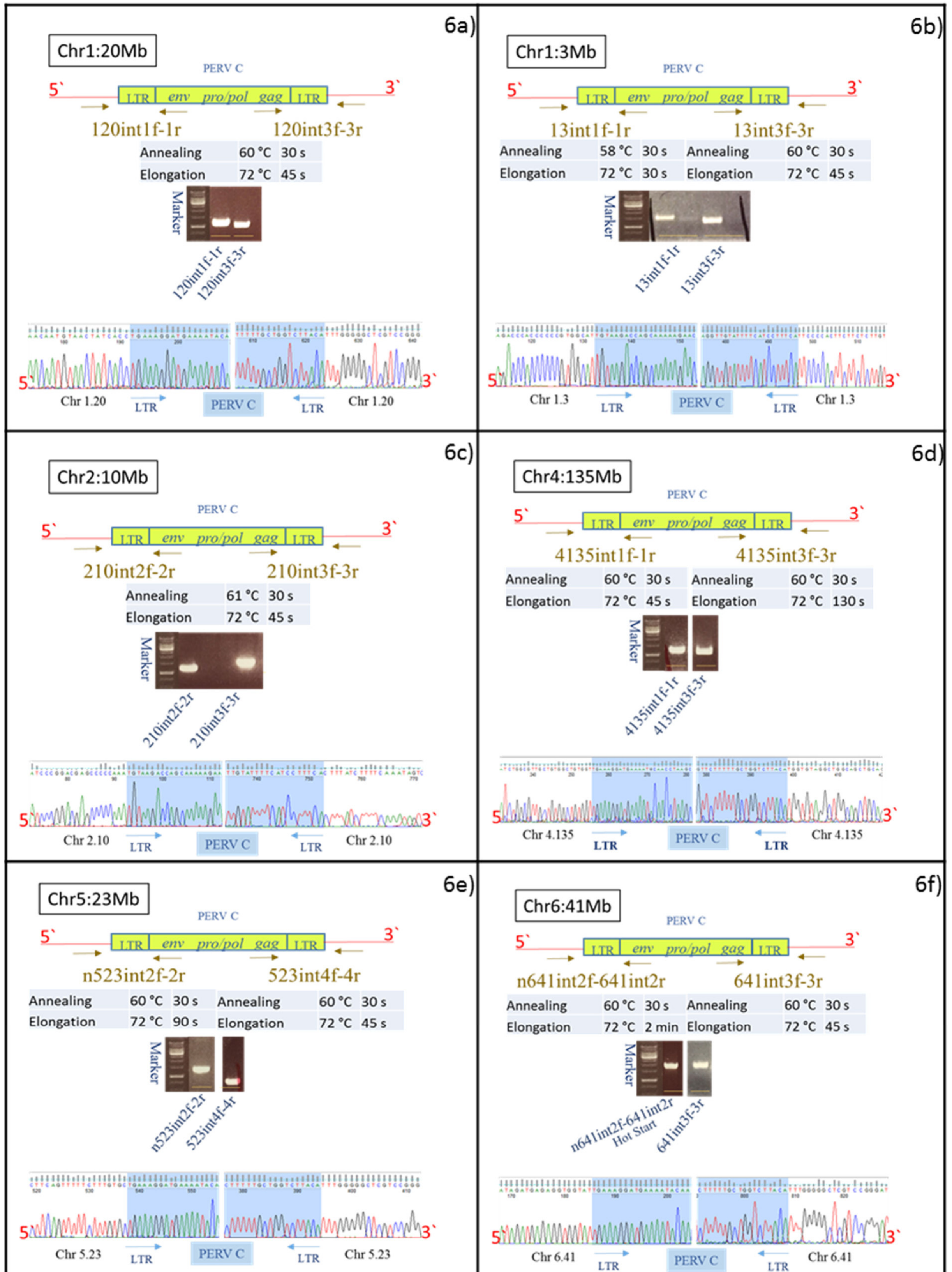
Figure 5: Localization and direction of PERV C loci within to annotated genes. *The elements are given as in the Fig. 4 and exons of porcine genes are confirmed by their homologies to the orthologous cDNAs from human and/or cattle.*

2. Screening for identified PERV C loci in our animals

Next goal was the establishing of reliable screening tools for each of the identified PERV C proviruses in our herd. Based on the integration sites of the 11 PERV C loci defined in paragraph 1.3, primer pairs were designed to establish specific PCRs for both sides of the PERV C loci. The optimized primer pairs were used also to confirm the LTR on the 5’ and 3’ end of all PERV C for a precise determination of proviruses integration time point, based on molecular clock hypothesis (TONJES & NIEBERT, 2003).

2.1. Optimizing PCRs and LTRs characterization

For each of the 11 PERV C loci 2 primer pairs on the 5’ end and 2 primer pairs on the 3’ end were designed and tested under different conditions. The initial annealing temperature was 58°C which was then modified during the optimization process, together with other PCR conditions such as elongation time, annealing time, $MgCl_2$ and primer pairs concentration. For some of PERV C loci it was necessary to design and optimize new primers. The optimal annealing temperature for the most of the primer pairs was 60°C, whereas the elongation time was substantially different for most of the optimized PCR. For every locus the best PCR for the detection of PERV C loci in our herd are reported in the figures 6. The sequences of 5’ and 3’ the LTRs were then compared to identify point mutations to determine the age of the provirus. Surprisingly, no difference was found between any of the LTR pairs and the LTR between the proviruses differed by 1 nucleotide at most. The PERV C LTR sequences were also (almost) identical to the LTRs in AF038600 which has been used as the PERV C reference. The pattern suggests a very recent integration into the pig genome. Based on general consideration there is assumed that the provirus integration occurred not more than 100.000-400.000 years ago (TONJES & NIEBERT, 2003).


6a)
Chr1:20Mb
PERV C
LTR env pro/pol gag LTR
120int1f-1r
120int3f-3r
Annealing 60 °C 30 s
Elongation 72 °C 45 s
Marker
Chr 1.20
LTR
PERV C
LTR
Chr 1.20
6b)
Chr1:3Mb
13int1f-1r
13int3f-3r
Annealing 58 °C 30 s
Elongation 72 °C 30 s
Annealing 60 °C 30 s
Elongation 72 °C 45 s
Chr 1.3
6c)
Chr2:10Mb
210int2f-2r
210int3f-3r
Annealing 61 °C 30 s
Elongation 72 °C 45 s
Chr 2.10
6d)
Chr4:135Mb
4135int1f-1r
4135int3f-3r
Annealing 60 °C 30 s
Elongation 72 °C 45 s
Annealing 60 °C 30 s
Elongation 72 °C 130 s
Chr 4.135
6e)
Chr5:23Mb
n523int2f-2r
523int4f-4r
Annealing 60 °C 30 s
Elongation 72 °C 90 s
Annealing 60 °C 30 s
Elongation 72 °C 45 s
Chr 5.23
6f)
Chr6:41Mb
n641int2f-641int2r
641int3f-3r
Annealing 60 °C 30 s
Elongation 72 °C 2 min
Annealing 60 °C 30 s
Elongation 72 °C 45 s
Hot Start
Chr 6.41

