

DONOR PIGS FOR XENOTRANSPLANTATION

FIELD OF THE INVENTION

This invention generally relates to novel strains of pig that are highly suitable for xenotransplantation. The first novel pig strain lacks functional porcine endogenous retroviruses so is suitable as a donor for tissue and/or cell xenotransplantation into a human recipient. These pigs can also be used as a foundation pig for further manipulation, for example, by gene editing of xenoantigens to produce a second novel strain of pig that is not only free of infectious porcine retroviruses but is also free of the main xenoantigens responsible for hyperacute organ rejection. These pigs can be used for whole organ, tissue and/or cell transplantation into a human recipient.

The invention also relates to methods for selecting pigs that lack infectious porcine endogenous retroviruses, and their use for tissue and/or cell xenotransplantation into humans, and to methods of gene editing of xenoantigens of the selected pigs to further enhance the immunological quality of the donor organs, tissues and/or cells to avoid xenotransplant rejection.

BACKGROUND

Xenotransplantation has the potential to solve the worldwide problem of human donor organ and tissue shortages, especially xenotransplantation using organs, tissues and/or cells from pigs.

Pigs are the closest in size and physiology to humans, making their organs, tissue and cells suitable as donors for human transplantation. Pigs are also easy to breed and manage in biosecure facilities. However, a major issue that has so far prevented their wide spread use in xenotransplantation is the fact that pigs are carriers of porcine endogenous retroviruses (PERVs). Most breeds of pigs have PERVs integrated into the genome of all their cells, and these viruses have been shown to be able to infect human cells *in vitro*. There is a risk therefore that PERVs in pig organs, tissues and cells can be transfected into human recipients, not only infecting the recipients but also potentially spreading infectious diseases to the general population which may cause disease characteristic of retroviruses, such as immunodeficiency and cancer.

There are three PERV subtypes, PERV-A, PERV-B and PERV-C. PERV-A and PERV-B are ubiquitous and can be transmitted to pigs and humans. PERV-C is only able to infect pig cells but can replicate and recombine with PERV-A to produce a highly

infectious hybrid PERV-A/C strain (Kimsa et al., 2014). The number of integrated PERVs varies in different pig breeds/strains and ranges from 1 to over 300 copies. Selection of pigs with a very low PERV copy number is one way to produce pigs for xenotransplantation.

- 5 Known selection methods of pig breeds for low PERV status has traditionally involved the use of polymerase chain reaction (PCR), using primers that are complementary to a variety of PERV DNA and RNA sequences to identify certain regions of the PERV virus (e.g. *gag*, *pol* or *env* gene sequences). The outcome of PCR testing can be a simple positive or negative result, or can enable the PERV copy number to be calculated. Pigs
10 that have naturally low PERV copy number include Auckland Island (AI) pigs, which have a PERV copy number of between 4 and 40. PCR selection methods have been used to select and breed an AI pig herd with a very low PERV copy number of between 0 and 30 for xenotransplantation (WO 2006/110054). The AI pigs in this herd were also pre-selected for other traits to minimise host rejection such as having blood type
15 O and being free of MHC Class I antigen, as well as being designated pathogen-free (DPF).

- These pigs provided one of the first animal populations suitable for xenotransplantation. However, this selection method cannot accurately identify those pigs that have full length PERV capable of replication from those that have inactive
20 PERV, i.e. PERV status or copy number determined by PCR is not associated with virus that is able to replicate. For example, PCR amplification of very small regions of *gag*, *pol* or *env* PERV sequences does not distinguish between full length or partial PERV sequences. A PCR-based test also does not distinguish between an intact gene and a gene that contains nonsense mutation(s) (stop codons). This means that a PERV
25 positive PCR test could be associated with a partial sequence that is not able to replicate, or a full length sequence that contains nonsense mutation(s) (false positives), and a PERV negative result could have missed some of the small *env* protein gene sequences (false negative).

- In addition, a copy number as low as 1 could mean that the organs, tissues and cells
30 transplanted into a human recipient may still have sufficient PERV to replicate and potentially cause disease. Indeed, to avoid this problem, pig cells have had to be protected from the recipient's immune system before implantation, for example by alginate encapsulation or by using an implant device.

- Elimination of PERV or at least PERV deactivation to eliminate the ability of PERV to
35 replicate and therefore be infectious, would be a more desirable goal.

Several methodologies to achieve this goal have been tried, including RNA interference (RNAi) vaccines, PERV elimination using zinc finger nucleases or TAL effector nucleases, but have met with limited success.

- More recently, the use of CRISPR technology has been suggested as a way of
- 5 inactivating genome wide PERV via gene editing. Yang et al (2015) succeeded in inactivating 62 PERV sequences in an immortalised pig cell line and Niu et al (2017) have, via CRISPR gene editing in primary fetal pig fibroblast cells and somatic-cell nuclear transfer cloning technology, succeeded in producing piglets with inactive PERVs. This involved inactivating 25 PERV sequences.
- 10 The use of CRISPR or any other gene-editing technology to inactivate all PERV genes, in conjunction with somatic-cell nuclear transfer cloning technology, appears to be seen as the best way forward to produce porcine organs for xenotransplantation that are human-compatible. For example, eGenesis (Cambridge, MA) are promoting the use of CRISPR Cas-9 technology to produce viable cloned porcine embryos that have
- 15 been gene edited so that all PERVs are inactivated.

PERV infection is not the only problem. Pigs should also be free of other pathogens that could be transmitted to human recipients, including lymphotropic herpes virus (PLHV), circovirus (PCV) and cytomegalovirus (PCMV), for example.

- Another major hurdle to be overcome is that of immunological incompatibility with the
- 20 recipient and therefore hyperacute organ rejection. Again, CRISPR or any other gene-editing technology can be used to edit xenoantigen genes (such as alpha 1,3 galactosyltransferase (GGTA1) and cytidine monophosphate-N-acetyl neuramic acid hydroxylase (CMAH) genes, etc) to either remove or deactivate unwanted xenoantigens to reduce immunological adverse reactions. In addition, human genes
- 25 that moderate the immune system can be added to pigs to help to avoid rejection (Revivicor, Blacksburg, VA).

- However, the combination of the use of gene-editing technology to deactivate/remove all PERV and xenoantigen genes (eGenesis talk about introducing "double digit" gene edits to reduce the likelihood of organ rejection (Weintraub, 2019)) can mean that the
- 30 resulting cloned animals have dozens of gene edits which may harm the animal and/or have unforeseen genetic consequences.

A better way of producing animals that are PERV-free without having to carry out such multiple gene edits would therefore be preferable and provide animals that do not have the potential problems that large number of gene edits is likely to cause.

It is an object of the present invention to go some way towards overcoming this problem and/or to provide the public with a useful choice.

In this specification where reference has been made to patent specifications, other external documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents or such sources of information is not to be construed as an admission that such documents or such sources of information, in any jurisdiction, are prior art or form part of the common general knowledge in the art.

10 SUMMARY OF THE INVENTION

The present invention provides a use of complete genome sequencing (CGS) as a tool to select PERV-C and PERV-A/C negative pigs, i.e. pigs that have no or inactive, non-replicating PERV C and PERV-A/C, for xenotransplantation of tissues and/or cells into human recipients; and/or as foundation pigs for further manipulation, for example by gene editing xenoantigens, to provide pigs that are not only PERV-A and PERV-A/C negative but are also free of xenoantigens that are responsible for hyperacute organ rejection so that this novel pig strain is suitable as donors for whole organ, tissue and/or cell xenotransplantation into human recipients.

In a first aspect, the present invention provides a method of selecting pigs suitable as donors for xenotransplantation of tissues and/or cells, or as foundation pigs for further manipulation, said method comprising the step:

- a) providing a designated pathogen-free (DPF) pig herd having a low PERV copy number of between 4 and 40;
- b) testing the PERV status of individual pigs of the herd using complete genome sequencing (CGS);
- c) identifying individual pigs that have PERV-C negative and PERV-A/C negative status;
- d) selecting said PERV-C negative and PERV-A/C negative pigs as donor pigs for xenotransplantation, or as foundation pigs for further manipulation.

The method preferably further comprises identifying the PERV-A and PERV-B status of the PERV-C negative and PERV-A/C negative pigs of step d) and further selecting pigs that have a very low number of full-length and potentially functional PERV-A and

PERV-B sequences of between 1-10, preferably between 1-5, and most preferably between 1-2.

Preferably the pigs are Auckland Island (AI) pigs, a swine breed that has designated pathogen-free (DPF) status and has a surprising low PERV copy number. More preferably, the AI pigs have been selectively bred for very low PERV copy number in a biosecure facility. More preferably, the AI pigs are PERV-null, i.e. any PERV sequences present in their genome are not able to produce infectious PERV particles (Garkavenko et al., 2008).

Other pig breeds/strains that are designated pathogen-free and have similar low PERV copy number and PERV-null status can also be used in the present invention.

In a second aspect the invention provides pigs selected by the method of the first aspect.

It was surprisingly found that full-length and potentially functional PERV-A/B sequences, in the PERV-C and PERV-A/C negative pigs selected by the method of the first aspect, were located on the Y-chromosome only so could be used to breed out functioning PERV. In a third aspect the present invention therefore provides a method of breeding a herd of pigs that have no functional PERV genomic sequences suitable as donors for xenotransplantation, or as foundation pigs for further manipulation, said method comprising the steps:

- a) selecting PERV-C and PERV-A/C negative male and female pigs using the CGS selection method of the first aspect;
- b) analysing the chromosomal location of any full-length, potentially functional PERV-A and PERV-B gene sequences in the male pigs of step a);
- c) selecting male pigs that have full-length, potentially functional PERV-A and/or PERV-B present in the Y-chromosome only;
- d) breeding the male pigs of step c) with the female pigs of step a) to produce progeny; and
- e) selecting female progeny that will lack the paternal full length and potentially functional PERV-A and/or PERV-B as future breeding stock to produce a herd of pigs that have no functional PERV genomic sequences suitable as donors of tissues and/or cells for xenotransplantation, or as foundation pigs for further manipulation.

The present invention also contemplates selecting the female progeny of step e) as suitable donors for xenotransplantation of tissues and/or cells, or as foundation pigs for further manipulation.

5 In a fourth aspect the invention provides a pig herd bred by the method of the third aspect.

The foundation pigs selected by the CGS selection method of the first aspect of the invention, or bred by the method of the third aspect of the invention can be further manipulated using gene editing technology to eliminate and/or deactivate one or more xenoantigens selected from GGTA1, CAMH, B4GalNT2, Neu5Gc, ASGR1 and SLA, for
10 example, to minimise the risk of hyperacute rejection of tissues and/or cells, as well as whole organs transplanted into human recipients. In a fifth aspect the invention therefore provides a method of providing donor pigs suitable for xenotransplantation of whole organs, tissues and/or cells into a human recipient comprising the steps:

- 15 (a) selecting PERV-C and PERV-A/C negative donor pigs using the CGS method of the first aspect;
- (b) establishing a PERV-C and PERV-A/C negative cell line from the pigs of step (a);
- (c) gene editing selected cells of step (b) to eliminate or deactivate one or more xenoantigen genes;
- 20 (d) optionally gene editing selected isolated cells of step (c) to express one or more human genes selected from A20, CD39, CD46, CD47, CD55, CD59, hemoxygenase-1 (HO-1), HLA-E, HLA-G, thrombomodulin (TM), CTLA4-Ig, and LEA29Y;
- (e) establishing a gene-edited cell line from the pig cells of step (c) or step (d);
- 25 (f) carrying out somatic cell nuclear transfer from one or more of said gene edited cells of step (e) into an oocyte from a PERV-C and PERV-A/C negative pig *in vitro*;
- (g) culturing to blastocyst stage;
- (h) transferring said blastocyst(s) to a female PERV-C and PERV-A/C negative pig
30 and growing to full term; and

- (i) providing donor pigs that are PERV-C and PERV-A/C negative and gene edited for one or more xenoantigens.

The one or more xenoantigens genes may be selected from GGTA1, CAMH, B4GalNT2, Neu5Gc, ASGR1 and SLA, or any other xenoantigen that would be useful to reduce immunological reactions, especially the possibility of hyperacute organ rejection, as would be understood by a skilled worker.

In a sixth aspect the invention provides pig cell lines that are PERV-C and PERV-A/C negative and gene edited to inactivate or delete one or more xenoantigens and optionally to express one or more human genes, produced by the method of the fifth aspect. The cell lines can be used to clone donor pigs for xenotransplantation of whole organs, tissues and/or cells into human recipients.

In a seventh aspect the invention provides donor pigs that are DPF, PERV-C and PERV-A/C negative and gene edited to inactivate or delete one or more xenoantigens and optionally to express one or more human genes, produced by the method of the fifth aspect. These donor pigs represent a novel pig strain, designated NZeno-1. These pigs also preferably have a very low number of full-length and potentially functional PERV-A and PERV-B sequences of between 1-10, preferably between 1-5, and most preferably between 1-2.

In an eighth aspect the invention provides tissues and/or cells from the pigs of the second, fourth, and seventh aspects for use in xenotransplantation.

