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Substituição gênica ortotópica de porco para humano baseada em CRISPR/Cas9 e recombinases para xenotransplante

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Programa de Ciências em Gastroenterologia

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Coorientador: Prof. Dr. Alfred Joseph Tector III

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RAFAEL MIYASHIRO NUNES DOS SANTOS

CRISPR/Cas9 and recombinase based pig-to-human orthotopic gene exchange for xenotransplantation

Thesis presented to the Faculty of Medicine of the University of São Paulo to obtain the degree of Doctor of Science

Program of Science in Gastroenterology

Advisor: Prof. Dr. Luiz Augusto Carneiro D'Albuquerque

Co-Advisor: Prof. Dr. Alfred Joseph Tector III

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2017

Dedication

To my parents, Jorge and Amélia, for providing me the necessary blocks for building a dream and pursuit it, all along my life.

To my fiancé Desirée, for giving me unconditional support when I take a leap of faith, assisting me in my dreams pursuit, even if “occasionally” it doesn’t seem rational. While we were separated by the work, we were always united by love.

To Rodrigo, my brother, and Carla, my sister-in-law, for their friendship.

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The completion of this thesis benefits from the help from my co-mentor - Prof. Dr. Alfred Joseph Tector III, who supported the research even when there was no light in the end of the tunnel.

Dr. Matthew Tector provided much needed guidance throughout the thesis, bringing light to my questions, even if in times this was by encouraging me to re-think from the beginning.

If the completion of this thesis was granted by Dr. Tector and Dr. Matt, I need to state the importance of my mentor, Prof. Dr. Luiz Carneiro D'Albuquerque, who guided my way from the residency, through the Resident Chief position in his service, and finally through the beginning of my research in USA.

Furthermore, I would like to show my gratitude to Prof. Dr. Flávio Henrique Ferreira Galvão, who was my first mentor during medical school, inspiring me into go further into research to become a better physician.

Epigraph

“ We cannot solve our problems with the same thinking we used when we created them”

Albert Einstein

SUMMARY

ABBREVIATION LIST

APC	Activated protein C
AVR	Acute vascular rejection
B2M	Beta-2-microglobulin
B4GalNT2	Beta-1,4-N-acetyl-galactosaminyl transferase 2
Bp	Base pairs
CGT	Clonal gene transplant
CMAH	Cytidine monophospho-N-acetylneuraminic acid hydroxylase
CNV	Copy number variation
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CDS	Coding sequence
CT	Cycle threshold
CVF	Cobra venom factor
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DR	Direct repeat
DSB	Double strand break
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
gDNA	Genomic DNA

gRNA	Guide RNA
GGTA1	Alpha-1,3-galactosystransferase
HA	Homology arm
HAEC	Human aortic endothelial cell
HAR	Hyperacute rejection
HLA	Human leukocyte antigen
HR	Homologous recombination
hTHBD	Human thrombomodulin
Hygro	Hygromycin
KO	Knockout
µg	Microgram
ml	Milliliter
MMF	Mycophenolate mofetil
ms	Milliseconds
ng	Nano gram
NHEJ	Non-homologous end joining
PAEC	Pig aortic endothelial cell
PAM	Protospacer adjacent motif
PBMC	Peripheral blood mononuclear cell
PDL	Population doubling level
PDT	Population doubling time
Pre-crRNA	Precursor CRISPR RNA
Puro	Puromycin

RNA	Ribonucleic acid
ROI	Region of interest
RVD	Repeat-variable di-residue
SCNT	Somatic cell nuclear transfer
SLA	Swine leukocyte antigen
TALE	Transcription activator-like effector
TALEN	Transcription activator-like effector nuclease
THBD	Thrombomodulin
TPAEC	Transgenic pig aortic endothelial cell
tracrRNA	Trans-activating CRISPR RNA
UTR	Untranslated region
ZFN	Zinc finger nuclease

FIGURE LIST

TABLE LIST

RESUMO

Santos RMN. Substituição gênica ortotópica de porco para humano baseada em CRISPR/Cas9 e recombinases para xenotransplante [Tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2017.

Modelos humanizados de porco são muito importantes para pesquisa biomédica e desenvolvimento de novas drogas e tratamentos. Além de ser um melhor modelo para doenças humanas do que animais de menor porte devido sua maior semelhança fisiológica, anatômica, de metabolismo e tempo de vida, o modelo suíno ainda permite suprimento ilimitado de órgãos para transplante. Apesar dessas vantagens, a expressão gênica inconsistente de animais transgênicos tornam a criação e avaliação desses animais muito dispendiosas, imprevisível e não permite a comparação de resultados de animais diferentes de maneira apropriada. Nesse estudo descrevemos uma nova técnica utilizando o promoter endógeno para a geração de um protocolo de substituição de genes com padrão clonal (transplante clonal de genes) sem clonagem de células, preservando a expressão genética e sua regulação intactas. Esse protocolo é reprodutível e pode ser aplicado para mais de um alvo genético, permitindo geração rápida de linhas transgênicas de animais (14-20 dias) com potencial de se tornar o novo padrão para geração de animais transgênicos de grande porte.

Palavras-chave: Transplante Heterólogo; Trombomodulina; Sistemas CRISPR-Cas; Linhagem Celular; Técnicas de Transferência de Genes; Transfecção; Suínos.

ABSTRACT

Santos RMN. CRISPR/Cas9 and recombinase based pig-to-human orthotopic gene exchange for xenotransplantation [Thesis]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2017.

Humanized pig models are very important for biomedical research, and drugs and treatment development. Not only it is a better model for diseases than smaller animals because of its closer physiology, anatomy, metabolism and life span, it also may provide unlimited organs for transplantation. In spite of all this advantages, inconsistent gene expression in transgenic animals make its generation and evaluation expensive, unpredictable and do not allow proper outcome comparison between different animals. In this report we describe a reproducible technique utilizing the endogenous promoter for generation of a clonal pattern gene replacement protocol (clonal gene transplant) without cell cloning, maintaining the normal gene expression and its regulation. This protocol is reproducible and applicable to more than one gene target, allowing fast generation of transgenic animals cell lines (as low as 14-20 days) and could become the new standard for transgenic large animal generation.

Keywords: Transplantation, Heterologous; Thrombomodulin; CRISPR-Cas systems; Cell Line; Gene Transfer Techniques; Transfection, Swine.

1. INTRODUCTION

1. Introduction

Pigs are considered better biomedical research models than smaller animals because of closer similarity with human size, life span, metabolism, physiology and anatomy (1-5).

Genetically engineered pigs are used vastly in several medical fields (6), including models for cystic fibrosis (5, 7, 8), diabetes models (9, 10), immunology (11-15), neurodegenerative diseases (2, 16-18), cancer (19-21), ophthalmology (22, 23) and cardiovascular diseases (24).

Also, in addition to that, its breeding pattern and gestation time associated with relatively fast time for archiving human adult size organs makes pigs the ideal animal for xenotransplantation (25).

Non-human primates were the first animals used on modern xenotransplant. In the 1960s, human-to-human kidney transplantation was starting, however, organs from deceased donors were extremely rare. Since chronic dialysis was not a possibility at that time, Keith Reemtsma started a non-human primate-to-human kidney xenotransplant program as the only solution for chronic renal failure. He performed 13 cases (using both kidneys from a single chimpanzee in each case) with survival up to 9 months with restricted immunosuppressive agents (26).

However, clinical human-to-human transplant success and better supportive care soon made xenotransplant interest to fall.

With the growing demands for organs, not fulfilled by the donor supply in spite of maneuvers to increase the pool of organs (marginal transplant (27), living

donor (28, 29), and donation after cardiac death (30, 31)), the enthusiasm in the chance of using cells or even organs from animals has reemerged again.

While non-human primates are genetically closer to human, there are many limitations to use them as adequate source of organs for transplantation, such as ethical concerns and difficulty in breeding, including other factors summarized on Table 1 (32, 33). Pig was then chosen to be the animal for xenotransplantation, because it has an unlimited availability, rapid growth to human size, and lower risk for zoonosis.

Table 1: Comparison between pig and baboon as organs source for xenotransplantation

Characteristic	Pig	Baboon
Anatomically similar	++	+++
Physiologically similar	++	+++
Immune system similarity	+	+++
Time to be mature to reproduce	4 - 8 months	3 - 5 years
Number of offspring	5 – 12	1 - 2
Length of gestation	4 months	6 months
Time to reach size for organ donation	6 months	9 years
Risk of xenozoonosis	Low	High
Availability	High	Low
Cost of maintenance	Low	High

Adapted from Cooper (2012) (33).

However, because of greater evolution distance between human and pigs, immunological incompatibility became significant limitation for xenotransplantation. The first barrier introduced was hyperacute rejection (HAR), caused by human preformed antibodies against pig antigens (34). These antibodies bind to pig endothelial cell surface, causing complement activation and cell damage with organ death within minutes to hours after a discordant xenotransplant (34, 35).

HAR was demonstrated in other discordant xenotransplant models as well. Galvão et al (2008) described a hyperacute rejection in discordant models of multivisceral transplantation, using canine and swine, allocated in four groups of experiments, canine donor and swine recipient, swine donor and canine recipients and two control groups comprising canine to canine and swine to swine transplantation. In the divergent models of transplantation, hyperacute rejection signs were apparent 15 minutes after reperfusion and critically damaged to all organs. Also, in divergent transplant, urine output decreased constantly and stopped within 15 minutes after reperfusion. However, in pig-to-pig and dog-to-dog multivisceral allotransplantation, normal aspect of transplanted organs and normal urine output was observed up to the end point. Pathological examination showed moderate to severe degree of hyperacute rejection in the majority of transplanted organs in discordant models (36).

For a prolonged time, HAR has been the major barrier to successful xenotransplantation. To overcome this issue, management of complement activation either by human complement regulators gene expression on xenograft or infusion of inhibitors of complement were attempted (37-39).

However, little success with these approaches was obtained. More recently, elimination of the xenoantigen by knockout of the gene responsible for its expression by molecular biology approaches was undertaken, so human pre-formed antibodies would not bind to pig cells, eliminating HAR (40).

Genome editing for knockout depends on DNA repair system after double strand breaks (DSBs) happen. There are two main kinds of DNA double strand breaks repair mechanisms in eukaryotic cells: homologous recombination (HR) (41) and non-homologous end-joining (NHEJ) (42).

HR uses similar DNA as a guide to repair the DSBs, and the outcome of this kind of repair is predicted (exact as the guide). For this reason, repairs through HR can be used to add exogenous DNA into the break point in the genome (43).

NHEJ ligates the disrupted segment and is associated by gain or loss of some nucleotides, therefore the consequence of NHEJ is variable: nucleotide deletions, insertions, or substitutions can happen at this location. This can eventually cause a frame-shift during translation, generating a non-functional truncated protein.

The initial method for generation of knockout pigs was by homologous recombination (HR).

Gene knockout generated through HR requires the creation of DNA insert with long sequences of identity with the gene to be disrupted (homology arms) in both ends, associated with an antibiotic selection marker to select cells with integrated insert. After introducing the DNA inside the nucleus, usually from a fibroblast, cells are selected with antibiotic in high doses to isolate the ones where

the insert DNA was integrated. This process generates several cell clones (individual cell populations originated from a single cell) in the dish, and expansion and genotyping of each is required.

The overall chance of monoallelic disruption in this model is around 0.01%, with a 0% chance of biallelic events (44). For this reason, hundreds of clones need to be individually evaluated to be used to generate a monoallelic-disrupted pig with somatic cell nuclear transfer (SCNT). SCNT is a technique that consists of taking an enucleated oocyte (egg cell) from a donor and implanting a nucleus from a somatic cell, generating a cloned organism from the last (45).

Once this animal reaches sexual maturity, it is bred with a wild type pig, generating several pigs (wild type and single allele disrupted) that eventually will need to be bred to generate complete gene disruption.

As we can see, generating a knockout animal was a very expensive (as much as US$300,000 per animal (46)) and time consuming process that would take at least 3 generations of pigs, that could take years for a single gene knockout.

This scenario was completely changed once the nucleases were developed. These new genetic tools have a DNA binding domain that identifies a specific DNA sequence and are associated with nuclease protein, being able to cause a targeted double strand break.

The association of nuclease-based genome editing with cloning techniques such as somatic cell nuclear transfer (SCNT) allowed transformation of knockout cell lines into cloned pigs (47).

Zinc finger nuclease (ZFN) was the first described. Zinc fingers can recognize different sets of nucleotide triplets major groove of DNA. Synthetic linker permitted the combination of multiple zinc fingers in an array, allowing them to recognize DNA sequences of 9-18 base pairs (bp) in length (48).

Since an 18 bp sequence of DNA can result in specificity within 68 billion bp of DNA, this approach granted targeted modifications for the first time (49, 50)

Different zinc-finger domains have been created to identify almost all of the 64 existing nucleotide triplets, allowing combination of these with each other to target any genomic sequence possible in an specific manner (49, 51-54).

Although great progress was obtained, ZFNs was not widely utilized for a few reasons.

One of those was that the specificity of the individual zinc finger array depended on context effects and sequences. In association to that, not all nucleotide triplets had a specific zinc finger discovered. Also, the construction of highly selective ZFN proteins is expensive, difficult, and time consuming (55-58).