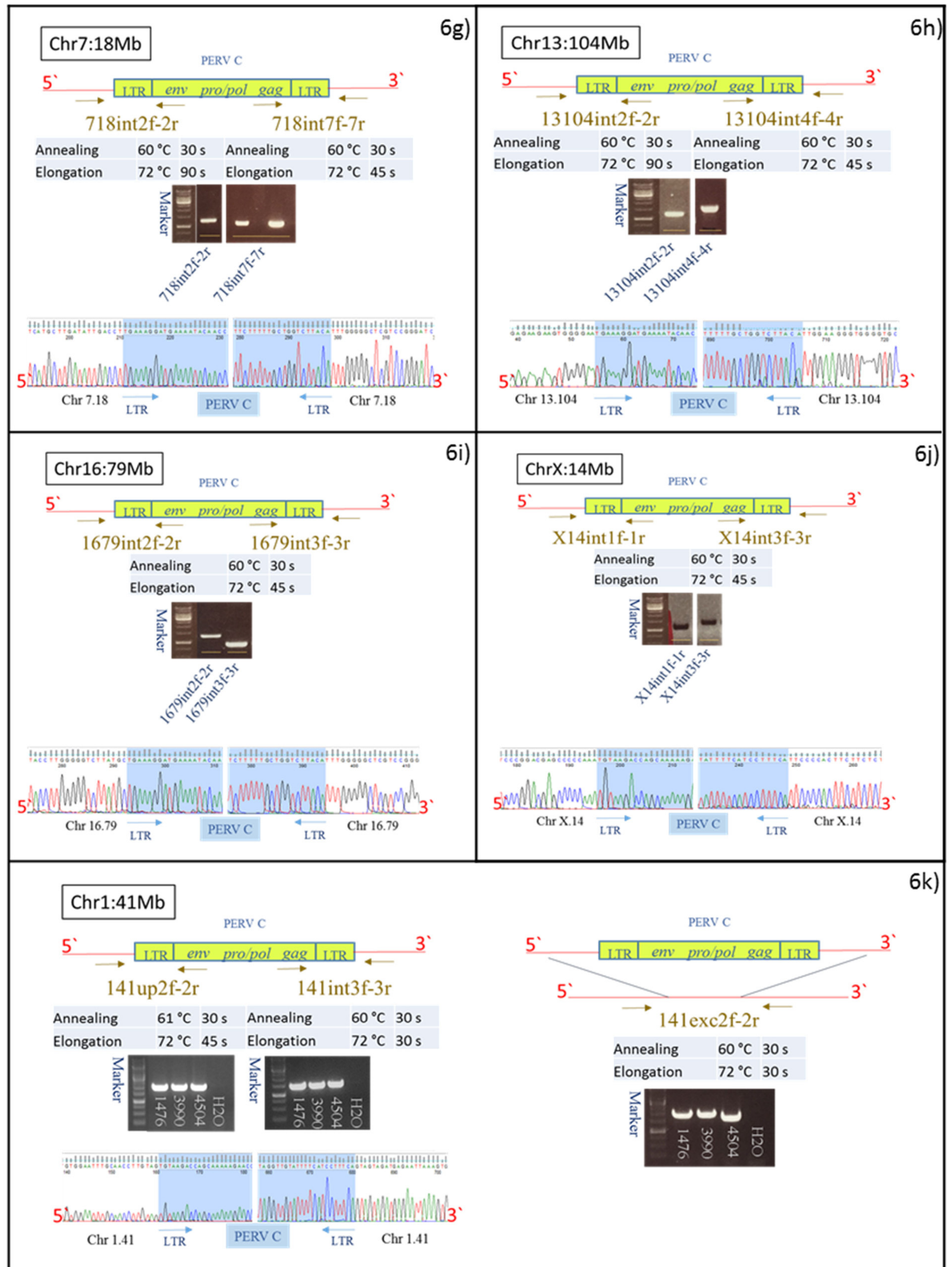


Figure 6: Site specific PCR for each PERV C locus. *Primer names, their position as*

well as the optimal annealing and elongation conditions indicated. A representative PCR figure is given and the Sanger sequencing electropherograms show the transition from the genome into the provirus. For chr1:41Mb an additional PCR was optimized for detecting alleles that do not contain the (right panel).

2.2. Xeno herd screening

The established PERV C loci PCR allowed to screen our existing xeno donor herd for the integration of the provirus. The screening for PERV integration sites is used in our lab as routine genotyping procedure for new litters (Tab. 5) in addition to the genotyping for genetic modifications. Up to now 278 animals with different transgenic background were screened initially by myself and meanwhile by lab-technicians. 32 of these animals did not have a PERV C integration in the 11 tested loci and in present xeno herd (20 animals) 4 of the animals are proven as PERV C free. The other 16 animals have PERV C in the chr1:20Mb, chr1:3Mb, chr1:41Mb, chr4:135Mb, chr7:18Mb, chr16:79Mb and in the chrX:14Mb. Thus, 4 of the initially identified proviruses, namely in chr2:10Mb, chr5:23Mb, chr6:41Mb, chr13:104Mb have been sustainably extincted from our herd.

Table 5: PERV C loci screening in our xeno herd. *Representative screening for PERV C loci in our xenotransplantation herd. Animal lacking any provirus integration are shown in orange. Their genotype is indicated in the right-most column (GTKO= homozygous KO of the GGTA1 locus; bLEA= homozygous knock in for bLEA locus; hTBM= homoozygous knock in for TBM locus).*

Nummer	chr1:41	chr1:20	chr1:3	chr2:10	chr4:135	chr5:23	chr6:41	chr7:18	chr13:104	chr16:79	chrX:14	
1476	pos	pos	neg	neg	pos	pos	neg	neg	pos	neg	neg	
1621	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	hTBM
1622	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	hTBM
4673	neg	pos	neg	neg	neg	neg	neg	neg	neg	pos	neg	
4675	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	
4678	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	
4679	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	GTKO; bLEA
5530	neg	pos	pos	neg	pos	neg	neg	neg	neg	pos	neg	
5568	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	
5570	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	GTKO
6109	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	
6110	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	
9948	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	GTKO;hCD46;hTBM
10041	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	
10042	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	

2.3. Side-finding: A new PERV-subtype in PERV C-free animal

Animals that were negative for any of the 11 characterized PERV C proviruses were tested further for the abundance of unknown PERV C loci. For this we established PCR specific for the *env* C gene, independent of the integration site. Interestingly, PERV C negative animals still revealed a PCR band albeit at a much lower intensity. Therefore, the PCR bands were excised and the DNA was sequenced. The obtained sequences were compared to the PERV A, B and C *env* reference sequences (Fig. 7). The sequences were highly homologous to A, B and C, but they did not fully correlate to any of the proviral subfamilies. Instead the sequences were (almost) identical to the Gene Bank annotations AF402663 and DQ996276 which have been characterized as a new variant of PERV C (HECTOR et al., 2007). The nucleotide sequence defines an amino acid sequence that is different from the protein sequences of PERV A, B and C. If this resembles a new tropism of PERV remain elusive as well as if this not yet described retrovirus is endogenous or represent an active provirus.

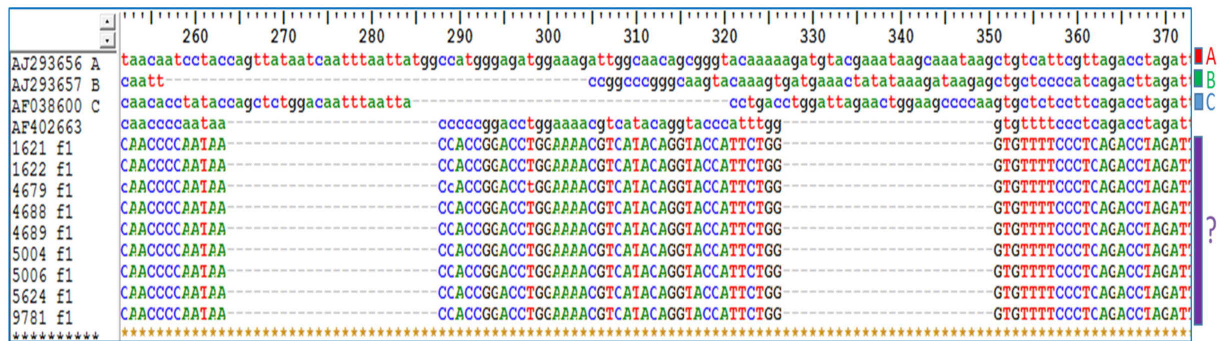


Figure 7: *Sequencing of env C PCR products*. *The sequences obtained are compared with the 3 subtypes of PERV and the Gene Bank annotation AF402663 . The different sequence starts at the 256bp position and end in the position 361bp.*

3. PERV C free animals by deleting a selected provirus

Due to the specific pattern of PERV C integration sites in each animal, selective breeding is a promising way to remove PERV C from donor animals for xenotransplantation. On the other hand, it is definitely a time consuming way, so sometimes it might be more efficient to excise PERV C from the genome of a precious animal. Therefore, the possibility to excise single chr1:41Mb provirus from a single individual (3990) representing the GTKO, hCD46, bLEA was examined in more detail. To achieve this goal, the chr1:41Mb site was characterized in detail, a CRISPR/Cas mediated gene editing approach was designed to excise this specific provirus.

3.1. Characterization of the chr1:41Mb integration site

First, the zygosity of the chr1:41Mb was determined in animal 3990. For this we used the above described primer pairs 1.41exc2, 1:41up2, 1:41int3 to produce PCR products and perform Sanger sequencing (Fig. 8). Importantly, PERV C is integrated in only one chromosome. Therefore, in the animal 3990 discrimination of the PERV containing and PERV lacking alleles through polymorphisms is important for designing sufficient gRNA for excision of the provirus as well as for screening cell clones.