In a ninth aspect the invention provides whole organs from the pigs of the seventh aspect for use in xenotransplantation.

The organs, tissues and/or cells for use in xenotransplantation may be selected from the group consisting of kidney, liver, lung, heart, brain, pancreas, muscle, blood, bone, testes and ovary.

The tissue and/or cells for use in xenotransplantation may be selected from pancreatic islets, hepatocytes, non-parenchymal liver cells, gall bladder epithelial cells, gall bladder endothelial cells, bile duct epithelial cells, bile duct endothelial cells, hepatic vessel epithelial cells, hepatic vessel endothelial cells, sinusoid cells, choroid plexus cells, fibroblasts, Sertoli cells, adrenal chromaffin cells and muscle cells.

In a tenth aspect the invention provides a method of treating a patient suffering from or predisposed to a disease, disorder or condition associated with a deficiency in or

absence of organ function, said method comprising transplanting tissue and/or cells of the eighth aspect, or whole organs of the ninth aspect to a patient in need thereof.

It is intended that reference to a range of numbers disclosed herein (for example, 1 to 10) also incorporates reference to all rational numbers within that range (for example, 1, 1.1, 2, 3, 3.9, 4, 5, 6, 6.5, 7, 8, 9 and 10) and also any range of rational numbers within that range (for example, 2 to 8, 1.5 to 5.5 and 3.1 to 4.7) and, therefore, all sub-ranges of all ranges expressly disclosed herein are hereby expressly disclosed. These are only examples of what is specifically intended and all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application in a similar manner.

As used herein the term '(s)' following a noun means the plural and/or singular form of that noun.

The term 'comprising' as used in this specification and claims means 'consisting at least in part of'. When interpreting statements in this specification and claims which include the term 'comprising', other features besides the features prefaced by this term in each statement can also be present. Related terms such as 'comprise' and 'comprised' are to be interpreted in a similar manner.

As used herein the term 'and/or' means 'and' or 'or', or where the context allows both.

The invention consists in the foregoing and also envisages constructions of which the following gives examples only.

As used herein the terms 'PERV-C negative' and 'PERV-A/C negative' means no full length proviral genome identified in pigs selected by the method of the invention. This can be distinguished from the terms "PERV-C negative" and "PERV-A/C negative" when assessed by conventional PCR which can only detect the presence of a small part of env region (PERV-C (A/C) positive) or absence of this region (PERV-C (A/C) negative) of PERV-C and recombinant PERV-A/C genomic sequences.

As used herein the term 'PERV-null' means the inability to produce infectious PERV particles.

The term "porcine" is used interchangeably with the terms "pig" and "swine" and refers to mammals in the family Suidae. Such mammals include wholly or partially inbred swine, preferably those members of the Auckland Island pig herd described herein.

The term "recipient" as used herein refers to a human suffering from or predisposed to a disease, disorder or condition associated with a deficiency in or absence of organ, tissue or cell function.

5 The term "gene editing" or "genome editing" is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in specific targeted sites in the genome of a living organism. Gene editing techniques include the use of engineered nucleases, or 'molecular scissors' that create site-specific double stranded breaks at desired locations in the genome. The induced double-strand breaks are repaired through non-homogenous end-joining or homologous recombination, resulting in
10 targeted mutations or gene edits. Engineered nucleases that can be used include zinc finger nucleases, transcription activator-like effector nucleases, engineered meganucleases and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas-9) system.

15 This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

20 **BRIEF DESCRIPTION OF THE DRAWINGS**

The present invention will now be described by way of example only and with reference to the accompanying drawings in which:

Figure 1 shows full PERV-C sequence BLAST hits against the *Sus scrofa* Male1 genome sequence;

25 Figure 2 shows a sequence alignment of selected regions of the *Sus scrofa* Male1 genome with known PERV sequences. Disagreements to the PERV-C reference KC116219 are highlighted in black;

Figure 3 shows a Maximum Likelihood phylogenetic reconstruction of 8 selected hits of *Sus scrofa* Male1 (black) with 9 known PERV sequences (red). The tree was rooted
30 with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

Figure 4 shows a Maximum Likelihood phylogenetic reconstruction of 18 genomic regions (inconsistently found across these PERV-A, B, C, and A/C references with a coverage threshold of 90%) of *Sus scrofa* Male1 (black) with 9 known PERV sequences (red). The tree was rooted with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

Figure 5 shows a Maximum Likelihood phylogenetic reconstruction of selected gag regions of the *Sus scrofa* Male1 genome (black) with 9 known PERV sequences (red). The tree was rooted with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

Figure 6 shows a Maximum Likelihood phylogenetic reconstruction of selected pol regions of the *Sus scrofa* Male1 genome (black) with 9 known PERV sequences (red). The tree was rooted with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

Figure 7 shows a Maximum Likelihood phylogenetic reconstruction of selected env regions of the *Sus scrofa* Male1 genome (black) with 9 known PERV sequences (red). The tree was rooted with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

Figure 8 shows full PERV-C sequence BLAST hits against the *Sus scrofa* Male2 genome sequence;

Figure 9 shows a sequence alignment of selected regions of the *Sus scrofa* Male2 genome with known PERV sequences. Disagreements to the PERV-C reference KC116219 are highlighted in black;

Figure 10 shows a Maximum Likelihood phylogenetic reconstruction of 8 selected hits of *Sus scrofa* Male2 (black) with 9 known PERV sequences (red). The tree was rooted with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

Figure 11 shows a Maximum Likelihood phylogenetic reconstruction of 18 genomic regions (inconsistently found across these PERV-A, B, C, and A/C references with a coverage threshold of 90%) of *Sus scrofa* Male2 (black) with 9 known PERV sequences

(red). The tree was rooted with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

5 Figure 12 shows a Maximum Likelihood phylogenetic reconstruction of selected gag regions of the *Sus scrofa* Male2 genome (black) with 9 known PERV sequences (red). The tree was rooted with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

10 Figure 13 shows a Maximum Likelihood phylogenetic reconstruction of selected pol regions of the *Sus scrofa* Male2 genome (black) with 9 known PERV sequences (red). The tree was rooted with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

15 Figure 14 shows a Maximum Likelihood phylogenetic reconstruction of selected env regions of the *Sus scrofa* Male1 genome (black) with 9 known PERV sequences (red). The tree was rooted with the PERV-B sequence. Numbers at the nodes indicate the ML support values. The scale bar represents the number of expected substitutions per site;

Figure 15 shows the morphology of NZK1 cells cultured on three different substrates;

20 Figure 16 shows the proliferation characteristics of NZK1 cells on three different substrates. TC = tissue culture;

Figure 17 shows the metaphase chromosomes of an NZK1 cell, a representative example of the total number of metaphase chromosomes released from an individual NZK1 cell at passage 3. Individual chromosomes are numbered;

25 Figure 18 shows the CRISPR target site in the GGTA1 gene. Cleavage by CRISPR is expected to delete a 152 bp fragment, as indicated, which leads to a functional disruption of the gene;

30 Figure 19 shows sequence verification of the AI pig target regions. Shown is the genomic sequence of the relevant target regions of the GGTA1 gene in AI pig cells in comparison to the published pig genome sequence;

Figure 20 shows sequence comparisons of wild type cells (WT) and pooled cells that were transfected (TC) with CRISPRs specific for GGTA1. The arrow indicates the

predicted CRISPR cleavage site and the bar below the sequence the region of overlapping sequences for the transfected cells;

Figure 21 shows bioinformatic identification of the different sequences present in amplified target sites of pooled CRISPR-transfected cells. Shown are the results from Tracking of Indels by DEcomposition (TIDE) analysis of cell transfected with two different CRISPRs for the GGTA1 gene. The bar graphs indicate specific percentages of sequences detected with 0 indicating the unchanged wild type sequence and 1 to 5 insertions of 1-5 base pairs and -1 to -10 deletions of 1-10 base pairs, respectively;

Figure 22 shows the characterisation of GGTA1-KO NZK1 cell clones. **A** shows PCR amplification products of the GGTA1 target region for individual cell clones (e.g. G53, G61, G12) and non-transfected control cells (WT). **B** shows a sequence comparison of wild type cells and cell clone G12. The region deleted in cell clone G12 is shown as a dashed black line highlighted in red. CRISPR binding and cleavage sites are indicated by black bars and arrows, respectively;

Figure 23 shows the CRISPR target site in the CMAH gene. Cleavage by CRISPR is expected to delete an 88 bp fragment, as indicated, which leads to a functional disruption of the gene;

Figure 24 shows sequence verification of the AI pig target regions. Shown is the genomic sequence of the relevant target regions of the CMAH gene in AI pig cells in comparison to the published pig genome sequence;

Figure 25 shows sequence comparisons of wild type cells (WT) and pooled cells that were transfected (TC) with CRISPRs specific for CMAH. The arrow indicates the predicted CRISPR cleavage site and the bar below the sequence the region of overlapping sequences for the transfected cells;

Figure 26 shows bioinformatic identification of the different sequences present in amplified target sites of pooled CRISPR-transfected cells. Shown are the results from Tracking of Indels by DEcomposition (TIDE) analysis of cell transfected with two different CRISPRs for the CMAH gene. The bar graphs indicate specific percentages of sequences detected with 0 indicating the unchanged wild type sequence and 1 to 5 insertions of 1-5 base pairs and -1 to -10 deletions of 1-10 base pairs, respectively;

Figure 27 shows the characterisation of CMAH-KO NZK3 cell clones. **A** shows representative gels with PCR amplification products of the CMAH target region for individual cell clones. Indicated are five cell clones (38, 86, 89, 90 and 104) which

have been deemed suitable as CMAH KO clones for the generation of KO pigs. **B** shows a sequence comparison of wild type cells and cell clone 90. The mutation in clone 90, a 1 bp insertion, at the cleavage site of CRISPR 68rev is highlighted by a black box. CRISPR binding and cleavage sites are indicated by black bars and arrows, respectively;

Figure 28 shows PCR amplification products of the GGTA1 and CMAH target regions for individual cell clones (e.g. 4.68, 4.10, 4.16)) and control cells (WT);

Figure 29 shows a sequence analysis of mutations in double KO cell clone 4.16. Shown is a comparison of the target sequences for GGTA1 and CMAH present in wild type cells and double KO candidate cell clone 4.16. Deleted regions are shown as dashed black lines highlighted in red. CRISPR binding and cleavage sites are indicated by black bars and arrows, respectively;

Figure 30 shows characterisation of GGTA1-KO NZK3 cell clones. **A** shows PCR amplification products of the GGTA1 target region for individual cell clones with cell clone GA2, GA5 and GA20 showing a single fragment with a putative 152 bp deletion. **B** shows a sequence comparison of wild type cells and cell clone GA5. The region deleted in cell clone G12 is shown as a dashed black line highlighted in red. CRISPR binding and cleavage sites are indicated by black bars and arrows, respectively; and

Figure 31 shows the analysis of NZK3 cells (A), GGTA1/CMAH double KO cell clones (B) and human HEK cells for the binding of a fluorescently labelled isolectin that has binding specificity for the α Gal epitope.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

The present invention provides novel strains of pig that are highly suitable for xenotransplantation. The first novel pig strain lacks functional porcine endogenous retroviruses so is suitable as a donor for tissue and/or cell xenotransplantation into a human recipient. These pigs can also be used as a foundation pig for further manipulation, for example, by CRISPR gene editing of xenoantigens to produce a second novel strain of pig that is not only free of functioning porcine retroviruses but is also free of the main xenoantigens responsible for hyperacute organ rejection. These pigs can be used for whole organ, tissue and/or cell transplantation into a human recipient.

More specifically, the present invention is directed to the use of complete genome sequencing (CGS) as a highly sensitive and specific tool to select donor pigs that have

no, or inactive, non-replicating PERV genomic sequences, for xenotransplantation of tissues and/or cells into human recipients, or as foundation pigs for further manipulation. CGS can identify all of the PERV sequences in the pig genome, i.e. both partial and full-length sequences, to identify and select for individual pigs that lack functional PERV sequences.

The present CGS selection method overcomes the problems of the prior art methods as the risk of false positives and false negatives associated with PERV status and/or copy number (via PCR) is eliminated, the risk of functional infective PERV sequences being transmitted to the recipient is eliminated, and the pigs have not had to undergo dozens of CRISPR gene edits to inactivate all of the PERV genes so that the potential problems associated with such multiple gene edits on the donor animal is also eliminated. The tissues and/or cells from the pigs selected by the CGS method of the invention can also be transplanted into human recipients directly, without the need for protective barriers against the recipient's immune system, such as alginate capsules, implantation devices etc, but they can also be used with these protective barriers if, after undergoing crossmatch testing, the donor tissue is considered at risk of hyperacute rejection.

Preferably, the present invention uses CGS to select for pigs that are PERV-C and PERV-A/C negative. There are three PERV subtypes, PERV-A, PERV-B and PERV-C which refer to differences in the envelope (env) region of PERV. The viral envelope protein is the major determinant of host range and is essential for infection. PERV-A and PERV-B are ubiquitous and infect humans and pigs while PERV-C only infects pigs, however, PERV-A and PERV-C can recombine to form a highly infectious variant PERV-A/C. Therefore, it is desirable for a donor pig to be PERV-C free or to have only non-replicating PERV-C to avoid any recombination with existing PERV-A, as well as being free of the recombined PERV-A/C.