The second nuclease described was transcription activator-like effector nucleases (TALENs). TALEs (transcription activator-like effector) were found in the plant pathogenic bacteria genus *Xanthomonas*, and contain DNA-binging domains. Each of these domains is comprised of a sequence of 33-35 amino-acid repeat that identifies a specific base pair. Its specificity is conferred by two hyper variable amino acids in those domains, known as the repeat-variable di-residues (RVDs) (59, 60). Similar to zinc fingers, several TALE repeats are combined together to recognize long DNA sequences.

While the single base recognition of TALE guaranties a more flexible design than the triplet of zinc-finger proteins, cloning and assembling repetitive sequences of TALENS is technically difficult (61-64).

Those nucleases started a great revolution, allowing easier targeted genome modifications, however, both of them had some negative aspects that impaired the scalability of the technique (65).

After that, Clustered regularly interspaced short palindromic repeats (CRISPR) /Cas9 system was described and filled the gap of affordability and scalability that the previous could not. CRISPRs were discovered in *E. coli* genome more than 30 years ago (66), being responsible for the acquired immune system, targeting infecting exogenous DNA via RNA-guided DNA break (67).

Type II CRISPR locus consists of four genes, comprising of the cas9 nuclease, in association to two noncoding CRISPR RNAs (crRNAs): trans-activating crRNA (tracrRNA) and precursor crRNA (pre-crRNA) array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (DRs). These noncoding RNA are matured by the RNAse 3, providing the targets (spacers) for the Cas9 nuclease (68).

Comparing to TALEN, CRISPR is faster and cheaper to assemble since the designed target component is RNA instead of protein (1-3 days against 5-7 days), being able to target multiple regions at once. It also presents some limitations such as more off-target effects (cleavage of DNA in the wrong sequence) and needs to target a region in DNA with PAM (protospacer adjacent motif) sequence (NGG) (43).

All these nucleases allowed generation of multiple knockout pigs with relative ease, generating animals with the same phenotype, including multiple gene knockouts in the same gestation (69), solving the problem of HAR in most cases by elimination of the xenoantigens responsible for it (70, 71).

The most well characterized xenoantigens on pig cells that could be disrupted to correct HAR are the carbohydrates galactose alpha 1-3 galactose (alphaGal) and N-glycolylneuraminic acid (Neu5Gc) (72, 73). With Alpha-1,3-galactosystransferase (GGTA1) and Cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH) gene knockout respectively, these carbohydrates are not present in the pig cell membrane anymore. Disruption of these genes let to less human antibody binding to pig than to chimpanzee's peripheral blood mononuclear cells (PBMCs) (74). This was a great leap forward, since chimpanzee kidneys were transplanted in the past without evidence of HAR, resulting in survival up to 9 months (26).

More recently, additional knockout of beta-1,4-N-acetyl-galactosaminyl transferase 2 (B4GalNT2) gene in pig reduced even more the genetic incompatibility between pigs and humans. The scarcity of antibody binding to PBMCs from GGTA1/CMAH/B4GalNT2 KO pig suggests that HAR could be avoided in most pig-to- human xenotransplants (75).

Now, after reducing or maybe eliminating HAR from the picture, a new barrier limiting graft survival became visible: acute vascular rejection (AVR) (76).

Days to weeks after xenotransplant, antibodies against the graft emerge, leading to endothelial cell activation and injury, causing vascular integrity disruption

and inducing a procoagulant status (76-78). Microthrombi formation and coagulation factors consumption leading to organ loss are presented in this syndrome (77-79), that can range from mild to disseminated intravascular coagulation (DIC) (80).

At first, the mechanisms for this coagulopathy were not clear and treatment was immunologically based, trying to eradicate elicited antibodies. This treatments included intravenous immunoglobulin, plasmapheresis, and immunoadsorption, however, they failed in preventing the coagulation issue in kidney xenotransplantation (80).

In xenotransplantation, the coagulation imbalance lean on endothelial cell activation and damage, in addition to incompatibilities of factors regulating coagulation.

One of the most important incompatibilities is the thrombomodulin/protein C pathway (81).

Thrombomodulin (THBD) is a 557 amino acid residues long type I transmembrane glycoprotein, containing five domains: the N-terminal lectin-like domain, six isolated epidermal growth factor (EGF)-like domains, serine/threonine (S/T)-rich domain containing chondroitin sulfate glycosaminoglycan, transmembrane domain, and the cytoplasmic tail (82, 83).

In the lack of THBD, thrombin activates fibrinogen to fibrin. Thrombin also activates additional coagulation factors (factors V, VIII, XI and XIII), and platelets. Nevetherless, in the presence of THBD, this binds to thrombin, preventing thrombin activation of these pro-coagulant molecules. In addition to that, THBD-Thrombin

complexes activate protein C, which inactivates coagulation factors Va and VIIIa, thus reducing extra thrombin formation. Consequently, THBD has a dual effect as an anticoagulant effect: inhibition of thrombin-mediated activation of coagulation factors and activation of protein C, an anticoagulant agent (82).

However, in spite of the fact that both pig and human thrombomodulin can bind human protein C, only the latter complex can activate human protein C (84). This explains at least part of the procoagulant events after pig-to-human xenotransplant, which could be ultimately correct by expression of human thrombomodulin in pig endothelial cells.

In fact, transgenic thrombomodulin pig increased survival results in both kidney and cardiac pig-to-non-human primate xenotransplant (85-87).

In cardiac xenotransplant, 3 groups of pig-to non-human primate xenotransplant were performed with GGTA1 KO in association with human CD46 (complement regulatory protein) transgenic pigs. In the first 2 groups, the only difference was the immunosuppressant protocol, and the last, human thrombomodulin was associated with the other previous 2 modifications.

The first group (GGTA1 KO + human CD46) was submitted to an immunosuppression protocol with induction therapy with Anti-thymocyte globulin (antibody against human T cells) + Rituximab (monoclonal antibody against CD20, which is primarily found on the surface of immune system B cells) + cobra venom factor (CVF). Maintenance therapy included CVF, anti-CD154, mycophenolate mofetil (MMF), and steroid tapper. Anticoagulation was performed with aspirin and heparin. With this protocol, mean survival was 71 (36-236) days (88), which was

similar to GGTA1 KO pigs alone (mean survival of 78 days, ranging from 16-179) (89).

When anti-CD40 (20mg/kg) was used instead of anti-CD154 in GGTA1 KO + CD46 pigs, the mean survival increased to 84 days (ranging from 30 to 149 days) (90).

Adding human thrombomodulin with the same drug therapy (with higher dose of anti-CD40 from 20mg/kg to 40mg/kg), mean survival went up to 200 days, ranging from 146 to 550 days (91).

The same good results were shown in kidney pig to non-human primate model with the addition of human thrombomodulin to the graft. Using a GGTA1 KO pig associated with human CD46 + CD55 + thrombomodulin + EPCR + CD39 resulted in a survival of 136 days (85), overcoming the previous longest survival of 90 days (92).

These results show the benefits of adding human thrombomodulin to the xenograft, supporting incorporation of human thrombomodulin in the already characterized knockout pigs for further testing under same drug regimen.

Unfortunately, gain of function mutation protocols did not evolve as much as loss of function mutations (knockouts).

This happens because for any loss of function mutation, any change to a gene that causes frame shift in the nucleotides will generate the same end result, a misread of the nucleotide generating an truncated and altered protein. The same is not true for gain of function mutations.

For gain of function mutations (transgenic animals), the phenotype of animal will vary according to promoter used, copy number of DNA insert in the genome, position of integration, generating a different animal every time a cell line is cloned to a animal.

The bottleneck for generation of transgenic pigs is the absence of embryonic stem cells (93, 94), which have long life span, allowing more complex procedures for target mutations, including screening of correct integrated cells. Because of that, most transgenic animal models are based on random integration of DNA being expressed by exogenous promoters.

Random integrated transgenic animals have unpredictable gene expression pattern with patchy expression based on integration location and copy number, and are susceptible to gene silencing over time (95, 96).

Screening of several founder pigs is the single option to find and establish animals with desired expression profile (97), however, the high cost of animal housing and screening limits a broader application of pig genetic engineering (97).

For those reasons, a more reproducible, consistent, faster and predictable model for generation of transgenic animals is necessary.

In our vision, an ideal transgenic animal for physiologic studies and xenotransplantation should present some characteristics.

First, the transgene should be expressed by the endogenous promoter of the host animal, so the expression parameters are the same and no methylation or copy number would affect its expression.

It also should have a bi-allelic substitution of the gene to be expressed; otherwise, the expression would be lower than it should be.

In addition to that, if a gene replacement is required, the host gene should be disrupted, otherwise the transgene could have its function limited by receptor competition with wild type gene.

And last, the final cell line ideally would not have any selection marker that could change the animal characteristics and impair future experiments in the cell line.

Some mice in locus knockin/knockout ideal models utilizing the endogenous promoter were developed before (98), but those models would not be applicable to pigs for a few reasons. Stem cell lines with unlimited number of doublings were used for the extensive screening process, and those are not available for pig (99, 100). In addition to that, several generations of mice were used to generate these animals, that started with single allele insertion with expression of selection markers, and crossbreeding with siblings and CRE-expressing mice were necessary for double allele insertion without selection markers (98).

Because somatic cells for nucleus transfer have a limited life span and small number of passages before senescence, optimized molecular biology technics should be employed to accomplish the ideal goals.

Cloning cells is also not desirable because of the length of the process, especially because most of the gene substitution protocols require at least two-step procedures that are usually not very efficient and requiring a time span greater than what a primary cell for nuclear transfer can withstand.

As described before, there is a lack of protocols for generation of pig somatic transgenic cell lines with a reliable and predictable expression pattern, not susceptible to positional, copy number and methylation effects. This lack of ideal models makes generation of suboptimal animals very expensive, not being ideal for comparison of different treatments, since each animal will present a different expression profile.

For this reason, we propose a new model for generation of transgenic animals, trying to achieve our vision of ideal animal described before, using a combination of CRISPR/cas 9 nuclease and PhiC31 recombinase for target gene insertion. In addition to that, since utilization of endogenous promoter for transgene expression is a key factor for the ideal animal generation, a new cell line with endothelial pattern of expression needs to be evaluated for the protocol, since the most commonly utilized fibroblast do not express endothelial specific genes.

2. OBJECTIVES

2. Objectives

- Test new cell line (pig aortic endothelial cell line) for generation of pig by somatic cell nuclear transfer
    - Test transfection efficiency
    - Test life span
    - Test ability to endure complex molecular biology protocols for generation of transgenic cell lines

- Develop new molecular biology model for gene replacement for generation of transgenic cell lines that will be suitable for generation of transgenic animals with the following characteristics
    - Expression pattern at least as much as human cells
    - Expression of transgene from pig endogenous promoter to avoid random integration expression and silencing due to position of insert
    - Maintained promoter regulation of gene expression
    - Reproducible
    - Generation of gene swap (pig to human) in primary cell life span

3.METHODS

3. Methods

Gene swap was achieved by removing the complete coding sequence from pig gene with dual CRISPR-Cas9, integrating an insert with a promoterless selection marker through homologous recombination at the same time in this location. This insert had the antibiotic selection marker surrounded by modified PhiC31 recombinase sequences (AttP and AttB) and was followed by a promoterless human version of the pig gene to be replaced (Fig. 1).

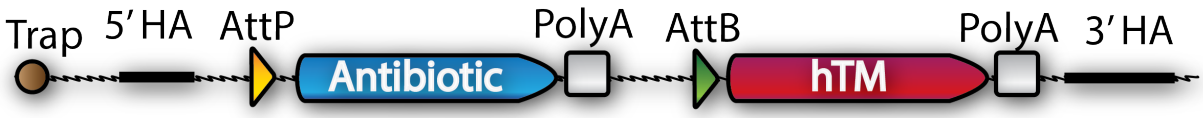


Figure 1 – Human thrombomodulin insert design
Trap: In-Frame Stop/off-frame Start codon trap.
5'-HA and 3'-HA: Homology arms identical to sequences in the targeted genomic locus.
AttP and AttB: modified PhiC31 recombinase sequences.
hTM: human thrombomodulin open reading frame.
Poly A: sequences driving the attachment of poly A tails to transcripts.