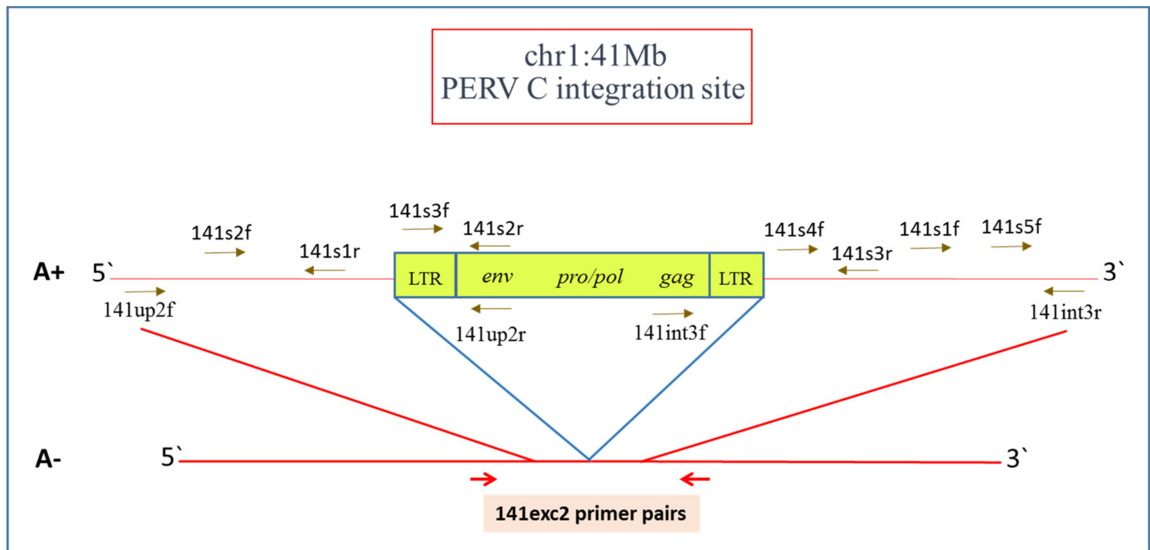
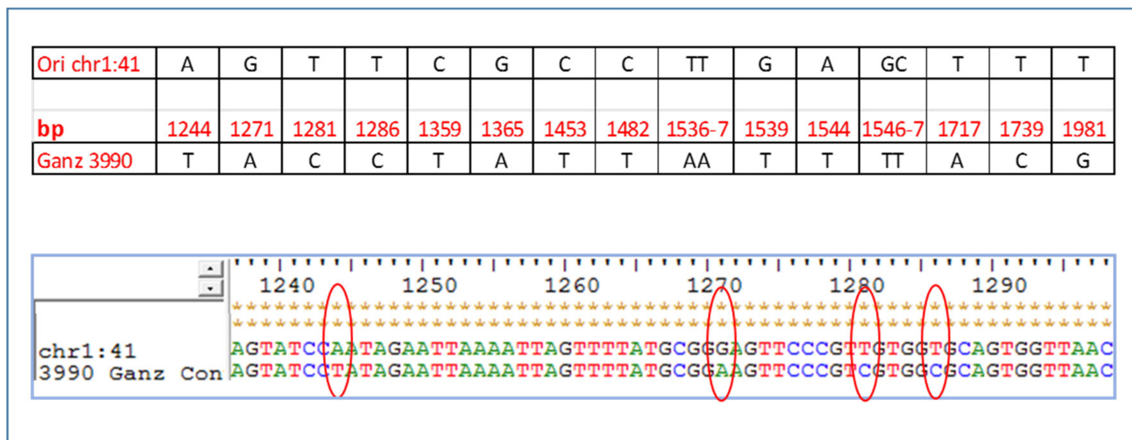


Figure 8: Amplifying and sequencing primers and their position. *All primers for characterizing the alleles with or without PERV C are indicated.*

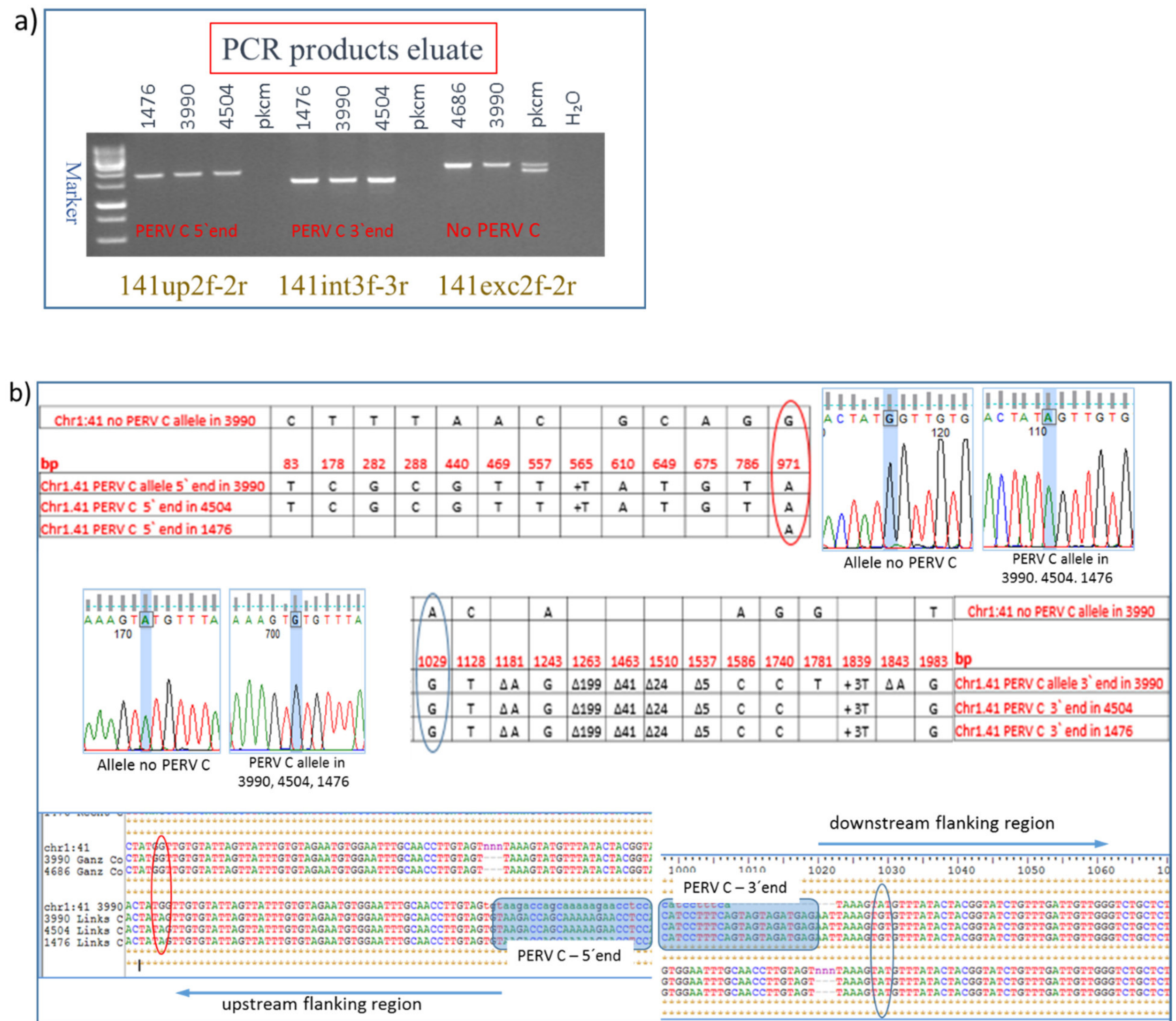
The allele without PERV C revealed 17 different polymorphisms which spread over 2kb when compared with the reference genome at chr1:41Mb (Fig. 9).



Ori chr1:41	A	G	T	T	C	G	C	C	TT	G	A	GC	T	T	T
bp	1244	1271	1281	1286	1359	1365	1453	1482	1536-7	1539	1544	1546-7	1717	1739	1981
Ganz 3990	T	A	C	C	T	A	T	T	AA	T	T	TT	A	C	G

Figure 9: Polymorphisms of the allele without provirus integration in the 3990 animal.

Similarly, the allele containing PERV C was sequenced in animal 3990 and the pigs 4504 and 1476 which contain this specific provirus as well. Overall the PERV-lacking and the PERV-containing alleles in pig 3990 differ by 13 polymorphic sites in the upstream region of the provirus and further 13 sites in the downstream region of the PERV (Fig. 10).