The present invention therefore provides a method of selecting pigs suitable as donors for xenotransplantation of tissues and/or cells, or as foundation pigs for further manipulation, said method comprising the step:

- a) providing a designated pathogen-free (DPF) pig herd having a low PERV copy number of between 4 and 40;
- b) testing the PERV status of individual pigs of the herd using complete genome sequencing (CGS);

c) identifying individual pigs that have PERV-C negative and PERV-A/C negative status;

d) selecting said PERV-C negative and PERV-A/C negative pigs as donor pigs for xenotransplantation, or as foundation pigs for further manipulation.

- 5 The method further comprises identifying the PERV-A and PERV-B status of the PERV-C and PERV-A/C negative pigs and selecting pigs that have a very low number of full-length potentially functional PERV-A and PERV-B sequences of between 1-10, preferably between 1-5 and most preferably between 1-2.

The donor pigs, prior to selection using the CGS method of the invention, must be
10 designated pathogen free (DPF). In particular, the pigs must be free from infectious microorganisms such as herpesvirus, PLHV, PCMV, hepatitis E virus (HEV), Toxoplasma, eperythrozoon, brucella, listeria, mycobacterium TB, leptospirillum, haemophilus suis, any virus causing porcine respiratory reproductive syndrome, any virus causing rabies, any virus causing pseudorabies, parvovirus,
15 encephalomyocarditis virus, any virus causing swine vesicular disease, porcine polio virus (techen), any virus causing hemagglutinating encephalomyocarditis, swine influenza type A, adenovirus, transmissible gastroenteritis virus and vesicular stomatitis virus. The pigs' pathogen free status should be regularly monitored using techniques known in the art such as immunological assays and PCR technologies.

- 20 The donor pigs are also preferably naturally low in PERV copy number or have been pre-selected and bred for low PERV copy number. For example, Auckland Island (AI) pigs are a swine breed that has designated pathogen-free status and has a surprising low PERV copy number. The AI pigs can be further selectively bred for very low PERV copy number in a biosecure facility using PCR/RT-PCR detection of PERV sequence and
25 calculation of PERV copy number, followed by selective breeding of swine with the lowest PERV copy number as described in WO 2006/110054. The AI pigs have also been shown to be PERV-null, i.e. any PERV sequences present in their genome are not able to produce infectious PERV particles (Garkavenko et al., 2008). Other swine breeds/strains that have the same designated pathogen-free status, low PERV copy
30 number, and PERV-null status could also be used in the present invention.

More preferably the donor pigs have also been pre-selected for other traits to minimise host rejection such as having blood type O and being free of MHC Class I antigen prior to selection using the CGS method of the present invention.

The present invention can use any one of the known techniques of complete genome sequencing (CGS) for detecting the PERV status of the donor pig. CGS is a method for determining the genome sequence of an organism. There are several methods known in the art for obtaining genome sequences. One common technique is whole-genome
5 shotgun sequencing (Venter, 1998). This typically involves several steps:

- obtaining a large number of short DNA sequences from random locations in the genome,
- assembling the short sequences into larger contigs (genome assembly), and
- analysing the data produced.

10 The present invention uses CGS to identify PERV sequences in pigs and analyse those sequences to determine whether the identified PERV sequences are functional, i.e. capable of replication. The identified PERV sequences are compared with reference sequences to determine whether they are full-length and to identify any mutations that may deactivate the PERV. The surrounding genomic context can also be analysed
15 to determine whether the PERV is likely to be transcriptionally silenced.

Pigs selected using the CGS method of the present invention are also provided by the present invention. These pigs can be used as foundation pigs for further manipulation, as described below, or for use as donors of tissue and/or cells for xenotransplantation.

20 These pigs have advantages over all current pig breeds purportedly suitable as xenotransplant donors, including those AI pigs selected for low PERV copy number described in WO2006/110054. In particular, by using the CGS method of the present invention the selected pigs can be verified to be truly PERV-C and PERV-A/C negative for the first time, i.e. they do not have any functioning genomic PERV-C and
25 recombined PERV-A/C sequences so can be used as donor pigs for tissue and/or cell xenotransplantation without fear of transmitting functioning PERV-C and PERV-A/C sequences to the recipient.

This major benefit of the selection method of the present invention can be seen in examples 1 and 2 of the present invention.

30 In example 1 of the invention, a male AI pig (Male1) having an apparent PERV copy number of 18 was in fact found to have only two full-length PERVs, one that groups with the PERV-A reference, and one that groups with the PERV-B reference. The

remaining 16 PERV sequences, including PERV-C sequences, were found to have multiple nonsense mutations (stop codons) in the *gag*, *pol*, and/or *env* genes. This pig was deemed PERV-C and PERV-A/C negative by conventional PCR methods and this status was verified by the CGS method of the invention. Furthermore, the CGS method of the invention determined that it contained only two potentially functional full length PERV sequences. The accuracy of this determination is a major benefit of the CGS method, as standard PCR-based testing only gives a positive or negative result that is prone to false-positives when PERV genes are present but contain nonsense mutations. When PCR is used to calculate PERV copy number, it can greatly overestimate the true number of functional PERV sequences.

However, as seen in example 2 of the invention, a second male AI pig (Male2) having a PERV-C positive status as determined by conventional PCR-based testing was in fact found to be PERV-C negative, and to have only a single full-length PERV that groups with PERV-A. As in example 1, the remaining PERV sequences, including PERV-C sequences, were found to have multiple nonsense mutations (stop codons) in the *gag*, *pol*, and/or *env* genes and are therefore inactive. This result was highly surprising. It was not expected that the selection method of the present invention would overturn the PCR based PERV-C positive status of an animal, especially given that the present method affirmed the PCR deemed PERV-C negative status of Male1.

The present invention has further unexpected advantages over the conventional PCR method of determining PERV-C and PERV-A/C status as, even when PCR identifies an animal as PERV-C positive, the present method can further identify whether or not the PERV-C sequences are complete, if they are not, as in the case of the Male2, there can be no PERV-A/C recombinants in the genome.

The selection method of the present invention is therefore highly sensitive and far superior to the previous PCR-based method that had identified donor pig Male2 as being PERV-C positive. Such a donor pig would not have been considered suitable for use in xenotransplantation for fear of transmission of PERV-C and the possibility of the highly infectious PERV-A/C recombinant sequence in the recipient.

The CGS method of the present invention can not only identify and analyse the functional status of all of the PERV gene sequences in the pig genome, but can also identify the PERV gene sequence locations. Surprisingly, in example 2, the single full-length and potentially functional PERV-A sequence found was located on the Y-chromosome. This surprising finding has unforeseen benefits as such animals can be used to breed out functioning PERVs in future offspring by selectively breeding such

pigs with female pigs that are PERV-C and PERV-A/C negative (as selected using the CGS method of the invention) and selecting female offspring as suitable donor pigs or for future breeding as their offspring will not have the functional paternal PERV-A. Such selective breeding can eventually produce a PERV-negative herd for
5 xenotransplantation, i.e. a herd of pigs that have no functional PERV genomic sequences.

The present invention therefore provides a method of breeding a PERV negative herd of pigs for xenotransplantation, said method comprising the steps:

- 10 a) selecting PERV-C negative and PERV-A/C negative male and female pigs using the CGS selection method of the invention;
- b) analysing the chromosomal location of any full-length, potentially functional PERV-A and PERV-B gene sequences in the male pigs of step a);
- c) selecting male pigs that have full-length, potentially functional PERV-A and/or PERV-B present in the Y-chromosome only;
- 15 d) breeding the male pigs of step c) with the female pigs of step a) to produce progeny; and
- e) selecting female progeny that will lack the paternal potentially functional PERV-A and/or PERV-B as future breeding stock to produce a herd of PERV negative pigs suitable as donors of tissues and/or cells for xenotransplantation, or as
20 foundation pigs for further manipulation.

The present invention also contemplates the selection of female progeny of step e) as suitable donors for xenotransplantation of tissues and/or cells, or as foundation pigs for further manipulation.

The present invention also provides a PERV-negative pig herd bred by the breeding
25 method of the invention. This pig herd can be used as foundation pigs for further manipulation or as a source of donor pigs for xenotransplantation of tissues and/or cells into human recipients. Depending on the results of crossmatch testing, the tissues and/or cells may be able to be transplanted directly, without the need for additional immune barriers such as alginate capsules or implantation devices, or such
30 immune barriers may need to be used to avoid hyperacute rejection of the implant.

As mentioned above, the present invention provides foundation pigs that are PERV-C and PERV-A/C negative (i.e. have no full-length or non-functional full-length PERV-C

and PERV-A/C genomic sequences) that have been selected by the CGS method of the present invention, and/or bred by the breeding method of the invention. These foundation pigs represent a novel strain of pig having a true PERV-C and PERV-A/C negative genomic status. These foundation pigs can be further manipulated using
5 gene editing technology to eliminate and/or deactivate one or more xenoantigens, thereby providing a novel pig strain (designated as NZeno-1) that is highly suitable for xenotransplantation of whole organs, tissues and cells into human recipients. As would be understood by a skilled worker, gene editing technology can be used to further improve the xenotransplantable status of the foundation pigs, i.e. to make
10 them more compatible to human recipients, i.e. by deactivating and/or eliminating the xenoantigens responsible for hyperacute organ rejection.

For example, suitable cells obtained from the DPF foundation pigs that have been selected for PERV-C and PERV-A/C negative status and preferably having a very low copy number of active PERV-A and PERV-B (1-2) using the CGS selection method of
15 the invention, and/or bred using the breeding method of the invention, can be used to establish a PERV-C and PERV-A/C free cell line. These cells can then be subjected to gene editing technology to knock out or deactivate genes related to xenoantigens known to trigger immune reactions in the recipient that cause hyperacute organ rejection. Hyperacute organ rejection is mediated by natural antibodies to pig
20 antigens, complement fixation to endothelial cells, and the rapid onset of intravascular coagulation. The major target of these antibodies is a specific carbohydrate epitope (α Gal) placed on branched sugar chains on cell surfaces by the enzyme alpha 1,3 galactosyl transferase (GGTA1). GGTA1 is not present in humans so the presence of these α Gal epitopes triggers this immune response. Removal of GGTA1 in donor pigs
25 will therefore be useful to avoid organ, tissue and/or cell rejection in human recipients.

Other targets to reduce xenoreactive antibody binding to the xenografted organ triggering hyper acute organ rejection include β 1,4 N-acetylgalactosaminyl transferase (B4GalNT2), CMP-Neu5AC Hydroxylase (CMAH), swine leucocyte antigen (SLA), N-
30 acetylneuraminic acid (Neu5Gc), ASGR1, as well as other target immunoreactive genes as would be understood by a skilled worker.

Other gene edits may be made to avoid organ, tissue and/or cell rejection in human recipients, such as expression of human A20, CD39, CD46, CD47, CD55, CD59, hemoxygenase-1 (HO-1), HLA-E, HLA-G, thrombomodulin (TM), CTLA4-Ig, and/or
35 LEA29Y, etc as would be understood by a skilled worker.

A cell line is established from these gene edited cells, and the gene edited cells are subjected to somatic-cell nuclear transfer to clone donor pigs that are ideally suited to xenotransplantation as, not only are they PERV-C and PERV-A/C negative, but they are also hypoimmunogenic as the gene editing will help to prevent adverse
5 immunological reactions when organs, tissues and/or cells of the cloned donor pigs are implanted into a human donor and avoid hyperacute organ rejection.

Suitable cells include fibroblasts, kidney-derived primary cells, or hepatocytes, for example. Other suitable donor cells should have at least the following properties: (1) exhibit a correct and stable karyotype, which could be simply determined by
10 counting the chromosome numbers in metaphase spreads, (2) show good proliferation, (3) have a long lifespan, and (4) should be transferable. Furthermore, an in vitro SCNT experiment gives information about developmental competence until blastocyst stage, as would be understood by a skilled worker. The cells are then gene edited, using CRISPR Cas-9 technology, and subsequent somatic-cell nuclear transfer
15 is carried out, using known methods (Kurome et al., 2015).

The present invention therefore provides a method of providing a novel strain of donor pigs suitable for xenotransplantation of whole organs, tissues and/or cells into a human recipient comprising the steps:

- 20 a) selecting foundation pigs that are PERV-C negative and PERV-A/C negative using the CGS selection method of the invention;
- b) establishing a PERV-C and PERV-A/C negative pig cell line from the pigs of step a);
- c) gene editing selected isolated cells of said pigs of step b) to eliminate or deactivate one or more xenoantigen genes;
- 25 d) optionally gene editing selected isolated cells of step c) to express one or more human genes selected from A20, CD39, CD46, CD47, CD55, CD59, hemoxygenase-1 (HO-1), HLA-E, HLA-G, thrombomodulin (TM), CTLA4-Ig, and LEA29Y;
- e) establishing a gene-edited cell line from the cells of step c) or d);
- 30 f) carrying out somatic cell nuclear transfer from one or more of the gene edited cells of step e) into an oocyte of a pig that is PERV-C negative and PERV-A/C negative;

- g) culturing to blastocyst stage;
- h) transferring blastocyst to a female PERV-C and PERV-A/C negative pig and growing to full term; and
- i) providing a novel strain of donor pigs that are PERV-C and PERV-A/C negative and gene edited for one or more xenoantigens, and optionally gene edited to express one or more human genes.