After antibiotic selection, PhiC31 recombinase expressing plasmid transfection allowed removal of selection marker and start of transgene expression (Fig. 2)

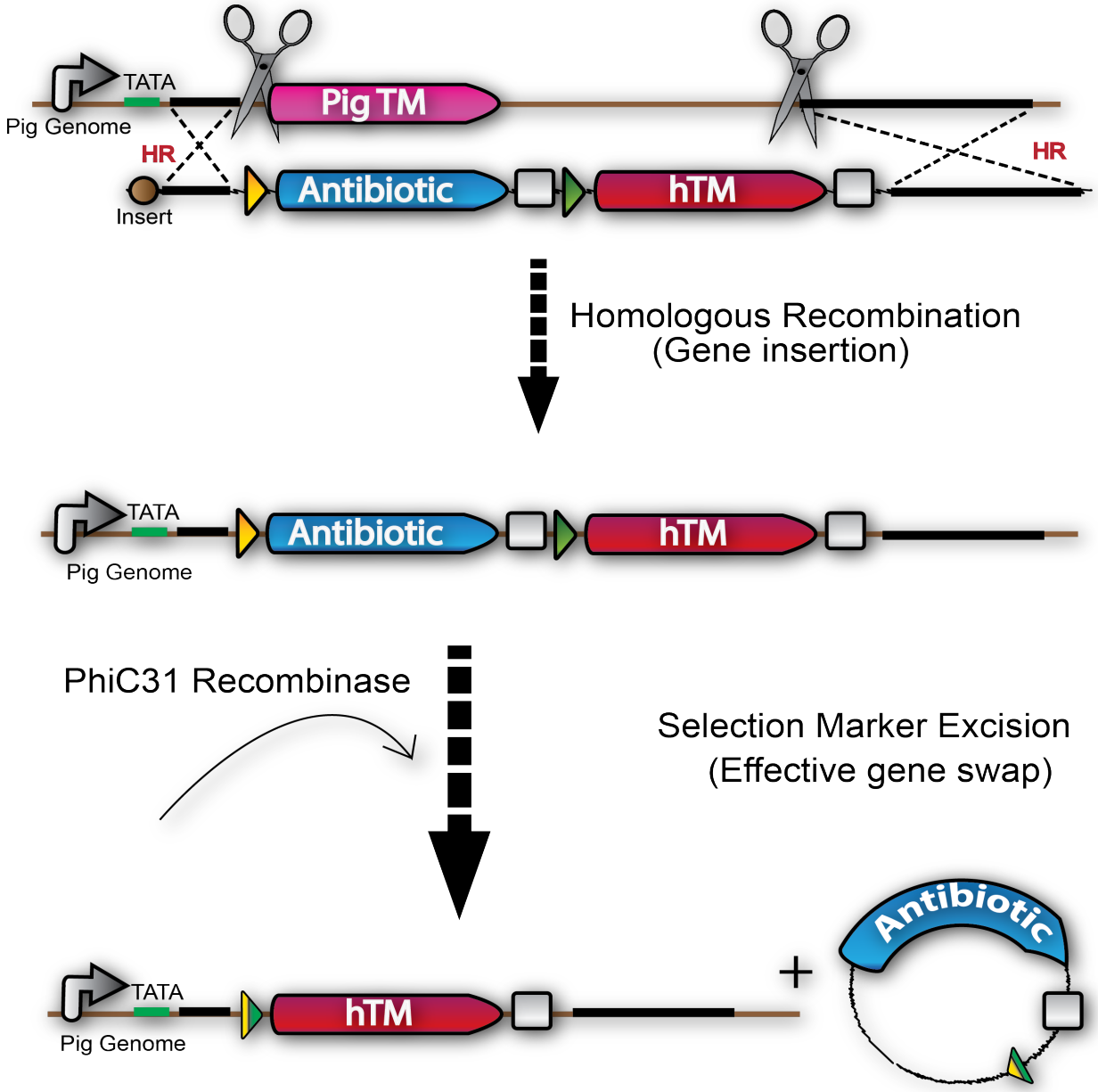


Figure 2- Gene swap experiment design
Cas9 cutting is directed to two sites flanking the pig thrombomodulin gene by two gRNA to increase the frequency of homologous recombination. Only correct insertion at the targeted locus allows antibiotic resistance gene expression. Cells that survive selection are transfected with the PhiC31 recombinase to excise the selection marker and initiate human thrombomodulin expression by the pig endogenous promoter.

The final pig to human gene replacement experiment was preceded by preliminary experiments to test feasibility and standardization of the most efficient protocols. These pre-experiments were divided in: Cell line experiments and integration validation experiments. Once these pre-experiments were performed, transgene expression and characterization of transgene profile experiments were performed.

Cell line experiments were performed for isolation, characterization, and optimization of transfection of the aortic endothelial cell line.

Integration validation experiments were performed to test the best protocols for correct bi-allelic integration of DNA into the cells.

Transgene expression experiments were performed after both of the former, to allow correct gene replacement (pig to human thrombomodulin).

Characterization of transgene expression profile experiments were performed to evaluate reproducibility of the model, correct integration copy number and overall copy number assays, pig knockout ratio, functional assay and persistence of promoter regulation.

3.1 Cell line experiments

3.1.1 Porcine aortic cell isolation and culture

All experiments were performed at IUPUI Xenotransplant lab (Science Building MS2009 & BS005). All animals used in this study were approved by the Institutional biosafety and Institutional Animal Care and Use Committee of Indiana University School of Medicine (Attachment 1). Porcine primary aortic endothelial cells (PAEC) were isolated from pigs procured during general anesthesia. The posterior lumbar arteries were ligated and the aortic lumen was filled with 0.025% Clostridium histolyticum's type IV collagenase (Sigma, St. Louis, MO), placing vascular clamps on proximal and distal ends. This sample was incubated at 37º C for 45 min. Enzyme activity was quenched by addition of 1/10 volume FBS. After enzyme inactivation, the sample was centrifuged at 400g for 5 min. The cell pellet was resuspended in RPMI medium with 10% fetal bovine serum, 100µg/ml Cornig endothelial cell growth supplement (Fisher Health Care), 1% penicillin + streptomycin (Invitrogen) and 1% amphotericin (Fisher Scientific) and cultured in the same media.

3.1.2 Growth curve

Growth curve was calculated as previously described (101). Briefly, to calculate the population doubling level (PDL), 100,000 cells were cultured in

collagen-coated 6-well plates. Cells were harvested every 48h. Cells were counted using a hematocytometer, and 100,000 cells were plated in collagen-coated culture dishes.

The PDL per passage was determined using the equation, PD =log(A/B)/log 2, in which A is the number of collected cells and B is the number of plated cells. These

PD values were used to plot the accumulated PDL curve. Population doubling time (PDT) value was calculated based on the PDL value divide by 48 hours.

3.1.3 Transfection optimization

Standard manufacture protocol for transfection optimization on Neon transfection system (Life Technologies, Grans Island NY, USA) was used. 250.000 cells were transfected with 400ng of pEGFP-N1 Plasmid (Clontech) and cultured for 24 hours.

After this period, cells were harvested and transfection efficiency was measured based on fluorescence using a BD Accuri C6 flow machine (BD Biosciences, San Jose, CA, USA) and visual mortality rate based on cells attached/cells floating. The best parameters of transfection were matched with visual mortality rate and if small mortality rate was noted for that parameter, voltage was increased until most cells were killed. Fluorescence was measured 24h after transfection.

3.2 Integration validation experiments

3.2.1 Selection of 3' CRISPR

Pairs of gRNA were picked so the complete coding sequence of pig thrombomodulin could be removed. Crispr/Cas9 plasmid Px330 (65) from addgene (plasmid #42230) was utilized for the experiments.

For this experiment, only one available 5' CRISPR at ATG region was possible (AGGAGCAGAACGCGGAGCATGG). To select the most efficient combination of CRISPR pairs, several sequences for 3' CRISPR were tested.

For this, the sequence after pig thrombomodulin gene was analyzed for chance of off-target effects (http://crispr.mit.edu).

The sequences with less chance of off-target effects were selected and transfected with the 5' sequence (1µg of each) in $0.5x10^6$ PAEC. The selected gRNA for 3' untranslated region (UTR) were:

1: AACCTTCTAACCTAACCGGT**TGG Score 95**

2: TTCTAACCTAACCGGTTGGC**AGG Score 95**

3: TCTAACCTAACCGGTTGGCA**GGG Score 93**

4: TTTGCCCCTGCCAACCGGTT**AGG Score 91**

5: TGCCAACCGGTTAGGTTAGA**AGG Score 90**

After 48 hours of transfection, cells were harvested and genomic DNA was isolated using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich St. Louis, MO).

Primers before and after the target regions were used to amplify both the complete region and possible deleted region, and the bands were compared to select the most efficient combination of gRNA (Fig. 3).

Forward primer: CACCAGGCACTTCCTTCCTT

Reverse Primer: CCTGTGTGGCAACGGTCTAA

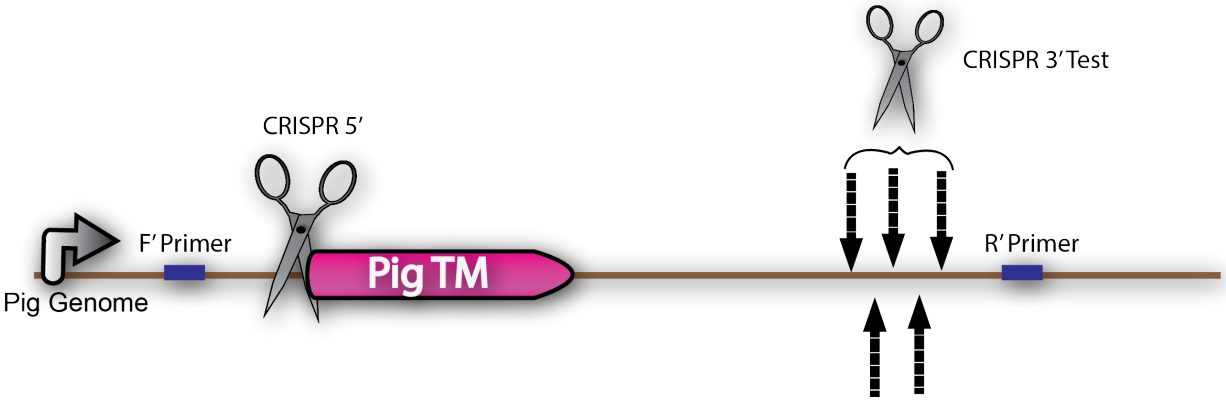


Figure 3 - CRISPR dual gRNA experiment design
Multiple gRNA tested for 3' UTR region of pig thrombomodulin in combination with the only available 5' target for complete coding sequence (CDS) removal.

All PCRs were performed using PWO SuperYield DNA Polymerase (Roche) and 50ng of gDNA as template.

Thermal cycling conditions were 94ºC x 2min (1 cycle), 94ºC x 15s, 54.9ºC x 30s and 72ºC x 3min 35s (40 cycles), 72ºCx 7min and 4ºC hold.

Expected size for unmodified region: 3574 bp.

Expected size for removed region: approximately 501 bp (Fig. 4).

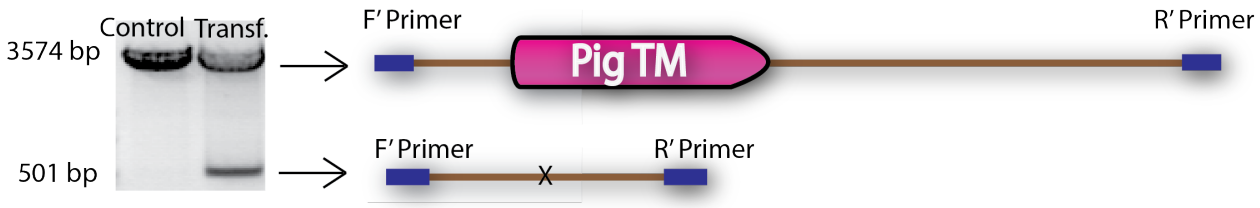


Figure 4 – Possible outcomes of CRISPR dual gRNA experiment design
Control: Untransfected cells presents only one band size after PCR amplification (3574).
Transf: Dual CRISPR transfected cells presents 2 bands. One of cells that were not affected by the nucleases (3574bp) and another smaller representing population cells that were affected by both CRISPR, resulting in deletion of the targeted segment (501 bp).

3.2.2 Size of 5' homology arm

For the selection of most efficient 5' homology arm for the experiment, two different sizes were compared. We utilized 60 bp and 2548 bp homology arms.

In order to compare the most efficient size of homology arms to get only corrected inserted DNA to be expressed, each size of homology arms were used to

create 2 different inserts, both promoterless expressing puromycin resistance gene linked with either GFP or mCherry by a optimized P2A sequence (Fig 5).