Chr1:41 no PERV C allele in 3990	C	T	T	T	A	A	C		G	C	A	G	G
bp	83	178	282	288	440	469	557	565	610	649	675	786	971
Chr1.41 PERV C allele 5` end in 3990	T	C	G	C	G	T	T	+T	A	T	G	T	A
Chr1.41 PERV C 5` end in 4504	T	C	G	C	G	T	T	+T	A	T	G	T	A
Chr1.41 PERV C 5` end in 1476													A

A	C		A					A	G	G			T	Chr1:41 no PERV C allele in 3990
1029	1128	1181	1243	1263	1463	1510	1537	1586	1740	1781	1839	1843	1983	bp
G	T	ΔA	G	Δ199	Δ41	Δ24	Δ5	C	C	T	+3T	ΔA	G	Chr1.41 PERV C allele 3` end in 3990
G	T	ΔA	G	Δ199	Δ41	Δ24	Δ5	C	C		+3T		G	Chr1.41 PERV C 3` end in 4504
G	T	ΔA	G	Δ199	Δ41	Δ24	Δ5	C	C		+3T		G	Chr1.41 PERV C 3` end in 1476

Figure 10: Polymorphisms of the alleles with provirus integration. *a) PCR products derived from the amplification of the 5´adjacent region (primer pair 141up2f-2r) and the 3´adjacent region of the provirus (primer pair 141int3f-3r) as well as the PCR product derived from the allele lacking the provirus (primer pair 141exc2f-2r) are shown.*

b) Positions and nature of 13 polymorphic sites in the upstream region (upper panel) and of 13 polymorphic sites in the downstream region (middle panel) are shown. Examples of polymorphic positions are indicated by electropherograms. The integration site of the provirus as well as the closest polymorphic positions are indicated in an alignment (lower panel).

3.2. Defining gRNAs

The polymorphic sites in the flanking regions of the chr1:41Mb locus allowed an optimal design of the gRNAs for the excision of the provirus through CRISPR/Cas9 technology. Three gRNA were designed, two upstream of the 5' LTR and one downstream of the 3' LTR. gRNA candidates were designed by Chop Chop and the gRNAs that presented high efficiency and specificity value were chosen for the excision of PERV C (Fig. 11). gRNA51 and gRNA52 are located respectively 0.717 kb and 0.776 kb upstream of the provirus and gRNA31 is located 0.592 kb downstream of the provirus. Thus, successful gene editing will result in the excision of the provirus and approximately 1.3 kb of its flanking regions.

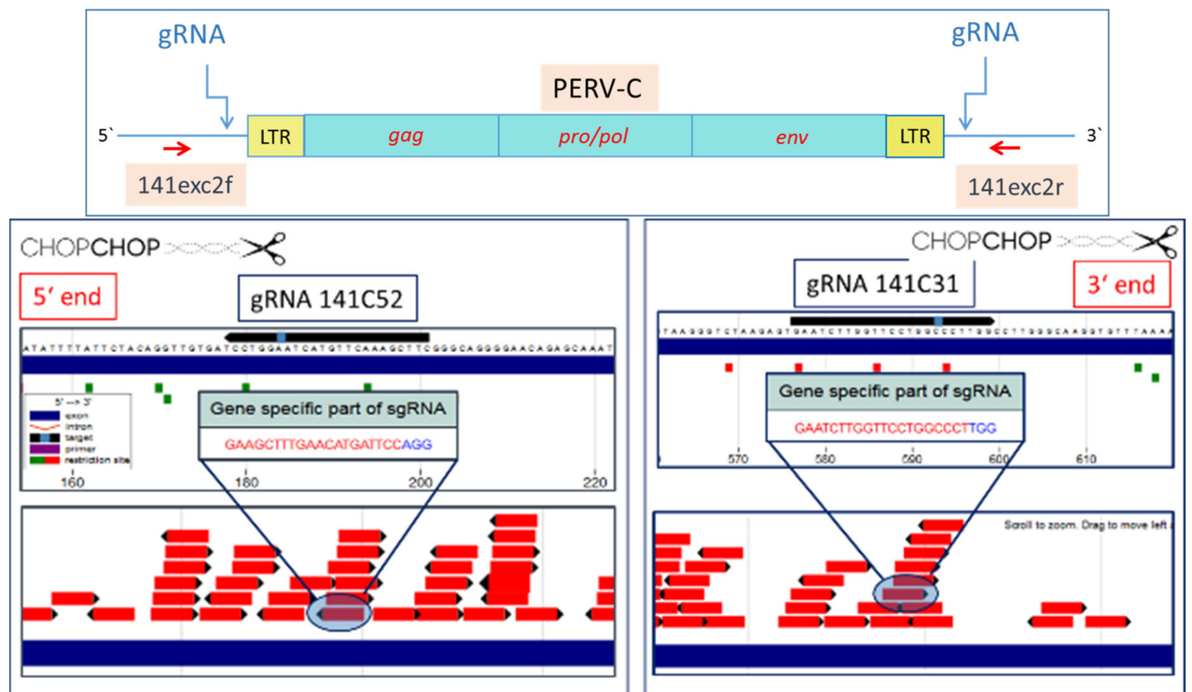


Figure 11: gRNAs position outside PERV C and example RNAs designing by chop chop.

Plasmid vectors expressing the respective gRNA under the control of a human RNA polymerase promotor U6 were synthesized according to (MALI et al., 2013) and transformed in TOP 10 *E.coli* cells in order to verify gRNAs sequence. All 3 gRNAs proved to be correctly synthetized at nucleotide level (Fig. 12).

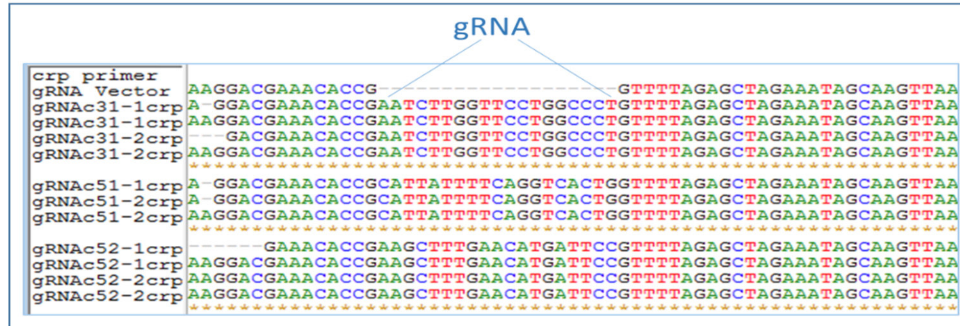


Figure 12: gRNAs nucleotide sequence. *For sequencing control gRNA primer (crp) forward and reverse were used.*

3.3. Producing and screening 3990 single cell clones

Transfection and production of single cell clones was performed with assistance by Eva Jemiller at our institute. After verification of gRNA sequences, 3990 cells were transfected with independent vectors encoding the Cas protein and the 3 gRNAs.

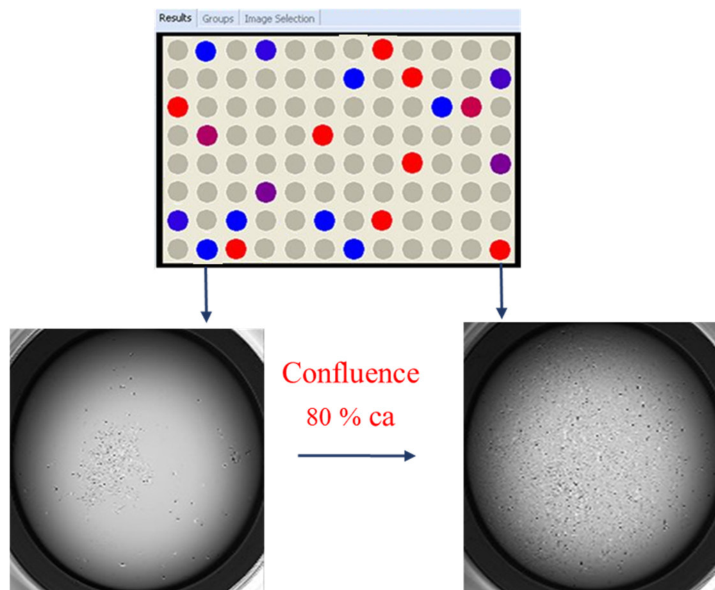


Figure 13: Example of single cell clone at 80 % of confluence.

After transfection, the cells were counted, diluted and seeded into 96 well plates in order to have 1 cell pro well. In a first experiment, cells were seeded into a total of 10 96 well plates. After reaching the 80 % confluence the single cell clones were splitted into 2 wells, one as a potential backup for somatic cell nuclear transfer and one for screening. In the first set of experiment 85 cell clones were obtained. After isolating genomic DNA from each cell clone, PCRs were performed to discriminate the allele with the provirus and the allele lacking the provirus (141int3f-3r,141exc2f-2r). Different patterns of screening products were obtained. A positive 141int3 PCR indicates a still abundant provirus at chr1:41Mb while a negative PCR indicates excision of provirus which should be accompanied by a 0.7 kb fragment in the 141exc2 PCR. For 19 clones PCR failed; 5 clones indicated excision of the provirus and 57 clones still retained the provirus at chr1:41Mb. If only a single 0.7 kb appeared in the 141exc2 PCR, also the PERV C lacking allele has undergone the excision of the 1.3 kb element between the gRNAs. If an additional 2 kb appeared, the PERV C-lacking allele underwent no or only minor modifications.