The one or more xenoantigen genes may be selected from GGTA1, CAMH, B4GalNT2, Neu5Gc, ASGR1 and SLA, and other xenoantigen genes that would be beneficial to deactivate, to improve acceptance by the recipient's immune system as would be understood by a skilled worker.

The present invention also provides pig cell lines produced by step e) of the above method. Examples of pig cell lines of the present invention are set out in examples 4-8 below. Specifically, cell lines from DPF AI pigs selected by the CSG method of the invention (i.e. PERV-C and PERV-A/C negative) were produced that have been gene edited using CRISPR Cas-9 to disrupt GGTA1 (examples 4 and 7); that have been gene edited using CRISPR Cas-9 to disrupt CMAH (example 5); and that have been gene edited using CRISPR Cas-9 to disrupt both GGTA1 and CMAH (examples 6 and 8). The cell lines of the present invention can be used to clone donor pigs using known somatic cell transfer techniques (example 9). The cloned donor pigs can then be used for xenotransplantation of whole organs, tissues and/or cells into human recipients. The cloned donor pigs represent a novel pig strain (NZeno-1) having PERV-C and PERV-A/C negative genomic status, a very low number (1-2) of full-length potentially functional genomic PERV-A and PERV-B sequences, having gene edits for one or more xenoantigens, and optionally having one or more gene edits to express human immune-compatible genes. The NZeno-1 donor pigs are also DPF and PERV-null making them particularly suitable for whole organ, tissue and/or cell xenotransplantation as they have been selected and manipulated to minimise the risk of hyperacute organ rejection.

The NZeno-1 novel donor pig strain of the present invention, produced by the combined method of CGS selection for PERV-C and PERV-A/C negative status and low (1-2) PERV A and B, being DPF and PERV-null, and having one or more of GGTA1, CAMH, B4GalT2, Neu5Gc, ASGR1 and/or SLA eliminated or deactivated via gene editing technology, are far superior to any currently available pigs in terms of their suitability as donors for xenotransplantation.

As discussed above, the current thinking in terms of producing pigs as a source of organs, tissues and/or cells for xenotransplantation is to use CRISPR Cas-9 technology to gene edit not only the xenoantigens but also all of the genome wide PERV genes. This would, however require dozens of gene edits being carried out on donor pig cells
5 (usually fibroblasts) prior to somatic cell nuclear transfer and cloning of the resulting embryos. For example, Yang et al (2015) succeeded in inactivating 62 PERV sequences in an immortalised pig cell line and Niu et al (2017) succeeded in inactivating 25 PERV genes to produce piglets with inactive PERVs. Additional gene edits on top of the PERV gene edits would be required to inactivate the xenoantigens
10 (eGenesis talk about introducing "double digit" gene edits to reduce the likelihood of organ rejection (Weintraub, 2019)).

As is now known, CRISPR Cas-9 technology can result in unwanted DNA deletions and rearrangements near its target site on the genome. CRISPR Cas-9 gene editing relies on the Cas-9 enzyme to cut DNA at a particular target site. The cell then attempts to
15 reseal this break using DNA repair mechanisms. The repair mechanisms do not always work perfectly and sometimes segments of DNA will be deleted or rearranged or other unwanted changes can occur. Clearly the more gene edits that are carried out on a single cell, the more the chances of these unwanted edits occurring increases.

20 The present method produces PERV-C and PERV-A/C negative pigs, without having to carry out gene editing and only contemplates using gene editing to eliminate or deactivate a minimum number of xenoantigen genes thereby avoiding having to produce cloned animals having dozens of gene edits which may harm the animal and/or have unforeseen genetic consequences.

25 The present invention therefore provides PERV-C and PERV-A/C negative pigs selected by the CGS selection method of the invention, preferably also having a very low copy number of active PERV-A and PERV-B of 1-2, as donor pigs for xenotransplantation of tissues and/or cells or as foundation pigs for further manipulation.

The foundation pigs of the invention may be further manipulated or modified by
30 undergoing gene editing to eliminate or inactivate one or more xenoantigens selected from GGTA1, CAMH, B4GalNT2, Neu5Gc, ASGR1 and SLA, and optionally to express one or more human genes selected from A20, CD39, CD46, CD47, CD55, CD59, hemoxygenase-1 (HO-1), HLA-E, HLA-G, thrombomodulin (TM), CTLA4-Ig, and LEA29Y, to produce a novel NZeno-1 strain.

The CGS selection method of the present invention used to identify PERV-C and PERV-A/C negative status can be carried out before or after gene editing of said one or more xenoantigens.

- 5 Usually, gene editing of one or more xenoantigens will be carried out on suitable cells of pigs that have been pre-selected as PERV-C and PERV-A/C negative using the CGS method of the present invention, whereby the gene edited cells are then subjected to somatic cell nuclear transfer to produce cloned pigs that are PERV-C and PERV-A/C negative and have had one or more xenoantigens eliminated/deactivated (novel NZeno-1 strain).
- 10 As the pigs used in the present invention are also designated pathogen-free, are PERV-null and may also have been pre-selected for blood group O and to be free of immunogenic antigens present at the cell surface such as MHC Class I antigen, the organs, tissues and/or cells of the present invention are particularly suitable for xenotransplantation. It is anticipated that the organs, tissues and/or cells of the novel
- 15 NZeno-1 strain of donor pigs of the present invention will be able to be transplanted into most human recipients in need of such transplants as the majority of the immune-mediated responses responsible for hyperacute organ rejection, and which are usually required to be tested for using complex crossmatching, will have been minimised by elimination or deactivation of the major xenoantigens responsible for
- 20 such immune rejection and by insertion of genes that express human coagulation factors and/or inhibitors of inflammation.

The present invention also provides tissues and/or cells isolated from one or more pigs selected by the CGS method of the present invention. The present invention also provides whole organs, tissues and/or cells isolated from the novel NZeno-1 strain of

25 donor pigs that, in addition to being selected by the CGS selection method of the invention, have also undergone CRISPR Cas-9 gene editing of one or more xenoantigens.

Preferably, the organs, tissues and/or cells are isolated from one or more pigs, wherein said one or more pigs are size matched for the recipient. For example,

30 organs and tissues for transplantation into adult human recipients are preferably isolated from adult pigs, and organs and tissues for transplantation into children are isolated from young/juvenile pigs as would be understood by a skilled worker. Cells for transplantation into adult or children human recipients are preferably isolated from young/juvenile pigs.

The organs, tissues and/or cells may be selected from the group consisting of kidney, liver, lung, heart, brain, pancreas, muscle, blood, bone, testes and ovary.

Preferably, the organs for whole organ xenotransplantation are selected from kidney, liver, lung and heart.

- 5 Preferably, the tissue and/or cells for xenotransplantation are selected from pancreatic islets, hepatocytes, non-parenchymal liver cells, gall bladder epithelial cells, gall bladder endothelial cells, bile duct epithelial cells, bile duct endothelial cells, hepatic vessel epithelial cells, hepatic vessel endothelial cells, sinusoid cells, choroid plexus cells, fibroblasts, Sertoli cells, adrenal chromaffin cells and muscle cells.
- 10 The invention also provides a method of treating a patient suffering from or predisposed to a disease, disorder or condition associated with a deficiency in or absence of organ function, said method comprising transplanting an organ, tissue and/or cells of the invention to a patient in need thereof.

Such transplantation will preferably be able to restore or augment cell, tissue or organ

- 15 function in a human recipient whilst minimising the risk of transmission of xenozyoonotic infectious agents, including PERV as well as minimising the risk of hyperacute organ rejection.

Preferred embodiments of the invention have been described by way of example only and modifications may be made thereto without departing from the scope of the

- 20 invention.

EXAMPLES

1. Example 1

This example describes methods for the analysis of PERV content of *Sus scrofa* by complete genome sequencing.

25 **1.1 Methods**

Sus scrofa Male1 is a male AI pig that is designated pathogen-free (DPF) and has been identified as PERV-C negative by PCR-based test.

- Complete genome sequencing of *Sus scrofa* Male1 was performed under contract by MacroGen Inc. The Binary Alignment Map (BAM) file (showing filtered DNA sequence reads that are mapped to a reference genome with duplicate reads removed) and
- 30 Variant Call Format (VCF) file (showing DNA sequence variants that are identified by

analysing the information taken from aligned reads) were obtained from MacroGen Inc. and used for further analysis. The files were used to create a consensus sequence using BCftools and the regions with no coverage from the consensus were replaced with the character "N". The full PERV-C sequence of KC116219 (8678bp; NCBI
 5 reference sequence) was BLASTed against the *Sus scrofa* Male1 genome using Geneious.

1.2 Results

1.2.1 Genome sequencing and identification of PERV sequences

A total of 27 genomic regions covering more than 50% of the query (KC116219) were
 10 detected (Figure 1), and only 8 covered more than 90%. These were chosen as candidates of potentially functional PERVs for further analysis (Table 1).

Table 1. Genomic regions of *Sus scrofa* Male1 that cover more than 90% of the PERV-C sequence (KC116219).

Sequence	Chromosome	Hit length	Hit start	Hit end	Query coverage	Query start	Query end
NC_010461.5	X	8740	73780807	73772068	99.16%	74	8678
NW_018085136.1	unplaced scaffold	8755	572572	563818	99.16%	74	8678
NC_010443.5	1	8722	262273484	262282205	99.03%	85	8678
NC_010450.4	8	8722	51596579	51605300	99.03%	85	8678
NC_010455.5	13	8711	142113106	142104396	99.03%	85	8678
NC_010445.4	3	8722	10677380	10668659	99.03%	85	8678
NC_010457.5	15	8699	66922015	66913317	99.03%	85	8678
NC_010462.3	Y	8154	25246277	25254430	92.54%	85	8115

1.2.2 Phylogenetic analysis

MAFFT was used to make an alignment (Figure 2) of the 8 hits with 9 existing PERV reference sequences for variants A, B, and C (variant C: KC116219, KC116221, AM229311; recombinant variant A/C: AY570980, AY953542; variant A: AJ279056, GU980187; variant B: EU523109, AJ133816) (Tang et al., 2016; Xiang et al., 2013).

20 In addition, equivalent analyses were performed using PERV-A, B, C, and A/C references as well. When the coverage threshold was raised to 60%, the same 25 genomic regions were found consistently in the BLAST results. However, when the coverage threshold was raised to 90%, 18 genomic regions were inconsistently found across these PERV-A, B, C, and A/C references.

25 A phylogeny of the 8 hits with greater than 90% coverage of the PERV-C sequence (KC116219) was constructed along with the 9 existing PERV reference sequences to identify the closest PERV variant of each genomic region by IQ-TREE. The phylogenetic

- results indicated that the 8 hits group with 2 PERV-A reference sequences, which means that all these 8 regions of the Male1 genome show greater similarity to PERV-A (Figure 3). Moreover, we also conducted a phylogenetic analysis of the 18 genomic regions that were inconsistently found across the PERV-A, B, C, and A/C references with a coverage threshold of 90%. The results revealed that 8 genomic regions (the same regions identified with greater than 90% coverage of the PERV-C sequence) again group with the 2 PERV-A reference sequences, and no genomic regions were grouped with PERV-A/C and PERV-C (Figure 4).

1.2.3 PERV gene analysis

- Protein coding regions of these 18 hits were identified by ORF Finder implemented by the NCBI website with standard genetic codes. The results showed that 16 sequences contained features associated with loss of function, and only two sequences (NC_010462.3 on chromosome Y and NC_010445.4_2 on chromosome 3) were found to include full lengths of the 3 retroviral genes (gag, pol, and env) (Table 2).
- Table 2.** Protein coding regions of selected 18 hits of *Sus scrofa* Male1. Two reference sequences of PERV-C (KC116219) and PERV-A (GU980187) are included.