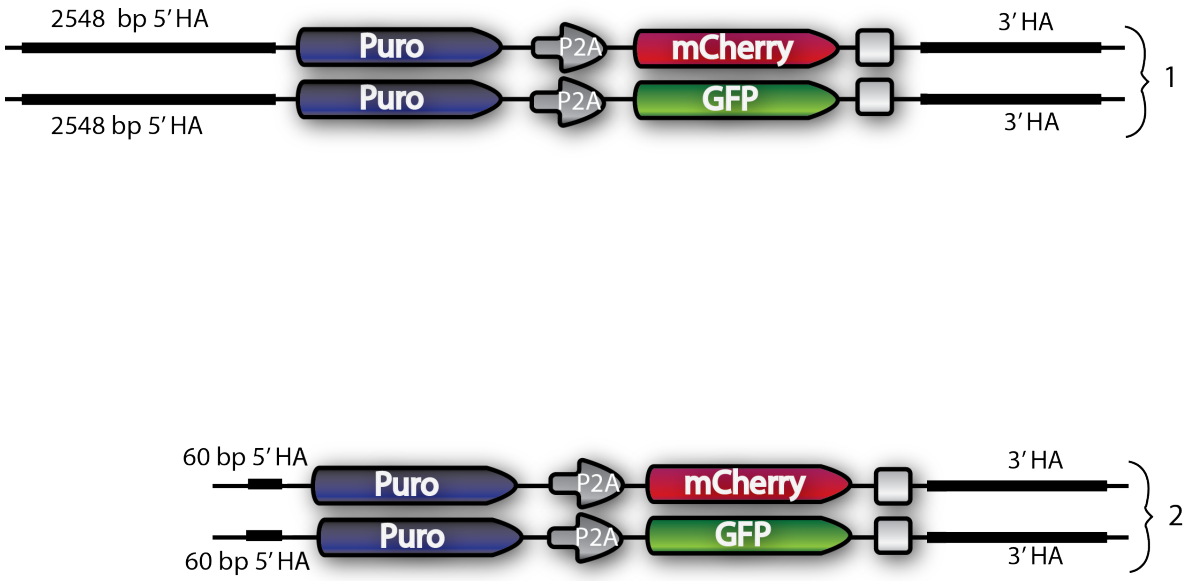


Figure 5 – Inserts for comparison of different 5' homology arm sizes
The only difference between Experiment 1 and experiment 2 inserts is the size of homology arm. Set 1 has a 2548 bp HA and set 2 has a 60bp HA. For each experiment two inserts were utilized. Both inserts with a promoterless puromycin resistance gene linked to a fluorescent protein marker gene through P2A sequence.

$1.x10^6$ PAEC were transfected with 2µg of each of the linearized inserts and 2.5µg of 5' and 3' CRISPR plasmids and selected with puromycin after 48h. After antibiotic selection, double positive cells (mCherry and GFP expressing cells) were sorted 3 times to ensure that all cells were expressing both inserts (two allele insertion) as shown in figure 6.

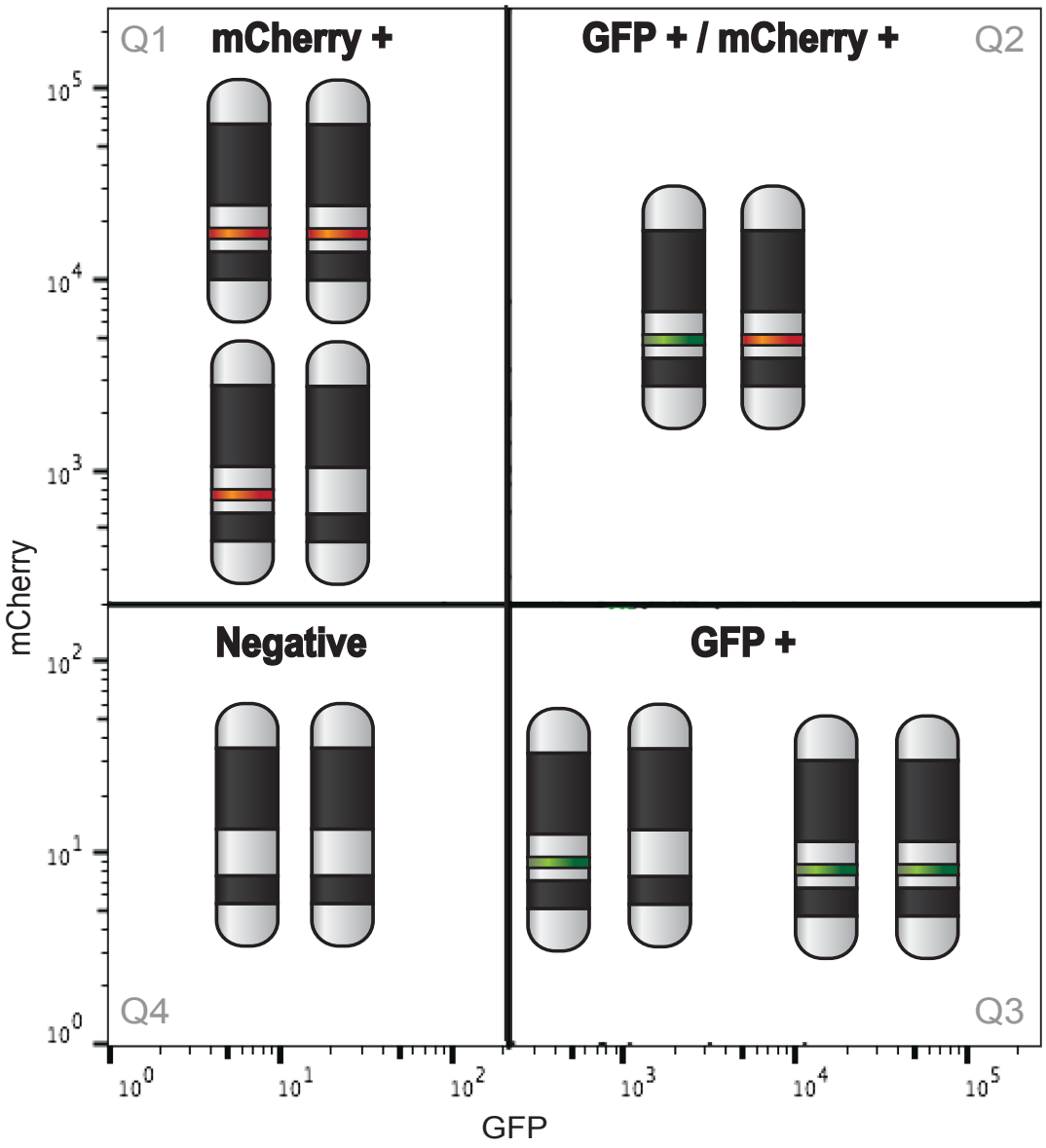


Figure 6 – Flow cytometry profile of possible correct insert integration
Representation of all possible correct integrations in thrombomodulin gene locus on pig chromosome 17 and its respective flow cytometry chart. Control cells have no integration of fluorescent protein gene (Q4). GFP positive cells (Q3) may present one or two alleles with the insert in the right location (indistinguishable by flow sort). mCherry positive cells (Q1) also present the same problem. Only simultaneous GFP and mCherry positive cells (Q2) are guaranteed to be double allelic insertion (excluding random integration gene expression).

After 100% of cells were double positive, genomic DNA was extracted and PCR amplification using primers to amplify from the pig promoter to the coding sequence of pig thrombomodulin were used in controls (non modified pig cells) and those cells. If correct integration of both inserts were made, no pig thrombomodulin should be detected in PCR amplification.

The same experiment was repeated with the same constructs but replacing the 2548 bp 5' Homology arm with a 60bp 5' homology arm and compared.

Primers for pig thrombomodulin amplification, from the promoter to the coding sequence were utilized for amplification of remaining pig thrombomodulin sequences:

5'- AAGCCTTCCTCCTTGACTC -3'

5'- TGTGGTTGTCCCCTGTAAC -3'

Thermal cycling conditions were 94ºC x 2min (1 cycle), 94ºC x 15s, 62.8ºC x 30s and 72ºC x 55s (40 cycles), 72ºCx 5 min and 4ºC hold.

Expected size: 889 bp.

3.2.3 Selection strategy of bi-allelic inserted cells

After determination of best 5' homology arm size, the best selection strategy for obtaining correct bi-allelic insertion was tested, utilizing the correct 5' Homology arm size.

For that, two different experiments were compared, each of those using 2 different inserts, which one for selecting correct insertion in each allele.

In the first, a single antibiotic (puromycin) was followed by P2A sequence to co-express either GFP or mCherry from the pig endogenous promoter (when correct inserted). The second experiment was similar to the first, however each insert expressed one different antibiotic selection marker (Puro→P2A→mCherry and Hygro→P2A→GFP), as shown in figure 7

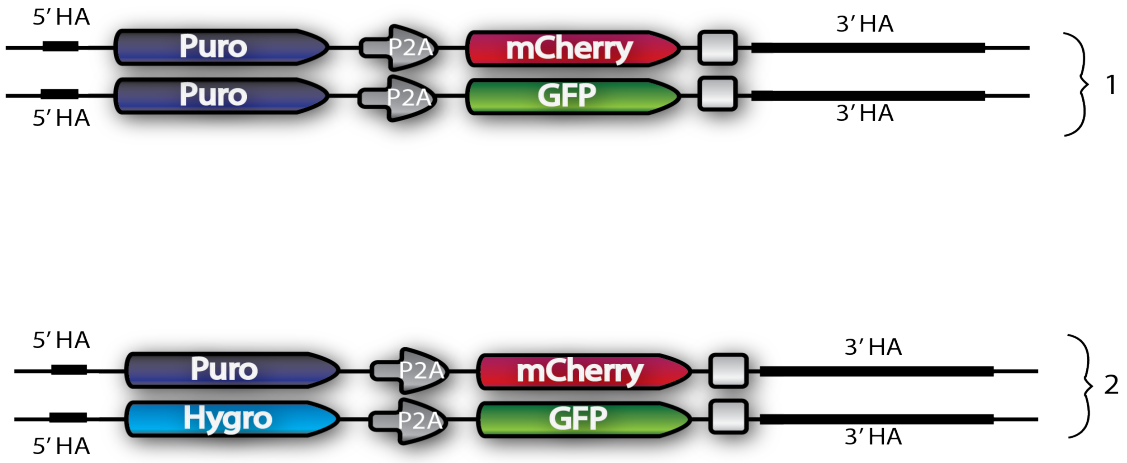


Figure 7 – Inserts for testing best selection strategy experiment
Experiment 1 utilizes the same antibiotic selection marker for both inserts (1), and biallelic inserted cells are selected by GFP and mCherry positive cells among puromycin selected cells. Experiment 2 utilizes 2 different antibiotic selection genes (2), one for each insert, not requiring sorting for biallelic selection.

For each of the previous mentioned experiments, transfection of $1x10^6$cells with 2µg of each of the linearized inserts and 2.5µg of 5' and 3' CRISPR plasmids was performed. 48 hours after transfection, cells were selected with puromycin (experiment 1) or both puromycin and Hygromycin (experiment 2).

Single antibiotic selected group were sorted 3 times for both GFP and mCherry positive cells and cloned one cell per well after that. Dual antibiotic selected cells formed isolated colonies, so individual clones were pick for further analysis.

Results were compared in terms of bi-allelic correct insert using GFP and mCherry specific primers inserted after the pig endogenous promoter in each individual colony resulted from both protocols.

mCherry insert primers:

5'- AGAATGCAGCATCAGCCCTT-3'

5'- GCCGTCCTCGAAGTTCATCA-3'

Thermal cycling conditions were 94ºC x 2min (1 cycle), 94ºC x 15s, 55ºC x 30s and 72ºC x 1min 55s (40 cycles), 72ºCx 7 min and 4ºC hold.

GFP insert primers:

5'- AGAATGCAGCATCAGCCCTT-3'

5'- GCATGGCGGACTTGAAGAAG-3'

Thermal cycling conditions were 94ºC x 2min (1 cycle), 94ºC x 15s, 54.6ºC x 30s and 72ºC x 1min 55s (40 cycles), 72ºCx 7 min and 4ºC hold.

3.2.4 5’ In-frame stop/Off-frame start trap

After definition of best homology arm size and best colony selection strategy for obtaining bi-allelic insertion, a 5’ in-frame stop/ off-frame start codon was placed before the 5’ homology arm of this inserts to avoid random integration gene expression (Fig. 8).

Figure 8 – In-frame stop/off-frame start trap sequence

Figure 9A-C shows all possible outcomes of location integration when incorporating the trap into the final experiment.