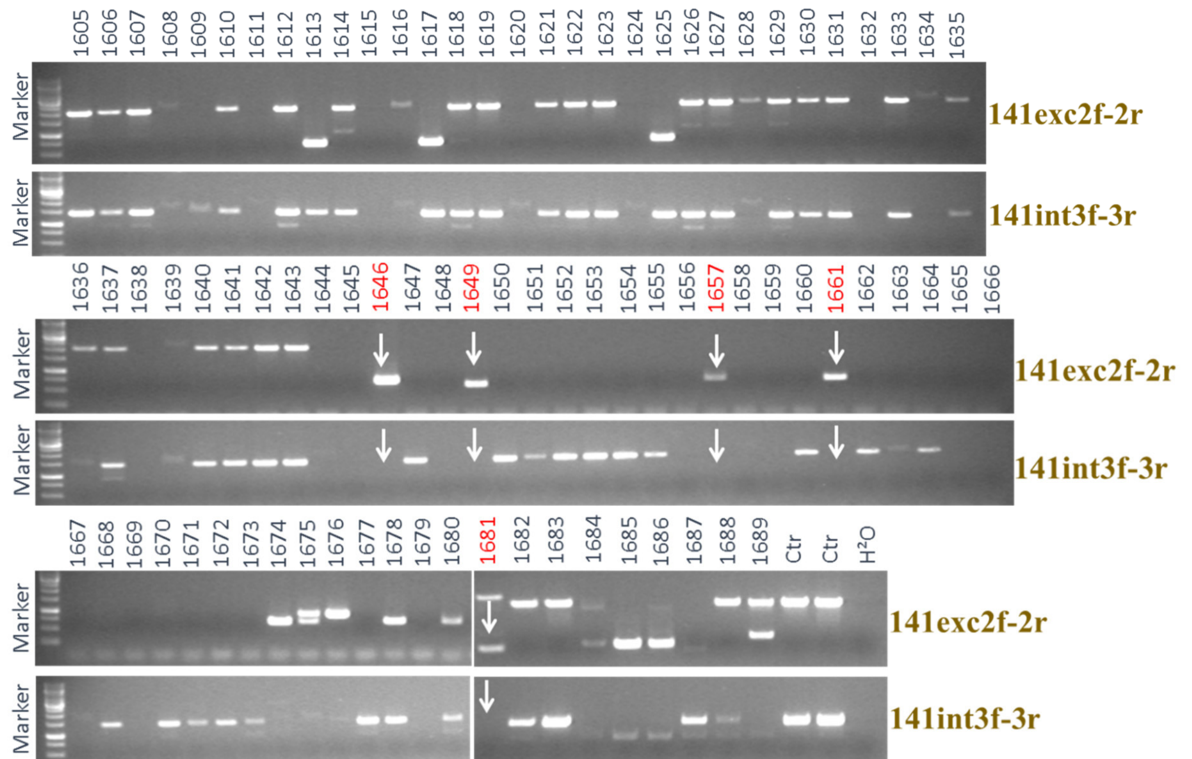


Figure 14: Single cell clones screening PCRs. *141exc2 primer pairs amplifying the*

entire PERV C locus and 141int3 primer pairs amplifying the transition region PERV C-chromosome on the 3' end.

The clones were further sequenced to verify the excision of PERV C. Three of them, namely 1657, 1646 and the 1681 demonstrated PERV C excision on the right allele and using the polymorphic sites between the allele with and without the provirus, further indicated that the provirus was excised by NHEJ (Fig. 15).

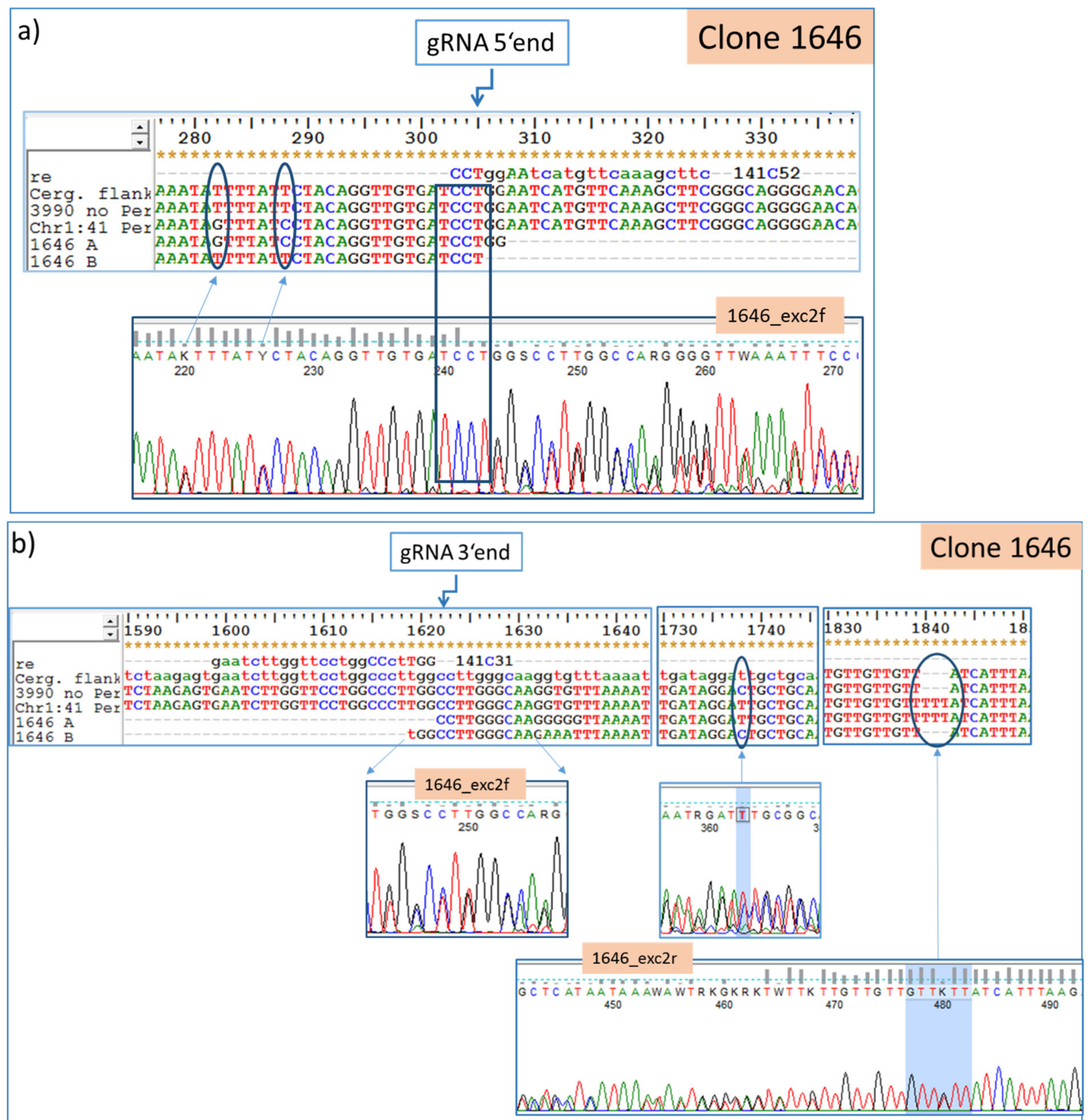


Figure 15: PERV C locus sequencing pattern of a typical clone that lack PERV C.

a) The region around the 5´ cutting site is shown in an alignment and in an electropherogram with the adjacent SNP and the different cutting sites on the two alleles. On the 5'end is shown the gRNA cutting site and 2 SNPs that discriminate the allele with PERV C from the one that lack it. In the elctropherogram is possible to see the 2 SNPs peaks as well as the starting point of overlapped sequence because of a frameshift due to the cutting site. Overlapping sequences happened since 2 alleles are sequenced in the same reaction/time. b) The corresponding region for the region around the 3´cutting site is shown in an alignment and representative electropherograms. On the 3'end is shown the gRNA cutting site, 1 SNP and in addition a INDEL of 3 nucleotides. In the allele with PERV C there is an insertion of 3 T in the position 1840 and this is the point where the sequence starts to overlap.