	Whole length (BP)	gag gene			pol gene			env gene		
		Size (bp)	Start codon	Stop codon	Size (bp)	Start codon	Stop codon	Size (bp)	Start codon	Stop codon
KC116219 (Reference, PERV-C)	8678	1566	ATG	TAG	3585	GGA	TAA	1917	ATG	TAG
GU980187 (Reference, PERV-A)	8939	1575	ATG	TAG	2931	ATG	TAG	1983	ATG	TAG
NC_010461.5	8740	several stop codons found			several stop codons found			several stop codons found		
NW_018085136.1	8755	several stop codons found			several stop codons found			several stop codons found		
NC_010443.5	8722	1575	ATG	TAG	several stop codons found			1614	ATG	TAG
NC_010450.4	8722	1575	ATG	TAG	several stop codons found			1965	ATG	TAA
NC_010455.5	8711	several stop codons found			3000	TTG	TAG	1965	ATG	TAA
NC_010445.4	8722	1575	ATG	TAG	several stop codons found			several stop codons found		
NC_010457.5	8699	several stop codons found			3411	ATG	TAA	several stop codons found		
NC_010462.3	8154	1575	ATG	TAG	3435	ATG	TAA	1962	ATG	TAG
NC_010446.5	8759	several stop codons found			3411	ATG	TAG	several stop codons found		
NC_010445.4_2	8760	1575	ATG	TAG	2925	TTG	TGA	1974	ATG	TAG
NC_010451.4	8757	1575	ATG	TAG	3411	ATG	TAG	several stop codons found		
NC_010453.5	8758	several stop codons found			3444	ATG	TAG	several stop codons found		
NW_018085086.1	8721	several stop codons found			several stop codons found			several stop codons found		
NC_010456.5	8679	several stop codons found			several stop codons found			1974	ATG	TAG

NC_010449.5	8826	several stop codons found	several stop codons found	several stop codons found
NC_010450.4_2	8741	May have, but many N found inside	several stop codons found	1974 ATG TAG
NC_010455.5_2	7707	several stop codons found	3444 ATG TAA	1983 ATG TAG
NC_010462.3_2	7698	several stop codons found	3114 ATG TGA	several stop codons found

Further analyses were focused on searching the three PERV genes independently. Each gene of the reference sequence KC116219 (gag 1566bp, env 1917bp, and pol 3585bp) was employed to run a search BLAST against the *Sus scrofa* Male1 genome:

- 5 gag: 32 genomic regions were found with query coverage $\geq 90\%$, but only 15 regions included the full-length gag gene. A total of 45 sequences with $\geq 70\%$ query coverage were selected for phylogeny analysis (Figure 5).

- pol: 25 genomic regions were found with query coverage $\geq 90\%$, but only 11 regions included the full-length pol gene. A total of 28 sequences with $\geq 70\%$ query coverage was selected for phylogeny analysis (Figure 6).

env: 15 genomic regions were found with query cover $\geq 90\%$, but only 4 regions included the full length env gene. A total of 17 sequences with $\geq 70\%$ query coverage was selected for phylogeny analysis (Figure 7).

- PERV long terminal repeats (LTR) analysis was also carried out and confirmed the results above (results not shown).

1.3 Conclusion

- For the *Sus scrofa* Male1 genome, 25 genomic regions covering at least 60% of the PERV sequence were found consistently in the BLAST results of four searches performed with the PERV-A, B, C, and A/C references (Figure 1). The 8 hits with greater than 90% coverage of PERV-C showed greater similarity to the PERV-A sequences than the PERV-B, C, or A/C sequences (Figure 3). The 18 genomic regions that were inconsistently found across the PERV-A, B, C, and A/C references using a coverage threshold of 90% did not group with the PERV-A/C or PERV-C sequences (Figure 4). Only two sequences (NC_010462.3 on chromosome Y and NC_010445.4_2 on chromosome 3) were found to include full lengths of the 3 retroviral genes (gag, pol, and env) (Table 2). Moreover, the sequence of NC_010462.3 was grouped with PERV-A, and the sequence of NC_010445.4_2 was closest to PERV-B. Furthermore, after building the phylogeny of three PERV genes (gag, pol, and env) independently, the results showed that no genomic regions were found to group with the reference genes from PERV-A/C or PERV-C (Figures 5-7).

These analyses showed that there is no evidence of functional PERV-A/C or PERV-C sequences in the *Sus scrofa* Male1 genome. This agrees with the PCR-based test that showed Male1 to be PERV-C negative.

From PERV analysis, without functional gene analysis (equivalent to PERV copy number analysis using RT-PCT) it appears that there are 18 PERVs in the genome of this animal. Such an animal would not have been deemed suitable for xenotransplantation as the risk of PERV-C and/or PERV-A/C infection in the human recipient would have been too high.

However, due to this new selection method, pigs that were previously thought to be unavailable for xenotransplantation due to the presence of active PERV, can now be used as donor pigs for xenotransplantation if the PERV copy number corresponds to non-functional sequences as identified by CGS, or can be used as foundation pigs for further manipulation by gene editing technology as described below.

2. Example 2

This example describes an additional instance of the analysis of PERV content of *Sus scrofa* by complete genome sequencing.

2.1 Methods

Sus scrofa Male2 is an AI male pig that is designated pathogen-free (DPF) and has been identified as PERV-C positive by PCR-based test. The complete genome sequencing of *Sus scrofa* Male2 was performed as described in Example 1 section 1.2.

2.2 Results

2.2.1 Genome sequencing and identification of PERV sequences

A total of 25 genomic regions covering more than 50% of the query (KC116219) were detected (Figure 8), and only 8 covered more than 90%. These were chosen as candidates of potentially functional PERVs for further analysis (Table 3).

Table 3. Genomic regions of *Sus scrofa* Male2 that cover more than 90% of the PERV-C sequence (KC116219).

Sequence	Chromosome	Hit length	Hit start	Hit end	Query coverage	Query start	Query end
NC_010461.5	X	8740	73779160	73770421	99.16%	74	8678
NW_018085136.1	Unplaced scaffold	8755	572583	563829	99.16%	74	8678
NC_010443.5	1	8722	262259539	262268260	99.03%	85	8678
NC_010450.4	8	8721	51599368	51608088	99.03%	85	8678
NC_010455.5	13	8711	142091853	142083143	99.03%	85	8678
NC_010445.4	3	8722	10677954	10669233	99.03%	85	8678

NC_010457.5	15	8699	66913201	66904503	99.03%	85	8678
NC_010462.3	Y	8156	25245370	25253525	92.57%	85	8117

MAFFT was employed to make an alignment (Figure 9) of the 8 hits with 9 existing PERV reference sequences for variants A, B, and C (variant C: KC116219, KC116221, AM229311; recombinant variant A/C: AY570980, AY953542; variant A: AJ279056, GU980187; variant B: EU523109, AJ133816) (Tang et al., 2016; Xiang et al., 2013).

In addition, equivalent analyses were performed using PERV-A, B, C, and A/C references as well. When the coverage threshold was raised to 60%, the same 25 genomic regions were found consistently in the BLAST results, with the exception that the sequence NC_010447.5 could not be detected using the PERV-B reference sequence. However, when the coverage threshold was raised to 90%, 18 genomic regions were inconsistently found across these PERV-A, B, C, and A/C references.

2.2.2 Phylogenetic analysis

A phylogeny of the 8 hits with greater than 90% coverage of the PERV-C sequence (KC116219) was constructed along with the 9 existing PERV reference sequences to identify the closest PERV variant of each genomic region by IQ-TREE. The phylogenetic results indicated that the 8 hits group with 2 PERV-A reference sequences, which means that all these 8 regions of Male2 genome show greater similarity to PERV-A (Figure 10). Moreover, we also conduct a phylogenetic analysis of the 18 genomic regions that were inconsistently found across the PERV-A, B, C, and A/C references with a coverage threshold of 90%. The results revealed that 8 genomic regions (the same regions identified with greater than 90% coverage of the PERV-C) group with the 2 PERV-A reference sequences, and no genomic regions were grouped with PERV-A/C and PERV-C (Figure 11).

2.2.3 PERV gene analysis

Protein coding regions of these 18 hits were identified by ORF Finder implemented by the NCBI website with standard genetic codes. The results showed that 17 sequences contained features associated with loss of function, and only the sequence NC_010462.3 on chromosome Y was found to include full lengths of the 3 retroviral genes (gag, pol, and env) (Table 4).

Table 4. Protein coding regions of selected 18 hits of *Sus scrofa* Male2. Two reference sequences of PERV-C (KC116219) and PERV-A (GU980187) are shown.

	Whole length (BP)	gag gene		pol gene		env gene	
		Size (bp)	Start codon	Stop codon	Size (bp)	Start codon	Stop codon
KC116219 (Reference, PERV-C)	8678	1566	ATG	TAG	3585	GGA	TAA
GU980187 (Reference, PERV-A)	8939	1575	ATG	TAG	2931	ATG	TAG
NC_010461.5	8740		several stop codons found			several stop codons found	
NW_018085136.1	8755		several stop codons found			several stop codons found	
NC_010443.5	8722	1575	ATG	TAG		1614	ATG
NC_010450.4	8721	1545	TTG	TAG		1965	ATG
NC_010455.5	8711		several stop codons found			1965	ATG
NC_010445.4	8722	1575	ATG	TAG		several stop codons found	
NC_010457.5	8699		several stop codons found			several stop codons found	
NC_010462.3	8156	1575	ATG	TAG		1965	ATG
NC_010451.4	8757	1575	ATG	TAG			TAG
NC_010446.5	8759		several stop codons found			several stop codons found	
NC_010445.4_2	8760	1575	ATG	TAG		May have, but many N found inside	
NC_010453.5	8756		several stop codons found			several stop codons found	
NW_018085086.1	8719		several stop codons found			several stop codons found	
NC_010456.5	8680		several stop codons found			several stop codons found	
NC_010449.5	8825		several stop codons found			several stop codons found	
NC_010455.5_2	7707		several stop codons found			several stop codons found	
NC_010462.3_2	7698		several stop codons found			several stop codons found	
NC_010459.5	7695		several stop codons found			several stop codons found	
					3054	ATG	TAG
					3054	ATG	TAG
					3444	ATG	TAA
					3114	ATG	TGA
					3117	TTG	TAA

Further analyses were focused on searching the three PERV genes independently. Each gene of the reference sequence KC116219 (gag 1566bp, env 1917bp, and pol 3585bp) was employed to run a search BLAST against the *Sus scrofa* Male2 genome:

- gag: 34 genomic regions were found with query coverage $\geq 90\%$, but only 14 regions included the full-length gag gene. A total of 46 sequences with query coverage $\geq 70\%$ were selected for phylogeny analysis (Figure 12).
- pol: 25 genomic regions were found with query coverage $\geq 90\%$, but only 11 regions included the full-length pol gene. A total of 27 sequences with query coverage $\geq 70\%$ were selected for phylogeny analysis (Figure 13).
- env: 16 genomic regions were found with query coverage $\geq 90\%$, but only 4 regions included the full length env gene. A total of 18 sequences with query coverage $\geq 70\%$ were selected for phylogeny analysis (Figure 14). PERV long terminal repeats (LTR) analysis was also carried out and confirmed the results above (results not shown).

2.3 Conclusion

- For the *Sus scrofa* Male2 genome, 24 genomic regions covering at least 60% of the PERV sequence were found consistently in the BLAST results of four searches performed with the PERV-A, B, C, and A/C references, with the sole exception that the NC_010447.5 sequence could not be detected by the PERV-B reference. Only one hit (NC_010456.5; chromosome 14, position 62,583,591-62,590,005) showed high similarity to the PERV-C gag gene, but this only partially matched the PERV reference sequence of KC116219 with 73.91% coverage (Figure 8). The 8 hits with greater than 90% coverage of PERV-C showed greater similarity to the PERV-A sequences than the PERV-B, C, or A/C sequences (Figure 10). The 18 genomic regions that were inconsistently found across these PERV-A, B, C, and A/C references using a coverage threshold of 90% did not group with the PERV-A/C or PERV-C sequences (Figure 11). Only one sequence (NC_010462.3 on chromosome Y) was detected to include full lengths of the 3 retroviral genes (gag, pol, and env) (Table 4). Moreover, the sequence of NC_010462.3 was grouped with PERV-A. Furthermore, after building the phylogeny of three PERV genes (gag, pol, and env) independently, the results showed that no genomic regions were found to group with the reference genes from PERV-A/C (Figures 5-7).

Surprisingly, these analyses show that *Sus scrofa* Male2 is in fact PERV-C negative, despite the previous positive PCR-based test. This new selection method shows that

there is no evidence of functional PERV-A/C or PERV-C sequences in the *Sus scrofa* Male2 genome, indicating that this individual is a candidate as a donor pig for tissues and/or cells for xenotransplantation into a human recipient, or as a foundation pig for further manipulation by gene editing technology as described below.

5 **3. Example 3**

This example describes the creation and characterisation of the NZK1 cell line derived from the kidneys of Auckland Island (AI) pigs.

3.1 Methods

3.1.1 Isolation of kidney derived AI pig cells

- 10 Kidney-derived primary AI pig cells were isolated from tissue samples from two individual animals. One was a male newborn piglet (#18/PA007) that accidentally died shortly after birth on 6 February 2018, with the resulting cell line denoted NZK1.

- Kidney cells were isolated based on methods from Richter et al (2012). Briefly, 1cm³ cubes of tissue from the cortex and medulla of the kidney were first washed and then
- 15 centrifuged after mincing. Afterwards, the tissue was resuspended in 15 ml Hank's Buffered Salt Solution with 0.1% (w/v) collagenase (type I or II; Invitrogen) and incubated at 37°C while stirring for 1 to 1.5 h. The supernatant, containing kidney cells, was filtered through a 100-µm mesh and washed with Dulbecco's Modified Eagle's Medium (DMEM) + GlutaMAX (Gibco) and centrifuged again (5–10 min,
- 20 180×g). The resulting cell pellet was seeded onto tissue culture plates or flasks. The remaining undigested tissue pieces were further treated with 0.25% trypsin/0.02% EDTA for 15 min at 37°C to harvest additional cells. Kidney cells were cultured in DMEM + GlutaMAX with non-essential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Gibco) and 10% fetal calf serum (FCS). For the first few days media included 1% (v/v)
- 25 Penicillin G/Streptomycin and Amphotericin B.