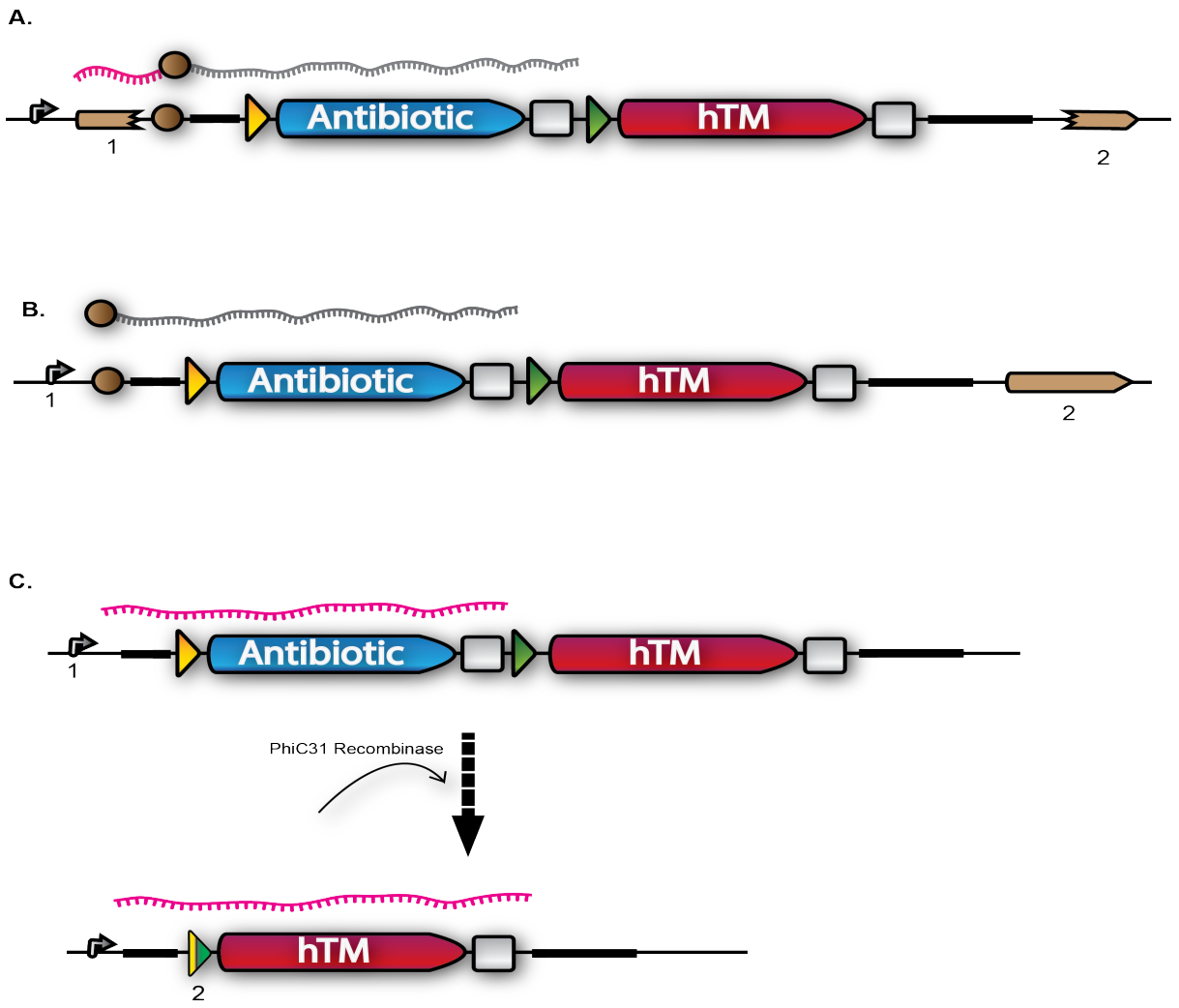


Figure 9 – Possible outcomes after trap insert integration
A) Insert integrated on a random gene. 1: Initial Part of random gene/ 2. Distal part of random gene. Transcription of original gene will be normal (pink RNA), however translation of fusion insert will not occur because of in-frame stop codon trap (gray RNA).

B) Insert integrated after a random promoter (1). Translation of the non-functional RNA (gray RNA) will generate a non-functional protein due to off-frame start codon trap.

C) Insert located in the correct place. 1: Pig endogenous thrombomodulin promoter. Trap will not be incorporated due to homologous recombination on arms will displace it. This will allow correct RNA transcription (pink RNA) and antibiotic selection marker translation after PhiC31 recombinase. After recombination, selection marker will be removed from the insert, inactivating the recombination site (2), and allowing hTM to start being expressed from pig endogenous promoter.

For trap test, the best protocol resulted from homology arm size and selection strategy experiments were repeated, including the above-described trap. For that, $1x10^6$ PAEC cells were transfected with 2.5µg of each CRISPR plasmid in association with 2µg of each linearized trap insert plasmid. 48h after transfection, cells were selected with the appropriate antibiotic selection and cloned according to the protocols with best efficiency determined in the pre-experiments.

All resulted clones were evaluated in regards to GFP and mCherry expression and compared to each other and a wild type PAEC negative control in flow cytometry study. Also, PCR identification of correct inserted DNA in each allele was evaluated using the same primers as in the best selection strategy experiment.

Trap sequence with 60bp 5' HA:

TAATGATAGAGCCACCATGGACCTGGCAGCTCCCTGCGCCTCTCAGCCCCGG
CCGGGCCCCTGCGCTTGGCGTGCTGACACC

3.3 Transgene expression experiments

Transgene expression experiments utilized the best results of integration validation experiments (selection cassette) for generation of final inserts.

In this experiment, a promoterless human thrombomodulin gene followed the selection cassette determined by the pre-experiments.

These selection markers were surrounded by modified PhiC31 recombinase sequences AttP and AttB for removal of them after PhiC31 expression. Once the selection marker was removed, the pig endogenous promoter would drive the promoterless human thrombomodulin, finalizing the gene swap in a two-step procedure (Fig 2).

In the first step of the procedure, $1x10^6$ PAEC were transfected with 2.5μg of each CRISPR plasmid in association with 2μg of each linearized insert containing the promoterless selection marker surrounded by recombinase sequences followed by an promoterless human thrombomodulin gene.

48 hours after transfection, cells were submitted to antibiotic selection for 8 days. After that period, colonies were combined together for transfection of PhiC31 recombinase expressing plasmid.

Recombination for selection marker removal and start of gene expression by the endogenous promoter were performed by transfection of $1x10^6$ antibiotic selected cells with a codon optimized PhiC31 recombinase with NLS (4μg) driven by CMV promoter modified from addgene plasmid pPGKphiC31obpA (plasmid#13795) (102).

In order to reduce possible incorrect recombination due to similarity between human and pig thrombomodulin in the first step, which would avoid colony formation during antibiotic selection, a pig codon optimized version of human thrombomodulin that had no similarity after blast alignment with pig version was generated (appendix).

Constructs using unmodified and customized human thrombomodulin were used and compared in terms of transgene expression, colony formation and bi-allelic insert ratio.

3.3.1 Flow cytometry

Flow cytometry was performed 2 days after PhiC31 recombinase transfection for evaluation of transgene expression.

Human and pig aortic endothelial cells were cultured to 80-90% confluence and harvested with trypsin 0.25% (Invitrogen). Cells were washed with flow buffer (PBS pH 7.2, BSA 0.5%). All incubation steps were performed in dark at room temperature for 30 min and were followed by 2 washing steps. $2x10^5$ cells were resuspended with mouse IgG1 isotype (1:75) (R&D systems, MAB002) or anti-human thrombomodulin antibody (1:500 Abcam PBS-01, ab6980). After this staining step, cells were incubated with Alexa Fluor 647 AffiniPure Goat anti-mouse IgG (1:750) secondary antibody (Jackson ImmunoResearch PA, USA Code 715-606-150, Lot: 116560). After washing, cells were resuspended with flow buffer and read in BD Accuri C6 flow cytometer machine (BD Biosciences, CA, USA).

3.3.2 Fluorescence-activated cell sorting (FACS)

All positive cells were sorted using BD FACS Aria sorter (BD Bioscience, San Jose, CA, USA). Staining protocol was the same as the one for flow cytometry, but the incubation period was performed at 4ºC. Untransfected PAEC

and selected PAEC before recombinase treatment were used as negative control. Human aortic endothelial cells (HAEC) were used as positive controls.

3.4 Characterization of transgene expression profile experiments

3.4.1 Mono-allelic vs. bi-allelic correct hTHBD insertion

3.4.1.1 Qualitative measurement of correct inserted hTHBD

To evaluate against one allele and two alleles insertion, gDNA PCR amplification of sorted TPAEC from the pig endogenous promoter to the 3' homology arm was performed. These primers can amplify all 3 potential outcomes to evaluate the proportion of each after the experiment: unmodified pig thrombomodulin sequence (3505 bp), correctly inserted human thrombomodulin (2525 bp) and removed coding sequence due to dual CRISPR (455 bp). Wild type PAEC were utilized as control.

Primers: (figure 10 shows primer binding location – blue primers)

5' – ACCCAGTAATCCGAGAAT– 3' (binds to region before 5' homology arm)

5' – GGCTGCTACTTCTTACTGAT– 3' (binds to region inside 3' homology arm)

Thermal cycling conditions were 94ºC x 2min (1 cycle), 94ºC x 15s, 55ºC x 30s and 72ºC x 2min 35s (40 cycles), 72ºCx 7 min and 4ºC hold.

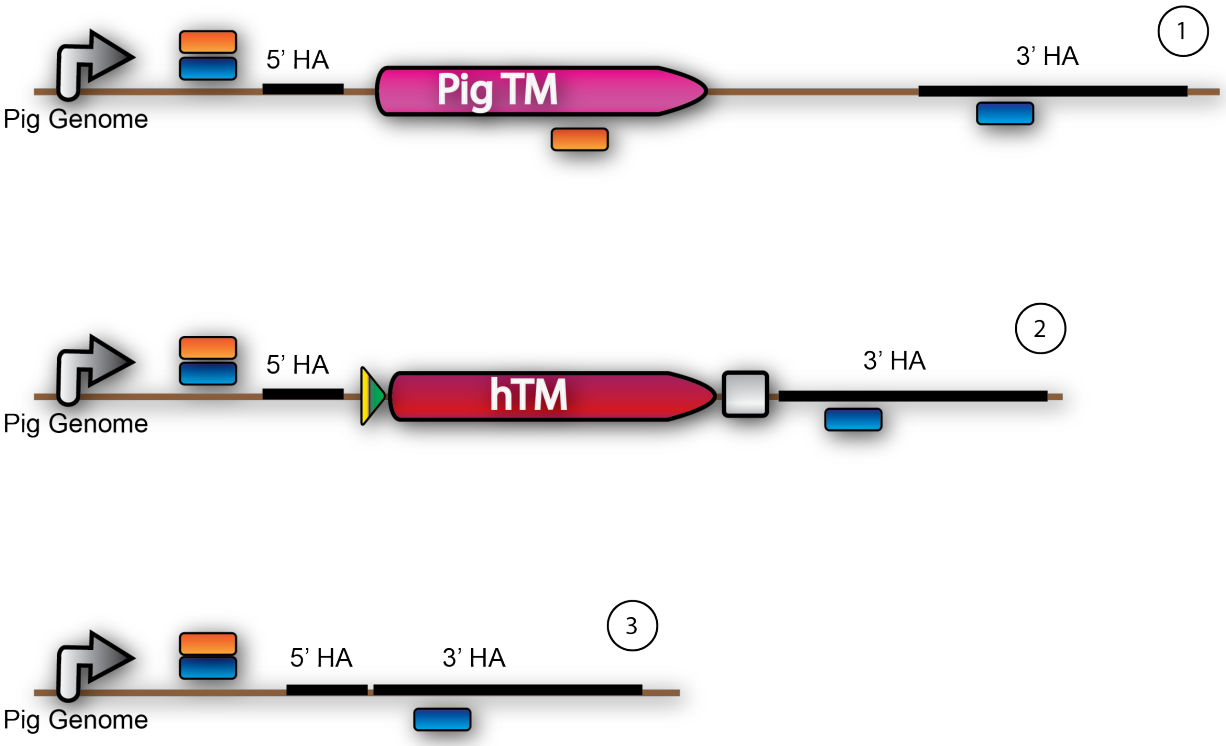


Figure 10 – Primer binding location for genotyping experiments
All possible outcomes after final experiment: 1- Unmodified pig thrombomodulin; 2- Pig-to-human gene replacement; 3- Removal of pig thrombomodulin without human version integration. Rectangles represent primers. Blue color: PCR primers for simultaneous amplification of all outcomes. Orange: Specific amplification of pig thrombomodulin.

To determine pig thrombomodulin expression in sorted human thrombomodulin expressing cells, specific primers for pig thrombomodulin were used to amplify from the promoter (before target region) to the pig coding sequence (Figure 10 – Orange Primers). After amplification, the band was extracted and cloned into PCR4blunt plasmid (Invitrogen, CA USA) and several

colonies were sent for sequencing to evaluate unmodified pig thrombomodulin sequence. Wild type PAEC were utilized as positive control.

Primers:

5’ – ACCCAGTAATCCGAGAAT – 3’

5’ – GCAGTAGCCGTTGTTGCA – 3’

Thermal cycling conditions were 94ºC x 2min (1 cycle), 94ºC x 15s, 53.7ºC x 30s and 72ºC x 50s (27 cycles), 72ºC x 5 min and 4ºC hold.

Expected size: 800bp.

3.4.1.2 Quantitative measurement of correct inserted hTHBD

For a quantitative measurement of single and double allele insertion in sorted TPAEC, digital droplet PCR was performed. PCR amplification of corrected inserted human thrombomodulin was performed.

Cells were harvested from 10cm plate and frozen at -80ºC until genomic extraction was performed (Genelute Mammalian Genomic DNA – Sigma-Aldrich St Louis MO, USA).

Digital droplet PCR samples were prepared as described previously (103). Briefly, 4.4µg of genomic DNA from sorted pig cells expressing human thrombomodulin and non-transfected aortic pig endothelial cells (negative control) were digested with 20 units of PvuII (NEB MA, USA) overnight.

The digestion was diluted 8-fold to 400µl with TE buffer then 33ng (3µL) was assayed per 20µL ddPCR reaction. Correct inserted human thrombomodulin was

detected using primers (forward primer) 5’ – AGAATGCAGCATCAGCCCTT – 3’, (reverse primer) 5’ – ACTGGCATTGAGGAAGGTCG – 3’ and (probe) 5’ – FAM – AGAGAAGCGGTTTTCGGGAGTAGT – BHQ1 – 3’ (Integrated DNA Technologies). Correct inserted copy number assay (Fig. 11, green primers/probe) was duplexed with pig Beta Actin gene (taqman assay Applied biosystems – Fisher Scientific Solutions Waltham, MA).

ddPCR Supermix for Probes (BioRad) was used for PCR amplification. Thermal cycling conditions were 95ºC x 10min (1 cycle), 94ºC x 30s, 60ºC x 120s and 68ºC x 30s (40 cycles), 98ºC x 10 min (1cycle), and 4ºC hold. Sample prepared in QX200 Droplet Generator and read in QX200 Droplet Reader (BioRad Hercules CA).