Interestingly, in 2 clones (1649 and 1661) the provirus has been removed rather by HR than NHEJ, as indicated by the polymorphic sites (Fig. 16).

Figure 16: PERV C locus sequencing pattern of a typical clone that lack PERV C not by NHEJ. *No overlapped sequence is observable.*

In a second experiment 62 further cell clones were obtained and screened with the same primer pairs as in the first set. Of these, 12 clones lack PERV C and 2 clones (573 and 586) shown 2 unspecific bands in the 141int3 PCR (Fig. 17).

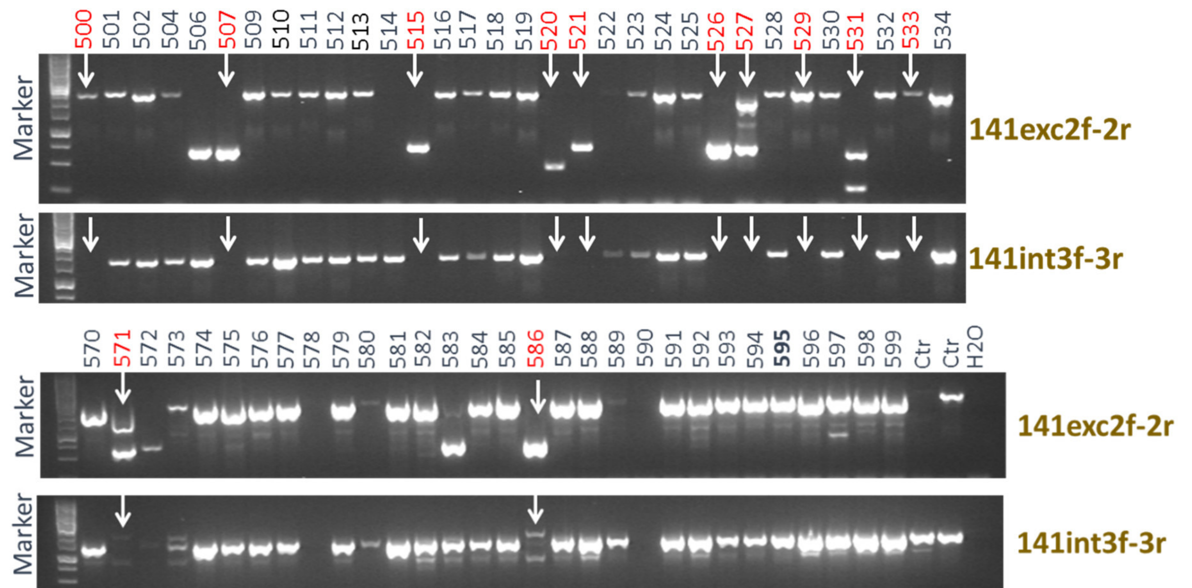


Figure 17: Single cell clones screening PCRs. *Second set of single cell clones screened with 141exc2 primer pairs amplifying the entire PERV C locus and 141int3 primer pairs amplifying the transition region PERV C-chromosome on the 3' end*

All 14 clones were further tested to confirm the excision of the provirus with two different PCR. One PCR using the primer pairs 141up2f-2r amplifies the region of the chromosomal flanking region and the LTR on the 5' end and the second PCR using envC1 primer pair amplifies any PERV C element. The clone 573 was positive for the 2 PCRs, indicating still the presence of the provirus. The clones 571and 586 were negative for both PCRs, indicating that only those have actively removed the PERV C provirus. The clones (572, 578 and 580) were positive for 141up2 PCR and negative for EnvC1 PCR (Fig. 18b) indicating that only the 3′-region of the chr1:41Mb has been removed from the genome. In other words, these clones cannot be used for generating PERV-C free animals as a truncated PERV-C is still present.

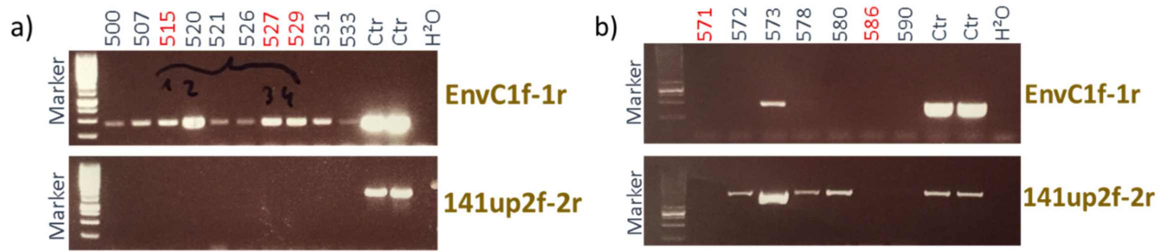


Figure 18: **PERV C *screening***. *PCRs of the single cell clones that shown PERV C lack on the first screening. The primer pairs used are EnvC1 and 141up2.*

The remaining single cell clones (Fig. 18a) lack PERV C as no PCR product was obtained amplifying with 141up2 primer pair. The unexpected finding of a faint band with the envC1f-1r PCR in clones 500, 507, 521, 526, 531, 533 with the above mentioned unspecific amplification of a non-PERV-C element (see paragraph 2.3.). For the clones 515, 520, 527 and 529 the more intensive bands from the envC1f-1r PCR were further examined by sequencing; all of them, except 520 again revealed a Sanger sequence that corresponded rather to AF402663 than to the references of PERV A, B and C (Fig. 19).

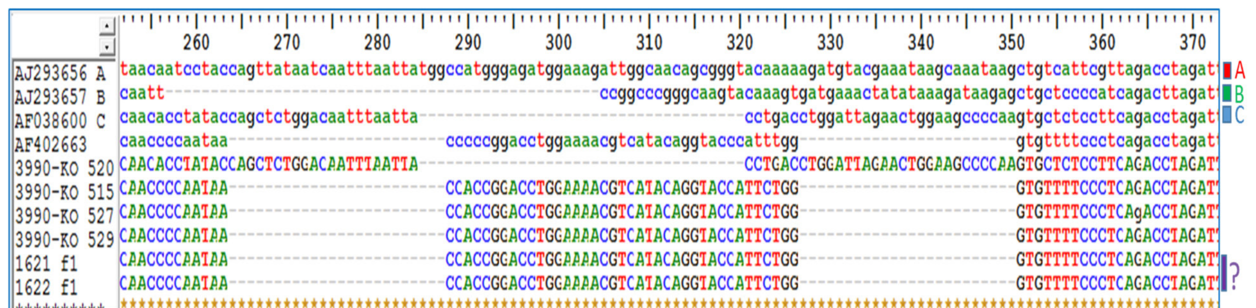


Figure 19: Sequencing of envC PCR products. *env C sequences of 520, 515, 527 and 529 cell clones compared to the sequence of PERV A, B and C.*

3.4. Perspective: SCNT approach

Somatic Cell Nuclear Transfer is a preferred method to produce genetically modified pigs at our institute. Of the147 single cell clones 15 lack PERV C at chr1:41Mb (10,2%). These cell clones can be used to generate GTKO. hCD46. bLEA triple modified pigs lacking PERV C. The generation of such pigs is an ongoing approach.

V. Discussion

With the increasing number of patients waiting for organ transplantations and the restricted number of human donors new alternative methods are needed to provide therapeutic options for the patients. Xenotransplantation using pigs as potential donor for organs, tissues and cells became a promising solution for closing the gap between the number of necessary allografts and the number of available donors, because of their organ size and physiological similarity to humans, their easy genetic manipulation and the high rate of reproduction (ONIONS et al., 2000; HRYHOROWICZ et al., 2017). Despite the enormous progress towards clinically applicable xenotransplantation in the recent past, questions regarding safety for the human recipient and the population has not yet been sufficiently clarified (COWAN & D'APICE, 2008; MATTIUZZO et al., 2008; NELLORE & FISHMAN, 2018). The removal of bacteria, fungi, protozoa and viruses from the source pig can be achieved with vaccines and drugs, designated pathogen-free (DPF) breeding conditions and early weaning (DENNER & MUELLER, 2015; EGERER et al., 2018), whereas this is not possible for PERV as they are stably integrated in the pig genome and are transmitted to the offspring according to Mendelian rules (ONIONS et al., 2000). Among the replication competent PERV, PERV C is the most critical subtype since it can recombine with PERV A, resulting in PERV A/C which are highly capable to infect human cells. Therefore, it will be essential to provide PERV C free donor animals for xenotransplantation. Different approaches were already tested to reduce the pathogenic potential of PERV C by producing neutralizing antibodies (KAULITZ et al., 2011a; WAECHTER & DENNER, 2014) or RNA interference, a strategy which silences the expression of a gene at the post transcriptional level. These approaches, however, only knockdown/reduce the expression of PERV without removing it physically from the porcine genome (MIYAGAWA et al., 2005; RAMSOONDAR et al., 2009; SEMAAN et al., 2012). In this doctoral thesis, 2 different strategies were tested to achieve the elimination of PERV C at the genomic level, namely selective breeding and gene editing by the CRISPR/Cas system. Targeted Locus Amplification sequencing was used to identify integration sites of PERV C in our herd which consists