The population doubling time of primary AI kidney cells over continuous passages was determined by culturing NZK1 cells on various substrates; including directly on tissue culture plastic, as well as coatings of 0.1% gelatin (Sigma) or 0.01% collagen (Sigma).

Mitotic cell spreads were prepared to determine chromosome numbers of NZK1 cells.

- 30 An additional cell line was established from the kidney of a female AI piglet using similar methods, denoted NZK3.

3.2 Results

After seeding 1×10^5 NZK1 cells / 3cm dish over repeated passages, the population doubling time (PDT) of NZK1 cells on tissue culture plastic steadily increased compared to cells grown on either gelatin or collagen coated dishes (Figures 15 and 16). The average PDT for the first five passages was 25.6h. Although PDT on gelatin and collagen dishes had increased to 44h between passages 15-20 of culture, it was 2.6-fold greater with cells grown on tissue culture plastic.

NZK1 cells at passage 3 displayed a mostly diploid chromosome number ($2N=38$, Figure 17).

4. Example 4

This example describes the creation of a line of Auckland Island (AI) pig cells with a functional disruption to the GGTA1 gene, and so lack the α Gal xenoantigen. This cell line was created from the NZK1 cells described in Example 3.

4.1 Methods

4.1.1 Nucleic acid constructs

Two guide RNAs (gRNAs) were designed using the CRISPOR design tool (Haeussler et al., 2016) with target specificity for GGTA1 exon 8 (65fw - CTCTCGTAGGTGAACTCGTC and 208rev - GATGCGCATGAAGACCATCG, respectively) beginning at position 261,513,520 and 261,513,686, respectively, of Ensemble gene ENSSSCG00000005518.

DNA oligo nucleotides for both strands of the gRNA were synthesised with short adapters, annealed and cloned into the expression vector pX330 according to published protocols (Ran et al., 2013).

4.1.2 Cell culture and transfection

Isolated kidney-derived primary AI pig cells as described in Example 3 were cultured in DMEM + GlutaMAX with non-essential amino acids, 0.1 mM 2-mercaptoethanol and 10% FCS on culture surfaces that were coated with 0.1% gelatine (Sigma-Aldrich) at 37 °C for 1 h prior to use. CRISPR plasmids were delivered into cells using the Neon transfection system according to the manufacturer's instruction (Invitrogen). Briefly, AI pig cells were harvested by trypsin digestion, washed with calcium and magnesium free Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldrich) and centrifuged. Approximately 2×10^6 cells were suspended in 120 μ l of resuspension buffer (Invitrogen) containing a total of 5 μ g of plasmid DNA when using a single and two CRISPR plasmids and 10 μ g with three and four CRISPR plasmids. AI pig cells were

electroporated with a 100 μ l tip using the Neon program A3 (1500 V pulse voltage, 20 ms pulse width, 1 pulse). After transfection cells were seeded into gelatine coated plates in media containing Pen Strep (Gibco) and cultured with 5% CO₂ at 37°C.

4.1.3 CRISPR activity assay

- 5 Kidney-derived primary AI pig cells transfected with expression plasmids for GGTA1-specific CRISPRs (65fw, 208rev) were harvested 48h after transfection. Approximately 1×10^4 transfected cells were incubated in 20 μ l lysis buffer (0.2 μ g/ml proteinase K (Qiagen) in PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3; Roche) for 30 minutes at 50°C followed by 10 minutes incubation at 95°C to inactivate the
- 10 reaction. The crude lysate (2 μ l) was mixed with 18 μ l Kapa 2G Fast Hotstart Ready mix (Kapa Biosystems) to PCR amplify the GGTA1 target locus with GGTA1 primers (GGTA1F - CCAGCAGTATTCTGGGGATAAGA) and GGTA1R - CCCAGAGGTTACATTTACCCCA3). PCR conditions were as follows: 95°C, 3 min; 95°C, 15 s, 60°C, 15 s, and 72°C, 1 s for 40 cycles; and a final extension step of 72°C for 5
- 15 min. PCR fragments were visualised after separation by electrophoresis on 0.8% agarose gels, isolated using a Nucleo-Spin Gel and PCR Clean-up kit (Macherey-Nagel) and sequenced. An overlay of multiple sequences starting around the CRISPR cleavage sites were interpreted as an indication of cleavage activity. The sequences results were then further analysed by Tracking of Indels by DEcomposition (TIDE; Brinkman et al., 2014) to quantify the reduction of the wildtype sequence and the presence of
- 20 several different sequences with small mutations in the amplified target regions.

4.1.4 Counterselection against α Gal and isolation of cell clones

- Kidney-derived primary AI pig cells transfected with GGTA1-specific CRISPRs were counter selected as previously described (Fujimura et al., 2008). Briefly, seven days
- 25 after transfection, cells were harvested and 3×10^6 cells incubated with 30 μ l of biotin-conjugated isolectin IB4 (IB4 lectin from Enzo Life Science, Farmingdale N.Y) in 600 μ l of PBS for 15 minutes. Subsequently, the cells were washed in 10 ml PBS to remove unbound isolectin conjugate and incubated with 600 μ l streptavidin-conjugated Dynabeads (Invitrogen) on ice, occasionally mixing the cell suspension. Following the
- 30 incubation, 5 ml PBS was added, the cell suspension transferred to a single well of a six well plate on a plate magnet and incubated for one minute. The IB4 lectin is specific for the α Gal epitope; cells that did not bind the Dynabeads remained in the supernatant representing cells that no longer express the α Gal epitope. The supernatant was aspirated and the Dynabead selection repeated two more times. Cells
- 35 from the final supernatant were seeded into a 35mm culture dish and cultured overnight. Mitotic cells were then manually picked with a glass capillary and individual

cells transferred into the wells of eight 96 well plates to isolate cell clones. After approximately 14 days individual cell clones reached confluency and were first transferred to a 48-well plate, then to a 12-well plate and 6-well plate for further expansion. When cell clones reached confluency in a 6-well or alternatively already at the 12-well stage, a small proportion of cells were removed for mutation screening and the remainder of cells cryopreserved to capture the cell clones.

4.1.5 Characterisation of cell clones for site-specific mutations and PCR analysis

Target regions were PCR-amplified with primers GGTA1F/R, visualised and sequenced as described above. All sequencing was done by a commercial service provider (Massey Genome Service, New Zealand).

4.2 Results

4.2.1 CRISPR design and testing for cleavage activity

Based on the public pig genome, the CISPOR design tool (Haeussler et al., 2016) was used to identify pairs of CRSIPRs to delete a 152 bp fragment that disrupts the reading frame of the GGTA1 genes in exon 8 (Figure 18).

The sequence for the target regions of the GGTA1 gene in the AI pig cells NZK1 was then determined. Comparison of the sequence with the public pig genome revealed the absence of any polymorphisms in the AI pig sequence (Figure 19). It confirmed the presence of unaltered CRISPR binding sites in AI pig cells and suitability of the two CRISPRs 65fw and 208 rev for editing of the AI pig cells.

The activity of the CRISPRs were verified by transfecting NZK1 cells with plasmids encoding each of the two CRISPRs and analysing the target locus for editing. Following transfection, cells were harvested and the GGTA1 target region was PCR amplified from their DNA. The sequence analysis of the amplified fragment revealed a unique sequence at the beginning that changed into multiple overlaying sequences from around the predicted CRISPR cleavage site onwards indicating the presence of CRISPR-induced mutations (Figure 20). This was further confirmed by Tracking of Indels by DEcomposition (TIDE) analysis of the sequences. This detected a strong reduction of the unaltered wildtype sequence from 100 % in NZK1 cells to 24.1 %-26.0 % in the PCR fragments of AI cells transfected with GGTA1-specific CRISPRs. (Figure 21). In addition, there were new sequence variants comprising small deletions and insertions in close proximity of the CRISPR cleavage site and the presence of several different sequences with small mutations. Taken together, this provided strong evidence for robust, site-specific cleavage activity of the two CRISPRs.

4.2.2 Isolation of NZK1 α Gal knockout cell clones

NZK1, kidney-derived primary AI pig cells as described in Example 3, were transfected with two plasmids expressing the GGTA1-specific CRISPRs 65fw and 208rev. After seven days of culture, cells were counter selected for the presence of the α Gal epitope.

- 5 From the selected cell population, mitotic cells were manually picked and individually transferred into 96 well plates to isolate cell clones. Following expansion of the cell clones into 12-well or 6-well plates, a small proportion of cells was removed for mutation screening and the remainder of cells cryopreserved to capture the cell clones.

- 10 Genomic DNA isolated from the cell clones was PCR amplified for the GGTA1 target region and the size of the amplified fragments analysed on agarose gels (Figure 22A). This revealed cell clones with two smaller fragments, indicative of the presence of two different deletion alleles while others had just a single fragment of a smaller size (such as cell clones G53, G61 and G12) potentially carrying two alleles with an identical deletion. Cell clones producing a single fragment were sequenced to determine the
15 exact mutations and confirm the presence of two identical deletion alleles (Figure 22B). All sequenced cell clones had two identical deletion alleles (homozygous, biallelic) with most possessing a 152 bp deletion that correlates with a precise excision of the intervening region between the two predicted CRISPR cleavage sites.

- 20 The results for the characterisation of the edited GGTA1 mutations in individual cell clones is summarised in Table 5.

Table 5. Summary of characterised GGTA1 knockout cell clones.

Clone ID	PCR	Sequence	Mutation
G3	1 smaller fragment	mixed sequence	N/A
G6	1 smaller fragment	biallelic deletion	152 bp deletion
G7	1 smaller fragment	mixed sequence	N/A
G12*	1 smaller fragment	biallelic deletion	152 bp deletion
G19	1 smaller fragment	biallelic deletion	152 bp deletion
G20*	1 smaller fragment	biallelic deletion	157 bp deletion
G21	1 smaller fragment	biallelic deletion	152 bp deletion
G23	1 slightly smaller fragment	mixed sequence	N/A

G53*	1 smaller fragment	biallelic deletion	152 bp deletion
G61*	1 smaller fragment	biallelic deletion	152 bp deletion
G62	1 smaller fragment	mixed sequence	N/A
G17	No fragment	N/A	N/A
G15	No fragment	N/A	N/A

4.3 Conclusion

The CRISPR design was effective and produced CRISPRs with site-specific cleavage activity for the target locus in the GGTA1 gene. The use of two GGTA1-specific primers generated many cell clones with a precise excision of the intervening sequence between the predicted cleavage sites of the two CRISPRs. Using this method, GGTA1 knockouts in the NZK1 cell line were generated. This novel cell line could be used for knocking out additional xenoantigens, for expressing human immune-compatible genes, and/or for somatic cell nuclear transfer to generate viable embryos and ultimately animals that could serve as a source of organs, tissues, and cells for xenotransplantation.

5. Example 5

This example describes the creation of a line of Auckland Island (AI) pig cells with a functional disruption to the CMAH gene, that lack the Neu5Gc xenoantigen.

5.1 Methods

5.1.1 Nucleic acid constructs

Two guide RNAs (gRNAs) were designed using the CRISPOR design tool (Haeussler et al., 2016) were designed to target exon 3 of CMAH (68rev - TAAGAATAAGAGCCGCCTGA and 165fw - TTGAGATTGGCAGCTTCGGC, respectively) beginning at position 19,917,647 and 19,917,702, respectively, of Ensemble gene ENSSSCG00000001099.

DNA oligo nucleotides for both strands of the gRNA were synthesised with short adapters, annealed and cloned into the expression vector pX330 according to published protocols (Ran et al., 2013).

5.1.2 Cell culture and transfection

Cell culture and transfection was performed as described in Example 4 section 4.1.2.

5.1.3 CRISPR activity assay

The CRISPR activity assay was performed as described in Example 4 section 4.1.3 using the CMAH-specific CRISPRs (68rev, 165fw) and CMAH primers (CMAHF – GAGCTGCCGTAAAGGAGCTT and CMAHR – CCTTGATGGGTAGGATGGCC3).

5.2 Results

5.2.1 CRISPR design and testing for cleavage activity

Based on the public pig genome, the CISPOR design tool (Haeussler et al., 2016) Was used to identify pairs of CRISPRs to delete a 88 bp fragment that disrupts the reading frame of the CMAH gene in exon 3 (Figure 23).