Human thrombomodulin total copy number primers for ddPCR were: Specific primers for human thrombomodulin F: 5’ –ACCCAGGCTAGCTGTGAGT – 3’ e R: 5’ – CGTTTTCGCACTCGTCGATG - 3’ (Gene bank NM_ 000361). Probe: 5’ – FAM –AGGCTACATCCTGGACGACGGTTTCATC – BHQ1. Total copy number assay (Fig. 11, yellow primers/probe) was duplexed with pig Beta Actin gene as well (taqman assay Applied biosystems – Fisher Scientific Solutions Waltham, MA).

Thermal cycling conditions were 95ºC x 10min (1 cycle), 94ºC x 30s and 60ºC x 60s (40 cycles), 98ºC x 10 min (1cycle), and hold 4ºC hold.

Assay was performed in IU Center for Diabetes and Metabolic Diseases Translation Core.

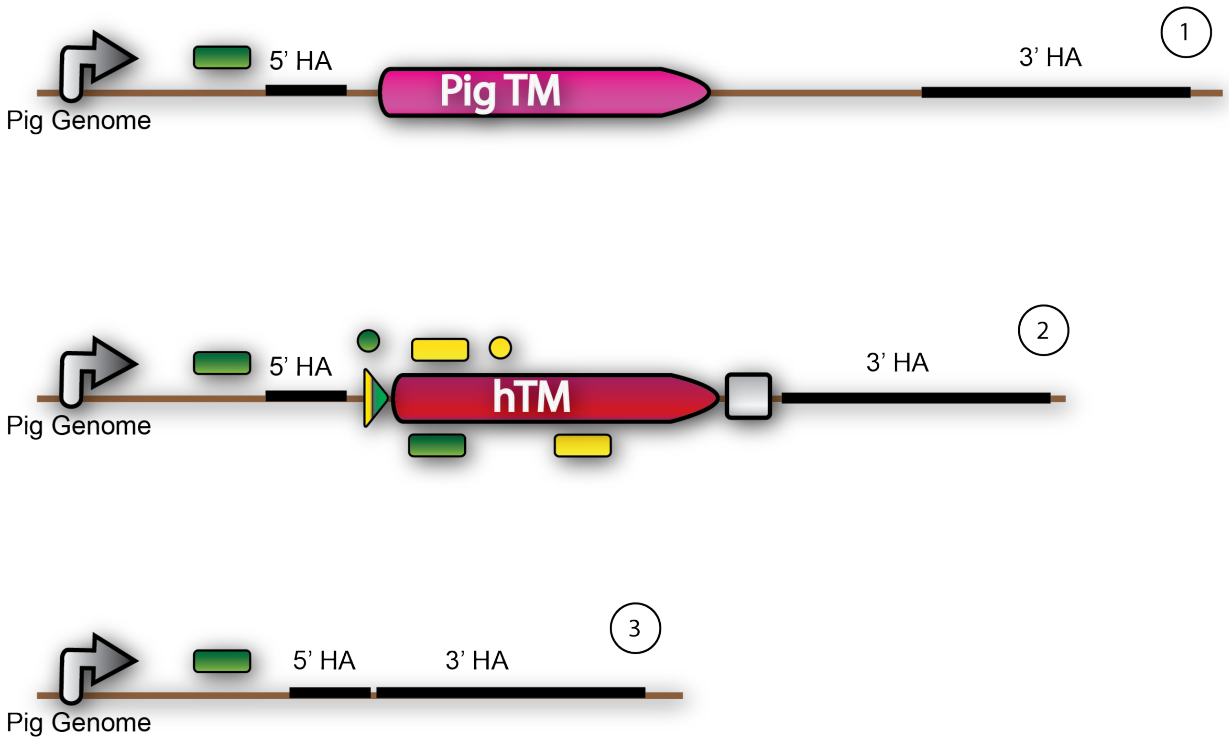


Figure 11 – Primer binding location for ddPCR and qPCR experiments
All possible outcomes after final experiment: 1- Unmodified pig thrombomodulin; 2- Pig-to-human gene replacement; 3- Removal of pig thrombomodulin without human version integration. Rectangles: primers/ Circles: probes. Green: primers and probe for correct inserted hTBHD ddPCR assay. Yellow: primers and probe for total hTHBD integration ddPCR assay and qPCR assay (without probe).

3.4.2 Sequencing

To evaluate if correct insertion of human thrombomodulin and correct removal of selection cassette was achieved on all cells, PCR from genomic DNA of

pig aortic endothelial cells was performed and the product was sent to sequencing (blue primers on figure 10).

5’ – ACCCAGTAATCCGAGAAT– 3’

5’ – GGCTGCTACTTCTTACTGAT– 3’

Thermal cycling conditions were 95ºC x 2min (1 cycle), 94ºC x 15s, 55ºC x 30s and 72ºC x 2m30s (40 cycles), 72ºCx 7min and 4ºC hold.

Expected size: 2509.

3.4.3 Functional assay (Activated Protein C Assay)

Activation of protein C by thrombin-mediated human thrombomodulin cofactor activity on PAEC, HAEC and TPAEC was measured by a specific chromogenic substrate as previously described (104). 20.000 cells were seeded in 96-well plates in RPMI supplemented with 10% FBS and Endothelial specific growth factor and evaluated 24 hours later after confluency. This media was then replaced by 50 µL of D-MEM (Lonza) supplemented with 3mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) with 200nM of human protein C (Haematologic Technologies) and either 0.1 or 1 IU/ml human thrombin (Haematologic Technologies, Essex Junction, VT, USA) for 30 min at 37ºC.

After incubation, 500nM of AT III (Assaypro, Winfield, MO, USA) with heparin (1µM) (Sigma-Aldrich) were added to each well to stop the reaction. Supernatants were frozen at -80ºC until analysis.

These supernatants were incubated with 50µL 3mM solution of S-2366 (Chromogenix, Bedford, MA) at room temperature and the amount of generated APC was measured in a dynamic plate reader at 405nm absorbance.

Purified APC (0-8µg/ml) was used as a calibration standard.

3.4.4 Promoter regulation assay

In order to evaluate if thrombomodulin expression regulation was maintained, sorted pig aortic endothelial cells expressing human thrombomodulin from the endogenous promoter were incubated with TNF-Alpha (R&D Systems, MN, USA)(10ng/ml) for 3 days. The same experiment was performed with human aortic endothelial cells for modulation comparison.

Regulation was evaluated by flow cytometry and qPCR with specific primers for human thrombomodulin (yellow primers in figure 11).

For qPCR evaluation, RNA was extracted from induced and non-induced pig aortic endothelia cells using RNA Easy Plus kit and QIAShredder (Qiagen) according to manufacture instructions.

After 3 days incubation with TNF-alpha, cells were pelleted and frozen immediately at-80ºC until RNA extraction.

RNA was converted to cDNA with Transcriptor First Strand cDNA Synthesis Kit (Roche) using both random hexamer primers and oligo dT according to manufacture instructions.

Specific primers for human thrombomodulin was used F: 5’ – ACCCAGGCTAGCTGTGAGT – 3’ e R: 5’ – CGTTTTCGCACTCGTCGATG - 3’ (Gene bank NM_ 000361) and pig B-Actin F: 5’ - TCCAGCCCTCCTTCCTGG - 3’ e R: 5’ - TCGCACTTCATGATCGAGTTG – 3’ (Gene bank U07786). FastStart Universal SYBR Green Master (ROX) (Roche- Indianapolis IN) was used on StepOne Plus Real-Time PCR System (Applied Biosystems – Fisher Scientific Solutions Waltham, MA).

Expression comparison between groups with and without TNF-treatment was made by delta delta CT method. The experiment was made with 3 samples from each group and they were repeated in triplicates.

3.5 β2 Microglobulin (B2M) experiments

In order to test if the protocol would work in other genes, B2M gene swap was tested. The same protocol was repeated for replacement of pig B2 microglobulin to human B2M but using the following DNA sequences instead of the described for thrombomodulin protocol

5’gRNA: AGCACCGCTCCAGTAGCGA**TGG**

3’gRNA: GGAGGCTCTCAGTCCAGTAC**AGG**

5’HA:

GCTATAAATGCGGGCAGGGAGCCGAGCTCTCATTCCACCGCCAGCACCGCT CCAGTAGCG

In the first step of the procedure, $1x10^6$ PAEC were transfected with 2.5µg of each CRISPR plasmid in association with 2µg of linearized insert containing a promoterless puromycin resistance gene surrounded by recombinase sequences followed by an promoterless human B2M gene.

After puromycin selection, surviving cells were sorted for SLA I negative cells to guarantee complete disruption of pig B2M cells using BD FACS Aria sorter (BD Bioscience, San Jose, CA, USA).

Staining for SLA class I was perfomed with mouse anti pig SLA class I clone JM1E3 (AbD Serotec) and isotype control Mouse IgG1 negative control (AbD Serotec), both diluted 1:50. After 30 min incubation on ice, 2 washing steps were performed and cells were sorted.

After this step, recombination for selection marker removal and initiation of human B2M gene by the pig endogenous promoter were performed by transfection of $1x10^6$ antibiotic selected cells with pCMV-PhiC31o-NLS (4µg).

Flow cytometry and sorting was performed using monoclonal mouse anti-human beta 2 microglobulin antibody [BM2-01] (Abcam) according to the following protocol: Primary antibody diluted 1:500 in flow buffer, 100µL for 200,000 cells, with 30 min incubation at room temperature. After this staining step, cells were incubated with Alexa Fluor 647 AffiniPure Goat anti-mouse IgG (1:750) secondary antibody (Jackson ImmunoResearch PA, USA). After washing, cells were resuspended with flow buffer and read in BD Accuri C6 flow cytometer machine (BD Biosciences, CA, USA) or sorted using BD FACS Aria sorter (BD Bioscience, San Jose, CA, USA).

After isolation of human B2M positive populations, screening of cells were performed:

Primers for correct inserted B2M amplification

5’ – TCCACCCAGTCCAACCTTTG – 3’

5’ – TGTCGGATGGATGAAACCCA – 3’

Thermal cycling conditions were 94ºC x 2min (1 cycle), 94ºC x 15s, 54ºC x 30s and 72ºC x 30s (40 cycles), 72ºCx 7min and 4ºC hold.

Expected size: 477 bp.

Probe for ddPCR:

5’- FAM - TCCACCTCACCCATCTGGTCCATC – BHQ1 – 3’

Primers for ddPCR correct inserted B2M

5’ – TCCACCCAGTCCAACCTTTG – 3’

5’ – TGTCGGATGGATGAAACCCA – 3’

ddPCR Supermix for Probes (BioRad- Hercules CA) was used for PCR amplification. Thermal cycling conditions were 95ºC x 10min (1 cycle), 94ºC x 30s, 60ºC x 120s and 68ºC x 30s (40 cycles), 98ºC x 10 min (1cycle), and 4ºC hold. Sample prepared in QX200 Droplet Generator and read in QX200 Droplet Reader (BioRad – Hercules CA).

3.6 Statistical analysis

Numerical variables were expressed by mean and standard deviation. Comparison among the three groups (PAEC, HAEC and TPAEC) was analyzed by One-way ANOVA and Bonferroni test for post-hoc multiple comparisons analysis for Activated Protein C formation functional assay. Statistical analyses were performed using SPSS statistics version 20.0 (IBM, Armonk, NY). Level of significance was set at $p<0.05$.

4. RESULTS

4. Results

Gene swap was achieved by removing the complete coding sequence from pig gene with dual CRISPR-Cas9, integrating an insert with a promoterless selection marker through homologous recombination at the same time in this location. This insert had the antibiotic selection marker surrounded by modified PhiC31 recombinase sequences (AttP and AttB) and was followed by a promoterless human version of the pig gene to be replaced. After antibiotic selection, PhiC31 recombinase expressing plasmid transfection allowed removal of selection marker and start of transgene expression.

In order to optimize steps for this procedure, validating experiments were performed to test the cell line and test protocols for correct gene integration.

After optimization of experimental design, human thrombomodulin was added to the insert for gene replacement using the validating experiments data.

4.1 Cell Line experiments

Pig aortic endothelial cell lines were utilized for the experiments, since the most commonly used fibroblast would not express endothelial gene from the endogenous promoter, rendering the experiments impossible.

4.1.1 Porcine aortic cell isolation and culture

After general anesthesia, pig aorta was resected. Cells were harvested according to methods section and were plated on a 15cm plate. After 3 days the dish was confluent, containing approximately $1.2x10^7$ cells. This procedure was repeated for wild type pigs, SLA I KO pigs, and GGTA1 + CMAH KO pigs.