of a mix of conventional pig breeds like German landrace, Swabian-Hall , Duroc and Pietrain. In 4 animals of our xenoherd with different genetically modified background we detected a total of 11 PERV C loci. None of these animals was PERV C free, but very importantly none of the 11 PERV C integrants was present in all of the animals. This finding is relevant for 2 reasons: First, the diversity of PERV C proviruses suggests that PERV C can be removed from the herd by selective breeding. Second, the diversity of PERV C load in the animals suggest a very recent timepoint for the integration of PERV C into the pig genome. This assumption is supported by the fact that the LTRs sequenced were identical within each provirus and differed only marginally between the LTRs of the proviruses integrated in the different loci. In this context it seems remarkable that one of the founder animals, the GTKO. hCD46 modified 9948 descends from a US-based entity (Revivicor). The genetic origin of this individual is not fully clarified, but based on personal communication this pig has been produced by cross-breeding US-based individuals (PHELPS et al., 2003) with Australian pigs (LOVELAND et al., 2004). Still, the only PERV C provirus identified in 9948 was almost identical to the other PERV that obviously descend from the Munich herd. The diversity of PERV integration sites suggest that the different proviruses descend from the different pig breeds that comprise our hybrid breed mixture.

Importantly, analyzing PERV C load or copy number at DNA level does not allow to say if these proviruses are biologically active and capable to infect. Therefore, it will be interesting to learn more about the capacity of the PERV C proviruses to infect new cells. Interestingly, in pig individual has been shown increase of proviral copy number in somatic cells compared to those in germinal cells, illustrating the general replication competence of PERV C. Whether these infection events are caused by PERV from the same individual or from exogenous viral particles remain unclear. Importantly, however, so far there has been no evidence documented that PERV C is nowadays still infecting the germ line (MOURAD et al., 2017; FIEBIG et al., 2018).

The animal 3990 contains only one single provirus integration site and became our target for the excision of PERV C by gene editing. The identified provirus was localized at chr1:41Mb using the pig reference genome within a large intergenic

region. This is highly relevant because the excision of the provirus using CRISPR/Cas9 eliminated also 1.3 kb of the surrounding genomic region. Although not finally confirmed, the intergenic localization of PERV C suggests that an effect on the overall function of the pig genome is very unlikely. This would have been not the case if any of the gRNA used for excision of the provirus bind in regulatory regions or even within a coding sequence.

All 11 PERV C loci were characterized using the flanking regions of the proviruses to localize the integration site and to use this DNA sequence for the establishment of site specific screening PCRs for every identified locus. A potential risk in the establishment of these PCRs was the possibility to amplify unspecific regions rather than PERV flanking elements. This cannot be excluded as PERV themselves as well as many of the flanking regions are repetitive elements and are present in high copy number in the porcine genome. To be sure and further verify the amplification specificity of the designed primer pairs, all PCR products were sequenced at a regular basis. In any case, the PCR products comprise the chromosomal flanking region and the LTR on the 5’ end and on the 3’ end of every provirus and thus resemble a robust screening tool for the 11 proviruses. As we have analyzed individuals only for the 11 previously detected PERV C loci, it might be that there are some additional proviruses present in our herd. Indeed, in some animals we found a clear PERV C signal when using the unspecific envC PCR, albeit animals were clearly negative for any of the 11 known proviruses. The significant number of animals that were evidently PERV C free when they appeared negative for the 11 PCR suggests, however, that this number is relatively low. Still it might be meaningful to re-analyze the xeno breeding herd at a later timepoint. Very surprisingly the PCR that we have initially designed as specific for *env* of PERV C resulted in a positive PCR signal in any animal, albeit at a much smaller intensity for some of the pigs such as 1621, 1622, 4679, 4688, 4689, 5004, 5006, 5624, and 9781. Upon sequencing, however, the less intensive band appeared as a different sequence, correlating rather to the Gene Bank entries AF40266 and DQ996276. Albeit this type of PERV *env* has been designated on a new variant of PERV C (HECTOR et al., 2007), I do not fully agree with this interpretation. The translated amino acid sequence might rather resemble a new PERV subfamily in the

porcine genome with a not yet determined tropism, as the PCR amplifies a stretch from the receptor binding domain (RBD) (ARGAW et al., 2008; ARGAW & WILSON, 2012) of the *env* gene. At the present time point, however, it is even unclear if the proposed new subfamily is even replication competent, as the respective Gene bank sequences do only comprise the *env* gene. In this context, it is of note that the quality of the pig reference genome is still not fully satisfactory. For the 11 identified PERV C integration sites the annotation of the nearest genes did not differ between the initial poor quality version 10.2 and the improved version 11.1. This is particularly true for the gene edited provirus at chr1:41Mb. The comparison of exon definition in the pig genome with BLAST matches of the corresponding human and bovine cDNA sequences further confirmed the characterization of the PERV integration sites.

Despite there is evidence that there are some additional, not yet characterized PERV C proviruses in any pig breeding herd, here I further demonstrate that PERV C can be removed effectively from donor pigs through selective breeding. However, the maintenance of the PERV C free animal status is an interesting aspect, because the young age of PERV C in the pig genome suggests that proviruses might still be active. New integration can therefore not be excluded, if PERV C free animals are maintained in a facility together with PERV C positive pigs. At best, de novo infections might be prevented by maintaining PERV C free pigs in a PERV C free stable.

As selective breeding requires time, space and economic resources the removal of PERV C by gene editing is a promising alternative way to generate PERV C free pigs. A ZFN-based approach was already used to knock out PERV from the porcine genome, but this attempt failed very likely due to the high copy number of PERV and the consequent introduction of multiple cutting sites in the genome resulting in DNA destabilization and high toxicity for the cells (SEMAAN et al., 2015). Recently, 2 other studies used the CRISPR/Cas technology instead (YANG et al., 2015; NIU et al., 2017). The principle aim was the inactivation of any PERV in the porcine genome by introducing deleterious mutations in the proviral *pol* gene but without discriminating between PERV A, B and C. It is of note that in the work performed by NIU et al. the cell line was wild type and not a cell line derived from a genetically modified pig useful for xenotransplantation. In contrast, in this doctoral thesis I worked on

multimodified pigs of our xenoherd with proven protective effect in xenotransplantation of heart (LANGIN et al., 2018) or islets (KLYMIUK et al., 2012). In addition, the PERV-inactivating approach introduced multiple modifications at once which might anyways not be necessary, as most of the proviruses in the porcine genome appear replication defective. Again this doctoral thesis represents an alternative approach as it was the primary goal to remove only the most critical PERV, namely the subfamily C. For removing the sole PERV C at chr1:41Mb the locus was examined at the nucleotide level on both, the PERV containing and the PERV lacking alleles. These data at first allowed the proper design of gRNAs upstream and downstream of the integration sites of the provirus. In addition, the polymorphic sites facilitated the discrimination of the two alleles in the target cell line and, eventually, allowed the identification of the proposed DSB-repair pathway. Indeed, the clones 1646, 1657, 1681 clearly showed to have repaired the DSB by NHEJ, whereas the clones 1649 and 1661 very likely have repaired the DSB by Homologous Recombination. This finding was not totally unexpected as the HR repair system was shown to be activated in pig primary cells (KLYMIUK et al., 2014). Still I would have expected HR-based repair to occur less frequently, in particular due to the fact that a 9kb element has been excised by recombination between the homologous alleles. The cell clones entirely lacking the provirus will be used for producing multi-modified PERV C free pigs by SCNT and subsequent embryo transfer in a recipient sow.

SCNT and embryo transfer are not very efficient techniques, characterized by poor pregnancy rates and frequent early neonatal death of the cloned piglets. Gene Editing might further impair the success of the cloning procedure due to the off-target effects. For generating new genetically modified pigs, however, SCNT still resembles a powerful method which is routinely used at the Institute of Molecular Animal Breeding and Biotechnologies (MABB).

In total, I have shown that both, selective breeding as well as Gene Editing, are sufficient methods to remove the critical PERV C proviruses from donor pigs for xenotransplantation. This is a major step forward on the road to the desired usage of porcine grafts for clinical purposes.

VI. Summary

Deleting PERV C infectious potential from donor pigs for xenotransplantation.