- 10 The sequences for the target regions of the CMAH gene in the AI pig cells NZK1 was then determined. Comparison of the sequences with the public pig genome revealed the absence of any polymorphisms in the AI pig sequences (Figure 24). It confirmed the presence of unaltered CRISPR binding sites in AI pig cells and suitability of the two CRISPRs 68 rev and 165fw for editing of the AI pig cells.
- 15 The activity of the CRISPRs were verified by transfecting NZK1 cells with plasmids encoding each of the two CRISPRs and analysing the target locus for editing. Following transfection, cells were harvested and the CMAH target region PCR amplified from their DNA. The sequence analysis of the amplified fragments revealed a unique sequence at the beginning that changed into multiple overlaying sequences from
- 20 around the predicted CRISPR cleavage site onwards indicating the presence of CRISPR-induced mutations (Figure 25). This was further confirmed by Tracking of Indels by DEcomposition (TIDE) analysis of the sequences. This detected a strong reduction of the unaltered wildtype sequence from 100 % in NZK1 cells to 10.5 % - 14.3 % in the PCR fragments of AI cells transfected with CMAH-specific CRISPRs
- 25 (Figure 26). In addition, there were new sequence variants comprising small deletions and insertions in close proximity of the CRISPR cleavage site and the presence of several different sequences with small mutations. Taken together, this provided strong evidence for robust, site-specific cleavage activity of the two CRISPRs.

5.2.2 Isolation of NZK3 CMAH knockout cell clones

- 30 NZK3, kidney-derived primary AI pig cells as described in Example 3, were transfected with two plasmids expressing the CMAH-specific CRISPRs 165fw and 68rev. After four days of culture, mitotic cells were randomly picked as doublets and individually transferred into 96 well plates to isolate cell clones. Following expansion of the cell

clones into 12-well or 6-well plates, a small proportion of cells was removed for mutation screening and the remainder of cells cryopreserved to capture the cell clones.

Genomic DNA isolated from 25 candidate cell clones was PCR amplified for the CMAH target region and the size of the amplified fragments analysed on agarose gels (Figure 27A). All 25 cell clones were subsequently analysed by sequencing (Figure 27B) and result summarised in Table 6.

Table 6. Summary of characterised CMAH knockout NZK3 cell clones. * indicates cell clones assessed as suitable candidates to generate live CMAH knockout pigs.

Clone ID	PCR	Sequence	Mutation
Several (4)	1 fragment	Wild type	no
Several (13)	1 or 2 fragments	mixed sequence	1 or 2 in-frame deletion or WT alleles
20	1 fragment	mixed sequence	TBD
34	1 fragment	mixed sequence	TBD
38*	1 fragment	biallelic homozygous	27 bp plus 164 bp insertion
86*	1 fragment	biallelic heterozygous	22 bp deletion; 1 bp deletion
89*	2 fragments	biallelic heterozygous	86 bp deletion; 1 bp insertion
90*	1 fragment	biallelic homozygous	1 bp insertion
104*	1 fragment	biallelic heterozygous	1 bp insertion; 10 bp deletion
105	2 fragments	mixed sequence	TBD

Of the cell clones analysed, five were deemed suitable for generating CMAH knockout pigs (indicated with * in Table 6). The best candidate was clone 90 which had a homozygous biallelic 1 bp insertion. Another homozygous biallelic cell clone (38) had a 24 bp and a 164 bp insertion at both of the CRISPR cleavage sites. The remaining three cell clones (86, 89 and 104) were heterozygous biallelic and possessed two different mutant alleles. Several cell clones (4) turned out to be unedited with both genes showing the wild type sequence. Another group of 13 cell clones were not analysed in further detail because they had one remaining wild type allele or possessed one or two in-frame deletion that are unlikely to disrupt the gene function. Other cell clones revealed mixed sequences despite the amplification of what appeared as a single PCR fragment. Our preliminary analysis of the mixed sequences indicated

that they are very unlikely suitable candidates and we therefore not further invested any effort into identifying the exact mutations present in these cell clones.

5.3 Conclusion

- The CRISPR design was effective and produced CRISPRs with site-specific cleavage activity for the target locus in the CMAH gene. Unlike the GGTA1 mutations generated in Example 3, the actual and predicted cleavage sites in the CMAH gene were different, resulting in many cell clones with in-frame deletions and no functional disruption. Further, we observed that the CMAH CRISPR 68rev had a higher activity compared to the second CMAH CRISPR 165fw.
- Overall, the editing efficiency was sufficiently high to allow CMAH KO cell clones to be isolated to produce a novel cell line. Moreover, homozygous biallelic mutants for the isolated cell clone knockouts, could be selected which will facilitate future breeding schemes. This novel cell line could be used for knocking out additional xenoantigens, for expressing human immune-compatible genes, and/or for somatic cell nuclear transfer to generate viable embryos and ultimately animals that could serve as a source of organs, tissues, and cells for xenotransplantation.

6. Example 6

- This example describes the creation of a line of Auckland Island (AI) pig cells that have functional disruptions to the GGTA1 and CMAH genes, and so lack the α Gal and Neu5Gc xenoantigens. This cell line was created from the NZK1 cells described in Example 3.

6.1 Methods

6.1.1 CRISPRs

- The GGTA1 CRISPRs are as described in Example 4 section 4.2.1 and the CMAH CRISPRs are as described in Example 5 section 5.2.1.

6.1.2 Cell culture and transfection

NZK1 cells as described in Example 3 were used. Cell culture and transfection methods are as described in Example 4 section 4.1.2.

6.1.3 Counterselection against α Gal

- Counterselection was used as described in Example 4 section 4.1.4.

6.2 Results

6.2.1 Isolation of NZK1 α Gal/CMAH double knockout cell clones

NZK1 cells were transfected with four plasmids expressing two GGTA1-specific CRISPRs (65fw and 208rev) and two CMAH-specific CRISPRs (165fw and 68rev). After seven days of culture, cells were counter selected for the presence of the α Gal epitope based on the assumption that cells with mutations in GGTA1 have a good chance to be also mutated at the CMAH locus and enrich for cells with mutations in both genes. From the selected cell population, mitotic cells were manually picked individually transferred into 96 well plates to isolate cell clones. Following expansion of the cell clones into 12-well or 6-well plates, a small proportion of cells was removed for mutation screening and the remainder of cells cryopreserved to capture the cell clones.

Genomic DNA isolated from the cell clones was separately PCR amplified for the GGTA1 and CMAH target regions using the methods described in Examples 4 and 5. The size of the amplified fragments for both genes was analysed on agarose gels (Figure 28).

The PCR analysis revealed cell clones with two fragments for one or both genes (e.g. 4.10) but also cell clone with single fragments for GGTA1 and CMAH (e.g. 4.68, 4.16). Of a total of 20 cell clones that were analysed by PCR, 12 were further characterised for their specific mutations by sequence analysis. Figure 29 depicts the result for cell clone 4.16 revealing a homozygous biallelic 152 bp deletion in the GGTA1 gene and two smaller deletions (9 bp and 22 bp), one each at the two CMAH-specific cleavage sites also present on both alleles (homozygous biallelic). Cell clone 4.68 was another cell clone with homozygous biallelic mutations (152 bp deletion in GGTA1 and 1 bp insertion in CMAH) that disrupts both genes. An additional cell clone (4.5) had disruptions for both genes but for each of the genes it had two different mutant alleles (heterozygous biallelic). A summary of the cell clone characterisation is provided in Table 7.

Table 7. Summary of characterised GGTA1 and CMAH double knockout NZK1 cell clones. * indicates cell clones assessed as suitable candidates to generate live GGTA1-CMAH double knockout pigs.

Clone ID	GGTA1 PCR fragments	Mutation	CMAH PCR fragments	Mutation
4.1	1 (mixed seq.)	N/A	1	large del.
4.2	1	7 bp del.	1 (mixed seq.)	N/A
4.3	1 (mixed seq.)	N/A	0	N/A
4.4	2	N/A	1 (mixed seq.)	N/A

4.5*	2	152bp and 402bp del.	1 (mixed seq.)	1bp ins./1bp conv./6bp del. 1bp ins./5bp del.
4.10	2	152bp and 13bp del.	2	87bp del. 304bp del.
4.13	1	N/A	2	N/A
4.15	2	N/A	1 (?)	N/A
4.16*	1	152bp del.	1	9bp del./22bp del.
4.18	1	N/A	1	87bp del.
4.52	1	2bp del.	1	87bp del.
4.68*	1	152bp del.	1	1bp ins.

6.3 Conclusion

GGAT1 and CMAH double knockouts in the NZK1 cell line were successfully produced, with the help of a counterselection procedure. Moreover, homozygous biallelic mutants were isolated for the isolated cell clone knockout to produce a novel cell line, which will facilitate future breeding schemes. For example, this novel cell line could be used for knocking out additional xenoantigens, for expressing human immune-compatible genes and/or for somatic cell nuclear transfer to generate viable embryos and ultimately animals that could serve as a source of organs, tissues, and cells for xenotransplantation.

7. Example 7

This example describes the creation of a line of Auckland Island (AI) pig cells with a functional disruption to the GGTA1 gene, and so lack the α Gal xenoantigen. This cell line was created from the NZK3 cells described in Example 3.

7.1 Methods

7.1.1 CRISPRs

The GGTA1-specific CRISPRs 65fw and 208rev are as described in Example 4 section 4.2.1.

7.1.2 Cell culture and transfection

NZK3, kidney-derived primary AI pig cells as described in Example 3, were transfected with two plasmids expressing the GGTA1-specific CRISPRs 65fw and 208rev. The transfection methods are as described in Example 4 sections 4.1.2.

7.1.1 Counterselection against α Gal

Counterselection was used as described in Example 4 section 4.1.4.

7.2 Results

7.2.1 Isolation of NZK3 α Gal knockout cell clones

After seven days of culture, cells were counter selected for the presence of the α Gal epitope. From the selected cell population, mitotic cells were manually picked
5 individually transferred into 96 well plates to isolate cell clones. Following expansion of the cell clones into 12-well or 6-well plates, a small proportion of cells was removed for mutation screening and the remainder of cells cryopreserved to capture the cell clones.

Genomic DNA isolated from the cell clones was PCR amplified for the GGTA1 target
10 region and the size of the amplified fragments analysed on agarose gels (Figure 30A). Of 28 cell clones we analysed by PCR we identified five cell clones (GA2, GA5, GA20, GA35 and GA65) with a single, smaller fragment, potentially carrying the homozygous biallelic 152 bp deletion alleles we had observed as a frequent editing outcome in our above experiments. Sequencing confirmed that all five cell clones carried the
15 homozygous biallelic 152 bp deletion in the GGTA1 locus (Figure 30B).

7.3 Conclusion

As in Example 3, GGTA1 knockouts were generated, this time in the NZK3 cell line. This novel cell line could be used for knocking out additional xenoantigens, for
20 expressing human immune-compatible genes and/or for somatic cell nuclear transfer to generate viable embryos and ultimately animals that could serve as a source of organs, tissues, and cells for xenotransplantation.

8. Example 8

This example describes the creation of a line of Auckland Island (AI) pig cells that have functional disruptions to the GGTA1 and CMAH genes, and so lack the α Gal and
25 Neu5Gc xenoantigens. This cell line was created from the NZK3 cells described in Example 3.

8.1 Methods

8.1.1 CRISPRs

The GGTA1 CRISPRs are as described in Example 4 section 4.2.1 and the CMAH
30 CRISPRs are as described in Example 5 section 5.2.1.

8.1.2 Cell culture and transfection

NZK3, kidney-derived primary AI pig cells as described in Example 3, was transfected with four plasmids expressing two GGTA1-specific CRISPRs (65fw and 208rev) and

two CMAH-specific CRISPRs (165fw and 68rev). The transfection methods are as described in Example 4 sections 4.1.2.

8.1.3 Counterselection against α Gal

Counterselection was used as described in Example 4 section 4.1.4.

5 8.1.4 Fluorescence-activated cell sorting (FACS) analysis of the α Gal epitope

Cells (1×10^6) from double KO cell clones were trypsinised, washed with PBS and incubated with FITC conjugated isolectin B4 as previously described (Hauschild et al., 2011). Subsequently, cells were analysed for fluorescence levels on a FACSverse (Becton Dickinson).

8.2 Results

8.2.1 Isolation of NZK3 α Gal/CMAH double knockout cell clones

After seven days of culture, cells were counter selected for the presence of the α Gal epitope and mitotic cells manually picked and individually transferred into 96 well plates to isolate cell clones. Expansion of the cell clones into 12-well or 6-well plates was hampered by contamination of the cultures which limited the number of cell clones to only seven that were available for analysis summarised in Table 8. Only one cell clone GC15 had mutations that disrupted both target genes, although possessing two different mutant alleles for GGTA1 (1 bp insertion plus a 5bp deletion on one allele and a 152 bp deletion on the other allele) and CMAH (11 bp deletion on one allele and a 107 bp deletion on the other allele). The other six cell clones had in-frame mutations and one or both genes excluding them as candidates for a cell clone with a functional KO of GGTA1 and CMAH.

Table 8. Summary of characterised GGTA1 and CMAH double knockout NZK3 cell clones. * indicates cell the cell clone assessed as a suitable candidate to generate live GGTA1-CMAH double knockout pigs.