4.1.2 Growth curve

Population doubling level and doubling time curves are shown in Figures 12A and 12B respectively. After growing cells 62 days, we found:

- No signs of senescence
- Population doublings: 86 times
- Doubling time: 17.2h

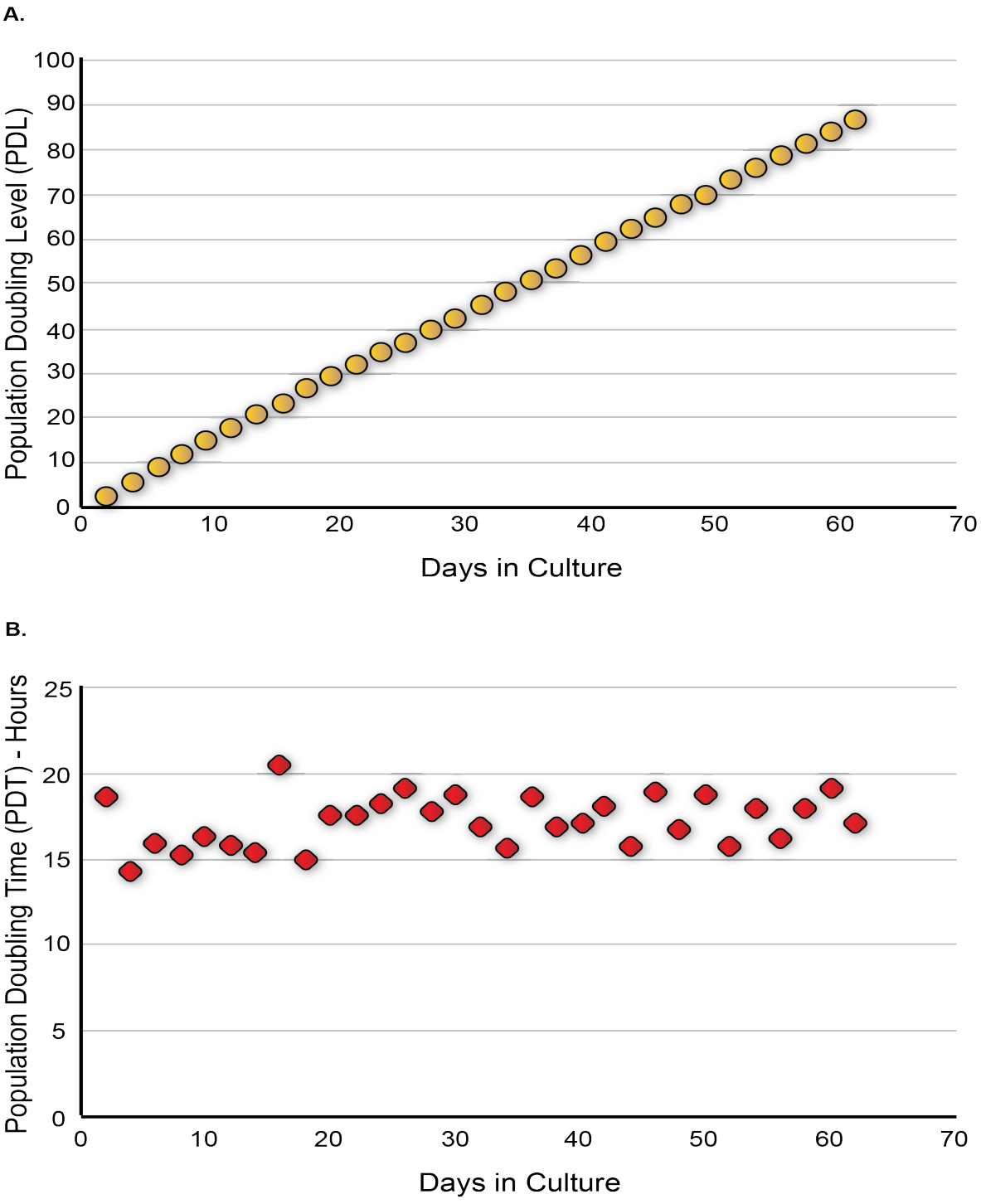


Figure 12 – Pig aortic endothelial cell line experiments results
A) Population doubling level (PDL).
B) Population doubling time (PDT). Mean PDT was 17.2h.

4.1.3 Transfection efficiency

After 30 different transfection assays with pEGFP-N1 plasmid (Clontech), 90.3% efficiency were obtained with 1500V, 30 milliseconds (ms) and 1 pulse. Table 2 and 3 show results for standard and extended parameters respectively.

Table 2 - Standard Neon parameters transfection efficiency

Sample	Voltage	ms	Pulse number	GFP Positive(%)
1	0	1	1	0
2	1400	20	1	4.2
3	1500	20	1	7.7
4	1600	20	1	11.6
5	1700	20	1	17.7
6	1100	30	1	1.5
7	1200	30	1	4.2
8	1300	30	1	4.2
9	1400	30	1	8.1
10	1000	40	1	1.5
11	1100	40	1	2
12	1200	40	1	3.9
13	1100	20	2	1.5
14	1200	20	2	3
15	1300	20	2	8.8
16	1400	20	2	12.7
17	850	30	2	0.8
18	950	30	2	0.7
19	1050	30	2	2.2
20	1150	30	2	5.8
21	1300	10	3	2.1
22	1400	10	3	4
23	1500	10	3	8.9
24	1600	10	3	15

Table 3 - Extended Neon parameters transfection efficiency

Sample	Voltage	ms	Pulse number	GFP Positive (%) 1	GFP Positive (%) 2
1	1300	30	1	11.6	65.1
2	1500	30	1	42.2	90.3
3	1600	30	1	46	89.9
4	1700	20	1	24.1	77
5	1800	20	1	44.7	89
6	1900	20	1	49.6	85.7

4.2 Integration validation experiments

These experiments were performed to test the best protocols for correct biallelic integration of DNA into the cells.

4.2.1 Selection of dual CRISPR gRNA

To remove the complete pig CDS, a dual CRISPR gRNA approach was used. Only one gRNA at ATG region was available (5'-AGGAGCAGAACGCGGAGCA-3'). To select the most efficient combination of gRNA pairs, several sequences for 3' CRISPR were tested.

Fig. 13 shows PCR amplification of each cell line treated with each dual gRNA. Based on these results, gRNA 1 was chosen for 3' CRISPR gRNA.

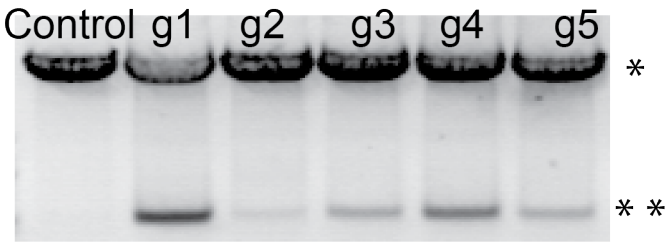


Figure 13 – Dual CRISPR gRNA experiment results
Comparison of different 3' gRNA with standard 5'gRNA for pig THBD coding sequence removal. gDNA PCR amplification before and after the target regions showing intact (*) and removed sequences (**). g1 had the best efficiency (**/*).

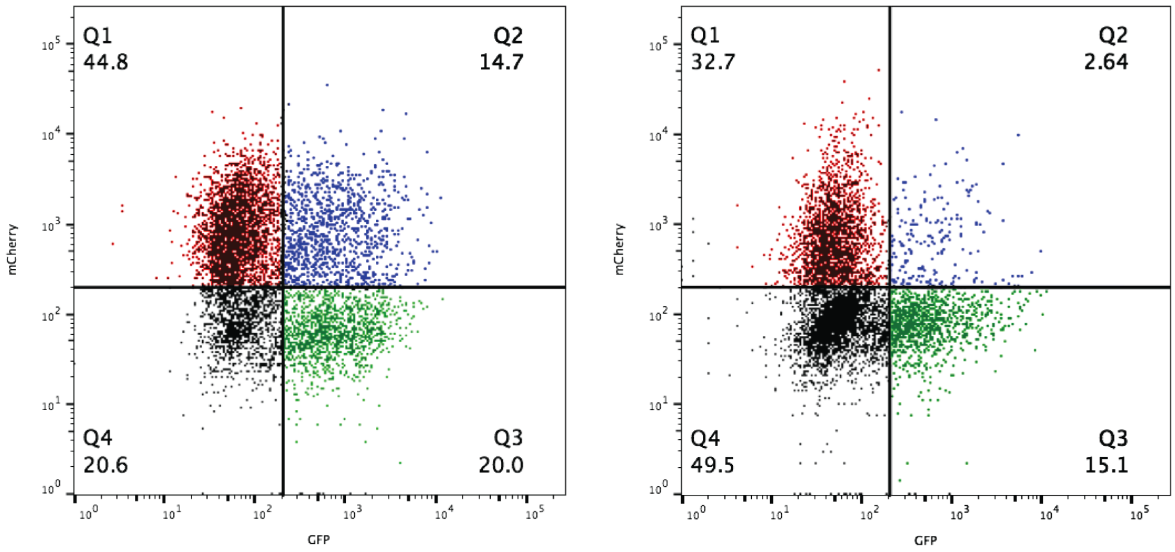
4.2.2 Homology arm size

Two different sizes of 5' homology arms (60bp vs. 2548bp) were compared to determine the most efficient for correct recombination.

Each tested size was used to create 2 different inserts, both promoterless containing a puromycin resistance gene linked with either GFP or mCherry by an optimized P2A sequence (Fig. 5) only to be expressed if inserted after a pig endogenous promoter. These inserts were transfected in combination with the best dual CRISPR combination, to replace pig CDS.

After sorting simultaneous positive GFP and mCherry cells after puromycin selection (Fig. 14A), PCR amplification of gDNA showed significantly less pig thrombomodulin remaining when a smaller homology arm was used (Fig. 14B).

A.



B.

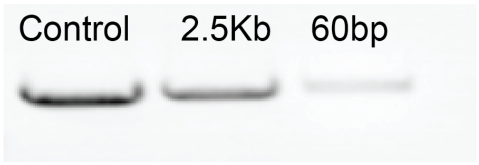


Figure 14 – Comparison of different 5' homology arm sizes results
A) Flow cytometry of puromycin selected cell in experiment 1 (left panel) and experiment 2 (right panel). Although experiment 1 (large HA) has more double positive cells (Q2), most of them are because of random integration.

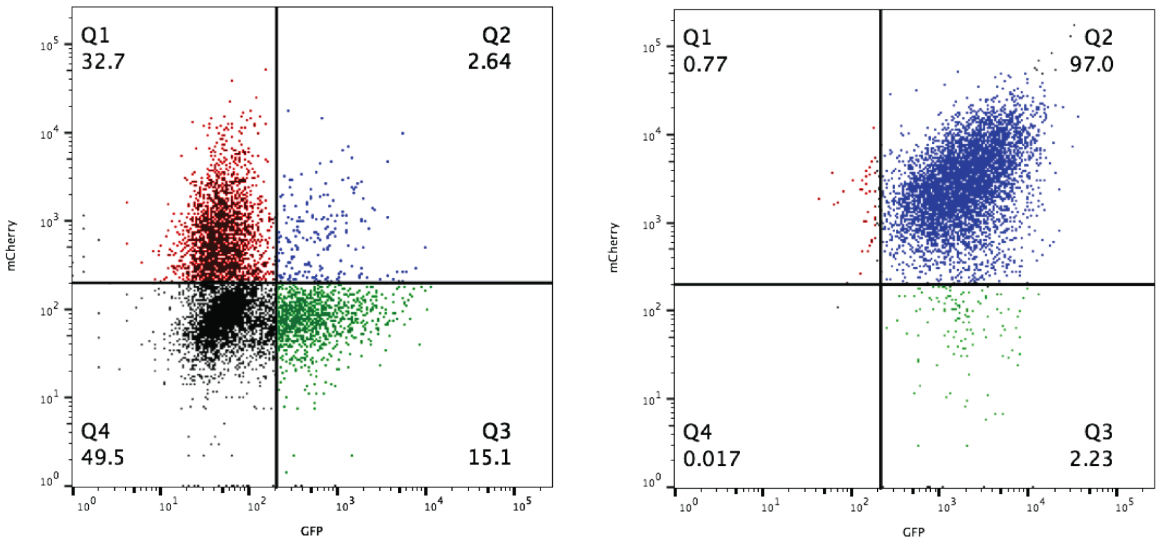
B) Comparison of correct integration efficiency in gDNA of antibiotic selected and sorted cells between 2.5 Kb and 60bp 5' homology arm inserts. The latter group presented better results seen as fainter band of pig THBD in gDNA PCR amplification.

4.2.3 Selection strategy for biallelic inserted cells

The smaller arm was used to compare the best selection strategy of biallelic insertion. Two inserts were transfected for each experiment, and only cells that expressed from both inserts were selected.

Two experiments were performed. In the first, a single antibiotic selection (puromycin) was followed by either GFP or mCherry linked through optimized P2A sequence. In the second, the cassettes had different antibiotic selection markers (puromycin and Hygromycin) (Fig. 7). Figure 15A shows flow cytometry of population cells surviving antibiotic selection and Figure 15B displays PCR amplification of gDNA from each of these groups showing that double antibiotic group presented significantly better results.