The increasing demand for cells, tissues and organs for transplantation and the limited number of human donors cause the necessity to find alternative sources. The pig is the most suitable donor for this aim since this animal has similar physiological characteristics with humans and its genome can be easily modified for different purposes. However, concerning genetic differences between species, xenotransplantation faces higher hurdles in immunological and safety terms compared to allontransplantation. Different genetic approaches facilitate the generation of genetically modified donor pigs in order to overcome xenograft rejection. The latest progress in xenotransplantation preclinical studies suggest that its application on human patients is realistic in a foreseeable future. Therefore, safety concerns like pathogens transmission (xenozoonosis) need to be elucidated and prevented. For bacteria, fungi and viruses, strategies like the use of antibiotics, anti-fungal treatments, vaccines, antiviral drugs, Cesarean delivery or early weaning and maintenance under designated pathogen-free conditions (DPF) are used for their eradication from donor pigs. However, these strategies do not work for removing viruses that are permanently integrated into genome like PERVs. In clinical application of xenotransplantation it is highly required to use porcine material derived from PERV-C free animals. Therefore, in this thesis two strategies are investigated to remove PERV C infectious potential from donor pigs for xenotransplantation namely selective breeding and excision of proviruses from the genome by CRISPR/Cas technology. At first, PERV C integration sites were identified by TLA-Sequencing in 4 chosen animals of our xenoherd. The sequences were then annotated to the pig reference genome and the flanking regions of every PERV C locus were then used to characterize the chromosomal localization of proviruses. Totally 11 PERV C loci were found. None of the animals was found to be PERV C free and PERV C load ranged from 1 to 8. For each PERV C locus site-specific PCRs were established to screen animals produced from our breeding herd. In a total of 278 examined animals, we found 36 PERV C free for the 11 tested loci. Interestingly, 4 of the identified proviruses have been extincted from our xenoherd

over time. Although selective breeding is a good way to remove PERV C from donor animals, it is time consuming. Alternatively, we aimed excising PERV C from the genome of a selected pig by gene editing. A single heterozygous provirus at chr1:41Mb was excised from a GTKO, hCD46, bLEA multimodified pig by CRISPR/Cas. At first polymorphisms were identified to discriminate the two different alleles. Two gRNAs on the chromosomal flanking regions of the provirus were defined for the excision of PERV C, one upstream of the 5’ LTR and one downstream of the 3’ LTR. Primary cells were then transfected with Cas9 and the two gRNAs. 147 single cell clones were screened for the abundance of the provirus by PCR and sequencing. 15 of them lacked the provirus and therefore will be used in somatic cell nuclear transfer (SCNT) to produce PERV C free animals. The production of PERV C free multimodified pigs by SCNT using the candidate clones is an ongoing approach at our Institute for Molecular Animal Breeding & Biotechnology (MABB).

In conclusion the results obtained in this research work demonstrate that both, selective breeding and excision of PERV C by CRISPR/Cas technology are two efficient ways to reduce or eliminate the pathogenic potential of PERV C from multimodified pig donors for xenotransplantation.

VII. Zusammenfassung

Eliminierung des infektiösen Potentials von PERV C aus Spenderschweinen für die Xenotransplantation.

Die zunehmende Nachfrage nach Zellen, Geweben sowie Organen zur Transplantation und die zugleich begrenzte Anzahl von menschlichen Spendern verdeutlichen die Notwendigkeit alternative Quellen zu finden. Das Schwein ist dafür am besten geeignet, da es die dem Menschen am ähnlichsten physiologischen Merkmale aufweist und sein Genom ohne große Schwierigkeiten für verschiedenste Zwecke modifizierbar ist. Hinsichtlich der genetischen Unterschiede zwischen Spezies gibt es bei der Xenotransplantation höhere immunologische und sicherheitsrelevante Hürden als bei Allontransplantation. Verschiedene genetische Ansätze ermöglichen die Generierung von genetisch modifizierten Spenderschweinen, um der Abstoßung von Xenotransplantaten entgegenzuwirken. Die aktuellen Fortschritte und Ergebnisse in vorklinischen Studien zur Xenotransplantation legen nahe, dass die Anwendung an menschlichen Patienten in naher Zukunft realistisch ist. Aus diesem Grunde müssen Sicherheitsbedenken, wie Übertragung von Pathogenen (Xenozoonose) genau erläutert und ausgeräumt werden. Vorgehensweisen, wie die Behandlung mit Antibiotika, Antipilzbehandlungen, Impfstoffen, antiviralen Wirkstoffen sowie die Haltung unter designiert Pathogen-freien Bedienungen (designated pathogen free, DPF) nach Kaiserschnitt und mutterloser Aufzucht werden zur Eliminierung von Bakterien, Pilzen und Viren bei Spenderschweinen angewendet. Diese Methoden sind jedoch nicht geeignet, Viren wie PERV zu entfernen, die stabil im Genome integriert sind. In klinischen Studien zur Xenotranplantation ist es unbedingt notwendig, Material von PERV C freien Schweinen zu verwenden. Daher werden in dieser Doktorarbeit zwei Strategien untersucht, um das PERV C Infektionspotenzial von Spenderschweinen für die Xenotranplanation zu beseitigen und zwar durch selektive Zucht sowie die Entfernung von Proviren aus dem Genom durch die CRISPR/Cas Technik. Zuerst wurden PERV C Integrationsstellen durch TLA-Sequenzierung in 4 ausgewählten Tieren aus unserer Herde identifiziert. Die Sequenzen wurden im Anschluss dem Referenzschwein annotiert und die flankierenden Regionen jedes

PERV C Lokus zur Charakterisierung der chromosomalen Positionen der Proviren verwendet. Insgesamt wurden 11 PERV C Loci gefunden. Keines der Tiere war PERV C frei und die Anzahl von Proviren reichte von 1 bis 8. Für jeden PERV C Locus wurden spezifische PCR´s etabliert, um die in unserer Zuchtherde produzierten Tiere zu testen. Insgesamt haben wir 278 Tiere untersucht. Davon waren 36 frei für die 11 getesteten PERV C Loci. Vier von den identifizierten Proviren wurden mittlerweile vollständig aus unserer Herde eliminiert. Selektive Zucht ist eine gute Möglichkeit, PERV C von Spendetieren zu entfernen, was allerdings sehr zeitintensiv ist. Alternativ beabsichtigten wir, PERV C aus dem Genome eines ausgewählten Schweines durch Gene Editing herauszuschneiden. Ein einzelnes heterozygotisches Provirus in chr1:41Mb wurde aus einem GTKO, hCD46, bLEA multimodifizierten Schwein durch CRISPR/Cas herausgeschnitten. Zuerst wurden Polymorphismen identifiziert, um die zwei unterschiedlichen Allelen zu diskriminieren. Zwei gRNAs wurden für das Herausschneiden des Provirus aus den flankierenden Regionen des Provirus bestimmt, eine oberhalb und eine unterhalb der Integrationstelle. Primäre Zellen wurden im Anschluss mit Cas9 und zwei gRNAs transfiziert. 147 Einzelzellklone wurden auf die Eliminierung des Provirus mittels PCR und Sequenzierung getestet. Fünfzehn dieser Zellen fehlte das Provirus und diese Zellklöne werden deshalb genutzt, um durch somatischen Zellkerntransfer PERV C freie Tiere zu generieren. Die Produktion von PERV C freien multimodifizierten Schweinen durch SCNT ist eine Routineprozedur an unserem Institut für Molekulare Tierzucht und Biotechnologie (MABB).

In dieser Arbeit wurde damit demonstriert, dass beide Verfahren, Zucht und Gene Editing, effiziente Strategien sind, um PERV C aus multimodifizierten Spenderschweinen für die Xenotransplantation zu reduzieren bzw. zu eliminieren.

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XI. Acknowledgements

At first I would like to express my gratefulness to Prof. Dr. Eckhard Wolf for giving me the opportunity to do my doctoral thesis at the Chair for Molecular Animal Breeding and Biotechnology (LMU-Munich) and for reviewing this manuscript.

My sincere gratitude goes to PD. Dr. Nikolai Klymiuk for the great supervision, constant support and help, and for his patience and encouragement during all this period.

I would like to thank all my colleagues at the Moorversuchsgut to senior scientists, particularly Dr. Andrea Bähr, to my fellow graduate students (Petra, Kilian, Erica, Arne, Stefanie, Melanie and Lina – thank you for the nice time spent together!) and to our technical assistants (Eva, Christian and Tuna – thank you for your help and patience).

Finally, I would like to express my deep gratefulness to Jörg, my husband, for supporting me the whole time as well as to my parents and my brother for encouraging me! Thank you for your moral support, patience and understanding!