Clone ID	GGTA1 PCR fragments	Mutation	CMAH PCR fragments	Mutation
GC15*	2 (hetero)	1 bp insertion plus 5 bp deletion; 152 bp deletion	2 (hetero)	11 bp deletion; 107 bp deletion
GC16	1 (homo)	36 bp deletion (in frame)	1 (homo)	~290 bp deletion
GC18	1 (hetero)	1 bp insertion; 3 bp deletion (in frame)	1 (homo)	1 bp insertion plus 6 bp deletion
GC21	2 (hetero)	1 bp insertion;	2 (hetero)	1 bp insertion plus 4

		152 bp deletion		bp deletion; 87 bp deletion (in frame)
GC24	1 (hetero)	Mixed sequence?	1 (hetero)	86 bp deletion; 87 bp deletion (in frame)
GC30	1 (homo)	1 bp insertion plus 5 bp deletion	1 (homo)	87 bp deletion (in frame)
GC31	2 (hetero)	136 bp deletion; ?	2 (hetero)	210 bp deletion (in frame); ?

Because of the availability of a single cell clone with only heterozygous biallelic mutations for both genes that would complicate subsequent breeding programmes due to the random segregation of the different alleles, we decided to repeat the

5 transfection and isolate additional KO cell clones. For this repeat, we transfected NZK3 with three plasmids expressing two GGTA1-specific CRISPRs (65fw and 208rev) and a single CMAH-specific CRISPRs (68rev). After seven days of culture, cells were counter selected for the presence of the α Gal epitope and mitotic cells were manually picked and individually transferred into 96 well plates to isolate cell clones. Following

10 expansion of the cell clones into 12-well or 6-well plates, a small proportion of cells was removed for mutation screening and the remainder of cells cryopreserved to capture the cell clones.

Genomic DNA isolated from 52 cell clones was separately PCR amplified for the GGTA1 and CMAH target regions and target regions sequenced for 21 clones to determine the

15 precise sequence change at the target loci. The analysis demonstrated that we had isolated four cell clones that had homozygous biallelic mutations, disrupting the reading frames for the GGTA1 and the CMAH gene (Table 9). All five cell clones are suitable candidates for the generation of double KO AI pigs.

Table 9. Double knockout cell clones with homozygous knockout mutant alleles for GGTA1 and CMAH.

Clone ID	GGTA1 PCR fragments	Mutation	CMAH PCR fragments	Mutation
3CG61	1 (homo)	152 bp deletion	1 (homo)	1 bp deletion
3CG72	1 (homo)	22 bp deletion	1 (homo)	1 bp deletion
3CG27	1 (homo)	154 bp deletion	1 (homo)	1 bp insertion
3CG12	1 (homo)	152 bp deletion	1 (homo)	71 bp deletion

8.2.2 Confirmation of the functional disruption of the GGTA1 gene

The use of two CRISPRs for the disruption of the GGTA1 gene resulted in most of our cell clones having a precise excision of a 152 bp fragment in exon 8. To verify that this mutation functionally disrupts the GGTA1 gene we have analysed cell from two double
5 KO cell clones (3CG33, 3CG55) with a homozygous, biallelic 152 bp deletion in GGTA1. The cells were incubated with an isolectin B4- FITC conjugate, that specifically binds the α Gal epitope. After removal of any non-bound isolectin conjugate, cells were analysed by flowcytometry for the binding or non-binding of the fluorescently-labelled
10 isolectin as an indication of the presence or absence of the α Gal epitope. In the absence of isolectin conjugate, wild type AI pig cells are non-fluorescent. Incubated with isolectin the cells become fluorescent indicated by a strong shift in the fluorescence intensity due to the binding of the isolectin conjugate to the α Gal epitope on the surface of the AI wild type cells (Figure 31A). In contrast, human HEK cells which do not express the α Gal epitope remain non-fluorescent independent of the
15 incubation with the isolectin conjugate (Figure 31C). Double KO cells with a 152 bp deletion in exon 8 of GGTA1 remain non-fluorescent in the presence of the isolectin conjugate and show the same pattern as human HEK cells, demonstrating the double KO AI pig cells no longer express the α Gal epitope (Figure 31B).

8.3 Conclusion

20 Using CRISPR, GGAT1 and CMAH double knockouts were generated in the NZK3 cell line with the help of a counterselection procedure to produce a novel cell line.

The predominant mutation in these GGTA1 KO cell clones was a 152 bp deletion in exon 8. Using flow cytometry, it was demonstrated that this mutation functionally
25 disrupts the GGTA1 gene and results in AI pig cells that no longer express the α Gal epitope. However, it was not possible to test for the functional disruption of CMAH because CMAH KO cells can take up and incorporate the CMAH-specific glycosylation Neu5Gc from the culture medium. Testing will become feasible once tissue or blood samples from CMAH KO fetuses and piglets are available. This novel cell line could
30 be used for knocking out additional xenoantigens, for expressing human compatible genes and/or for somatic cell nuclear transfer to generate viable embryos and ultimately animals that could serve as a source of organs, tissues, and cells for xenotransplantation.

*All gene editing was carried out under contract by AgResearch Limited, New Zealand.

9. Example 9

This example describes the development of porcine somatic cell nuclear transfer (SCNT) for generating blastocysts from gene-edited cell lines.

9.1 Methods

5 Ovaries were sourced from pre-pubertal gilts slaughtered at the Ruakura abattoir (AgResearch, New Zealand). Cumulus-oocyte complexes (COCs) were recovered by follicular aspiration and matured in vitro (IVM) for 44h. Two IVM systems to mature porcine oocytes were compared: biphasic (ESOF and LSOF) IVM1/2 medium containing 1mM dibutyl cyclc adenosine monophosphate for the first 20-24h (Bagg
10 et al., 2006; Somfai and Hirao, 2011) and FLI medium supplemented with FGF2, LIF and IGF1 for the entire IVM period (Yuan et al., 2017).

COCs were denuded by vortexing with 0.1% hyaluronidase (Sigma) at 2000rpm for about 60-90 sec. Oocytes were washed twice in HEPES-buffered TCM199 medium (H199) + 10% FCS. Denuded oocytes were incubated for 10 min with 0.2M sucrose in
15 H199 + 10% FCS. Oocytes that maintained a regular, round shape (but smaller diameter) along with a first polar body (1PB) were selected for use. Those oocytes that displayed a very irregular shape after sucrose treatment were discarded, as these are of poorer developmental quality (Dang-Nguyen et al., 2018; Lee et al., 2014).

For zona-free SCNT, pronase was used to remove the zona pellucida. Oocytes were
20 washed thoroughly before staining DNA with 5µg/mL H33342 plus 7.5µg/mL cytochalasin B (all Sigma) for 5 min. A 24µm outer diameter blunt-ended pipette was used for UV-assisted enucleation of metaphase II stage oocytes at 35°C.

Donor cells (e.g. NZK1 kidney fibroblasts) were trypsinised to a single cell suspension after 5-7 days in medium with 0.5% FCS. Single cells were adhered to each
25 enucleated cytoplasm with the aid of 20µg/mL phytohemagglutinin in H199 + 3 mg/mL BSA. Electrical fusion was achieved with two direct current pulses of either 1.2kV/cm for 30µs or 2kV/cm for 10µs in fusion buffer without calcium at approximately 48h after the start of IVM. Following fusion, reconstructed embryos were held in embryo culture medium without Ca + 10% FCS + 5µg/mL cytochalasin B at 38.5°C in 5% CO₂
30 in air for 2-4 h before activation.

To maintain high levels of maturation promoting factor-cyclin B complex in oocytes aiding nuclear reprogramming, some experiments compared the addition of 5µM of the proteasome inhibitor MG132 (Sigma). This was added to media formulations from the time of sucrose selection of oocytes until activation of NT reconstructed embryos
35 (Le Bourhis et al., 2010).

Activation of reconstructed NT embryos was initiated with three direct current pulses of 1.0 - 1.5kV/cm for 80µs each, in buffer containing 0.1mM calcium, ~50-52h after the start of IVM.

Within 5 min of electrical stimulation, reconstructed embryos were transferred to culture medium (e.g. ESOF) supplemented with 2mM 6-dimethylaminopurine + 5µg/mL cytochalasin B and cultured individually in 5µL drops for 3h at 38.5°C in 5% CO₂. Afterwards, embryos were washed thoroughly in HEPES SOF before in vitro culture for 6-7 days. Approximately 10-12 zona-free embryos were group-cultured in microwell depressions made in 20µL drops of ESOF (or alternative medium) for four days in a low oxygen atmosphere (5:7:88, CO₂:O₂:N₂) in a modular incubator at 38.5°C. Media formulation was changed to LSOF (or alternative medium) from Day 4 to 7. On Day 6, any late morula and early blastocyst-stage embryos were transferred individually to 5µL drops of LSOF (+ 5% FCS if required) under oil and culture continued. Embryo development was assessed on Day 6 or 7 and the numbers of nuclei in resulting blastocysts determined in selected experiments.

9.2 Results

The FLI media system resulted in significantly more usable oocytes after IVM compared to IVM1/2. However, development of SCNT embryos, but not parthenogenetically activated oocytes (PG), was three-fold greater with the biphasic IVM system (Table 10). Zona-free embryo development of SCNT embryos was substantially less compared to the PG controls.

Table 10. Comparison of two IVM systems for the development of porcine embryos derived from either somatic cell nuclear transfer (SCNT) or parthenogenetic oocyte activation (PG). nIVM=number of oocytes matured in vitro. 1PB=first polar body in oocytes. nIVC=number of embryos cultured in vitro. ^{ab}P<0.001. ^{cd}P<0.05

IVM system	nIVM	Round cytoplasm + 1PB	Embryo type	Fusion	nIVC	Cleavage	Blastocyst Development Day 7
IVM1/2	1231	52% ^a	SCNT	77%	199	93%	6% ^c
			PG	n/a	247	94%	14%
FLI	1271	63% ^b	SCNT	73%	288	92%	2% ^d
			PG	n/a	300	96%	17%

A preliminary experiment examining the addition of 5µM of the proteasome inhibitor MG132 between oocyte selection and activation of NT reconstructs was unable to show any benefit in terms of in vitro development following SCNT or PG (Table 11).

Table 11. The addition of the proteasome inhibitor MG132 prior to activation did not increase porcine embryo development following somatic cell nuclear transfer (SCNT) or parthenogenetic oocyte activation (PG). nIVC=number of embryos cultured in vitro. ^{ab} $P < 0.05$.

Media system	Embryo type	Fusion	nIVC	Cleavage	Blastocyst Development Day 7
Control	SCNT	37%	57	93%	13% ^a
	PG	n/a	96	100%	23%
MG132	SCNT	49%	81	99%	11% ^b
	PG	n/a	102	100%	23%

5

Focus was on developing a zona-free SCNT procedure in pig as experience in other species has demonstrated advantages in simplifying embryo reconstruction and increasing laboratory throughput. However, rates of embryo development are still lower in our current zona-free in vitro culture system in pig compared to more conventional zona-intact procedures, as demonstrated with parthenogenetically activated porcine oocytes (Table 12).

10

Table 12. Greater in vitro embryo development with zona-intact compared to zona-free parthenogenetically activated oocytes (PG). nIVC=number embryos cultured in vitro. ^{ab} $P < 0.0001$.

PGs	nIVC	Cleavage	Blastocyst Development Day 7
Zona-intact	344	96%	20% ^a
Zona-free	203	94%	7% ^b

15

9.3 Conclusion

It is known that the development of zona-free porcine SCNT embryos to the blastocyst-stage on days 6-7 of *in vitro* culture increased from 5% to 15% by selecting better quality oocytes after treatment with sucrose (Dang-Nguyen et al., 2018). Despite using this oocyte selection procedure here, development results following zona-free SCNT were generally low and variable, especially compared to parthenogenetically activated oocyte controls. The *in vitro* maturation of oocytes in a biphasic system over a 44-hour period, incorporating the use of dbcAMP, increased the

20

rates of blastocyst development of SCNT embryos compared to the FLI oocyte maturation system (6% vs. 2%).

Calcium-free cell fusion buffer was used to prevent premature oocyte activation which is an obstacle to reprogramming a differentiated nucleus following SCNT. Preliminary investigations with MG132 addition to maintain maturation promoting factor-cyclin B complex, to facilitate epigenetic reprogramming of donor nuclei and improve SCNT embryo development, showed no benefit at the concentration and time period examined here.

A zona-free SCNT protocol in pig was initially favoured as in other species this is technically easier and results in greater throughput compared to more conventional zona-intact methods. However, unlike the situation in cattle, the present zona-free embryo culture system in pig still compromises *in vitro* developmental potential compared to a zona-intact embryo culture system. Thus, preliminary experiments have been carried out producing zona-intact SCNT pig blastocysts utilizing electrode needles to improve cell fusion efficiencies. To improve the success of *in vivo* development of porcine SCNT embryos in future work, the current recommendation is to transfer reconstructed 1-cell embryos to the oviducts of recipient gilts rather than *in vitro* blastocysts transferred to the uteri. To manage the logistics of embryo transfer this may require embryo vitrification.

The present inventors contemplate using the blastocysts produced by the methods described herein, having one or more xenoantigen knockouts, to be implanted into female PERV-C and PERV-A/C negative foundation pigs selected by the CGS method of the invention, to produce a novel cloned PERV-C, PERV-A/C and xenoantigen negative pig strain (designated NZeno-1) as donors for xenotransplantation.

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