A.



B.

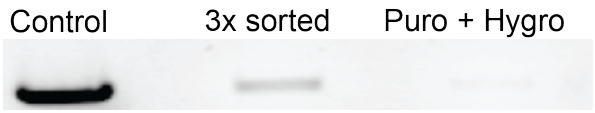
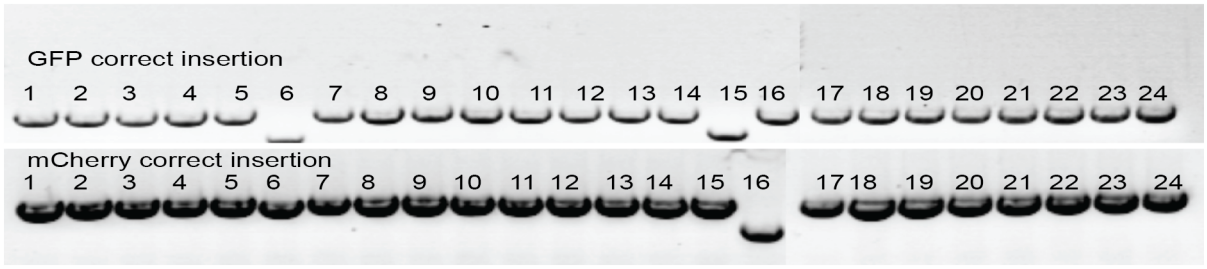


Figure 15 – Comparison of different selection strategies results
A) Flow cytometry of puromycin selected cell in experiment 1 (left panel) and puromycin + hygromycin in experiment 2 (right panel).

B) Comparison of selection strategy for correct biallelic insertion between single against dual antibiotic selection in gDNA of selected and sorted cells. The latter group presented better results seen as fainter band of pig THBD in gDNA PCR amplification.

When cloning where performed, colony screening showed 88% bi-allelic correct insertion (Fig. 16A) with single antibiotic selection followed by 3 sets of cell sorting of both GFP and mCherry positive cells. In the group with dual antibiotic selection, 100% biallelic integration was observed (Fig. 16B).

A.



B.

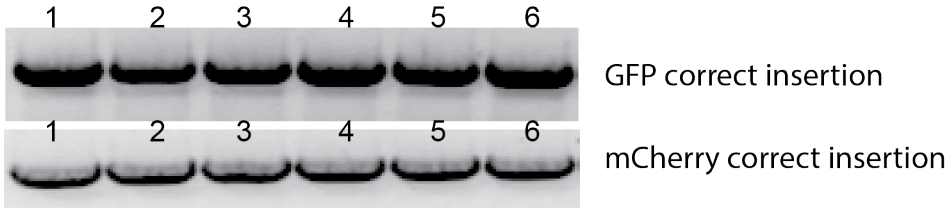


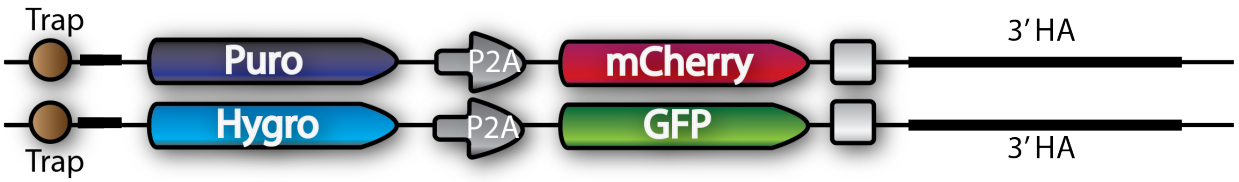
Figure 16 – Colony screening comparison of selection strategy experiments
A) Correct integration of GFP (upper line) and mCherry (lower line) inserts in clones with single antibiotic selection group (1 through 24) showing 100% single allele correct integration, 94% overall correct insertion and 88% bi-allelic correct insertion.

B) Correct integration of GFP (upper row) and mCherry (lower row) inserts in clones with dual antibiotic selection group (1 through 6) showing 100% correct biallelic insertion.

4.2.4 Trap test

When adding an in frame stop/off-Frame start trap to the small 5' homology arm cassette with dual antibiotic selection protocol (Fig. 17A), colonies in the trap group showed 100% bi-allelic correct insertion (5/5) and similar GFP and mCherry expression, demonstrating that any eventual random integrated cassettes were not adding fluorescence to the colonies (Fig. 17B).

A.



B.

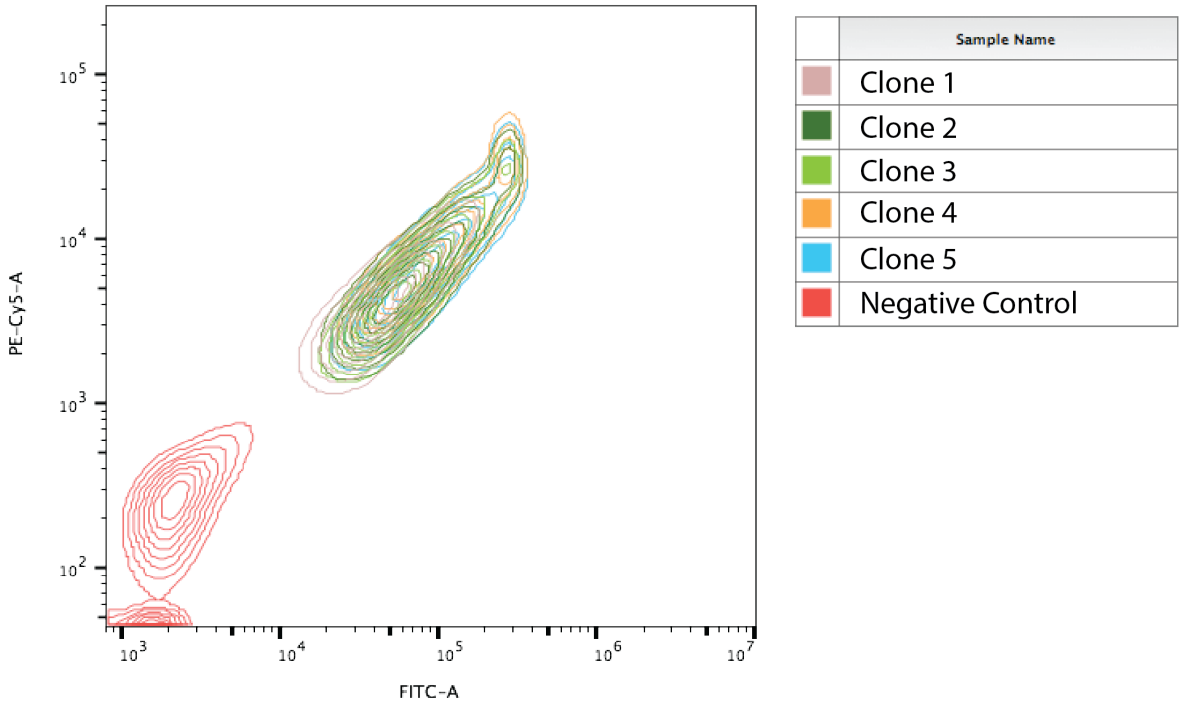


Figure 17 – Inserts for trap experiment and dual color flow cytometry of resulted colonies
A) Insert for testing Trap efficiency in avoiding random integration gene expression. Structure is the same as inserts in dual antibiotic selection experiment with the addition of in-frame stop/off-frame start trap.

B) GFP and mCherry expression pattern in clones with 60bp 5' homology arm associated with In-frame stop/off-frame start trap and dual antibiotic selection regimen. Clones show same expression pattern, which indicates that random integration do not affect gene expression.

4.3 Gene replacement experiments

After all validation experiments were performed, the final construct was made using this data to replace pig for human thrombomodulin (hTHBD) (Fig 1).

Although dual antibiotic selection was possible during integration experiments, it was not possible once hTHBD was cloned in the cassettes (no clones survival)

A hypothesis that the high similarity between pig and human thrombomodulin was impairing homologous recombination at the arm region was made. A codon modified hTHBD with no homology after blast homology to pig thrombomodulin was used, but this also resulted in no colony formation.

For this reason, cells were transfected with hygromycin only cassettes.

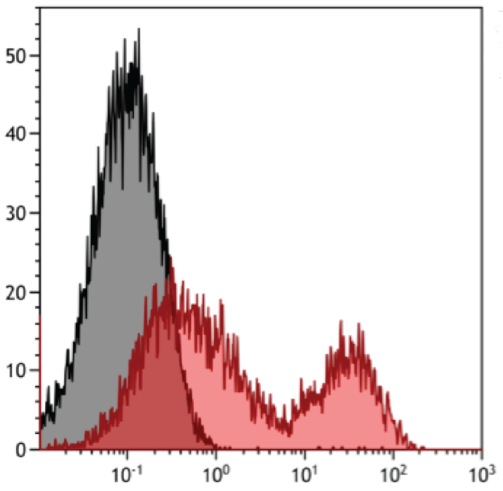
72h after single insert transfection in combination with both tested CRISPRs, single antibiotic selection was started, and 8 days later, cell clones were combined and transfected with pCMV-NLS-PhiC31o.

4.3.1 Flow cytometry

Flow cytometry was performed 48h after PhiC31 transfection, and a high number of cells expressing hTHBD were observed (Fig. 18A). These positive cells were present in a clonal population pattern, with narrow base and the results were similar in different experiments using puromycin or hygromycin and modified human thrombomodulin.

To test reproducibility, the same experiment was repeated using Puromycin as antibiotic resistance gene, in different cell lines (primary and immortalized aortic endothelial cell lines) and with swine codon optimized version of human thrombomodulin, and results were similar for all the occasions as shown in picture 18B.

A.



B.

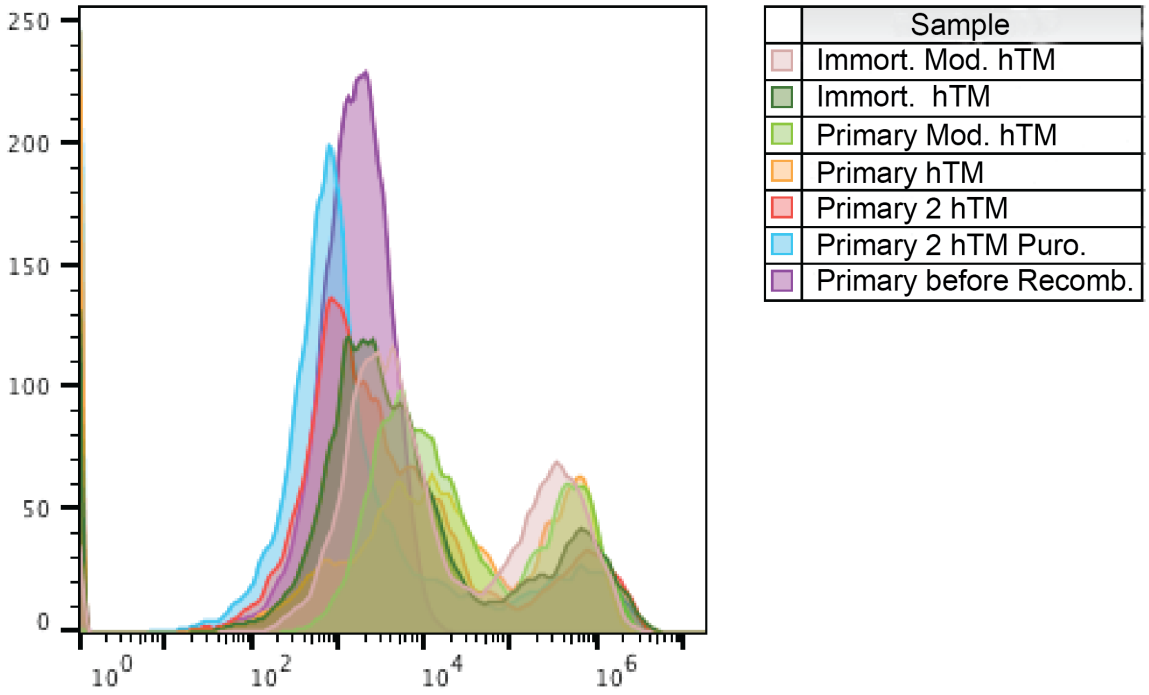


Figure 18 – Human thrombomodulin expression in PAEC
A) Human thrombomodulin expression before (black) and after (red) pCMV-NLS-PhiC31o plasmid transfection in hygromycin selected PAEC.

B) Protocol reproducibility test with different cell lines, antibiotic selection markers and versions of human thrombomodulin showing similar results.

4.3.2 FACS

FACS was performed in the PhiC31 transfected cells. After cell sorting, transgenic pig aortic endothelial cells (TPAEC) presented higher hTHBD expression and a narrower expression profile than human cells (Fig. 19A)

In order to evaluate random integration gene expression (defective trap), a clone selected with bi-allelic inserted dual antibiotic non-modified human thrombomodulin cassette (selection with lower antibiotic dose, requiring screening of dual integrated cells) was transfected with pCMV-NLS-PhiC31o. Two days after clone transfection, expression of this clone showed similar expression pattern as non-clonal sorted cells (Fig. 19B).

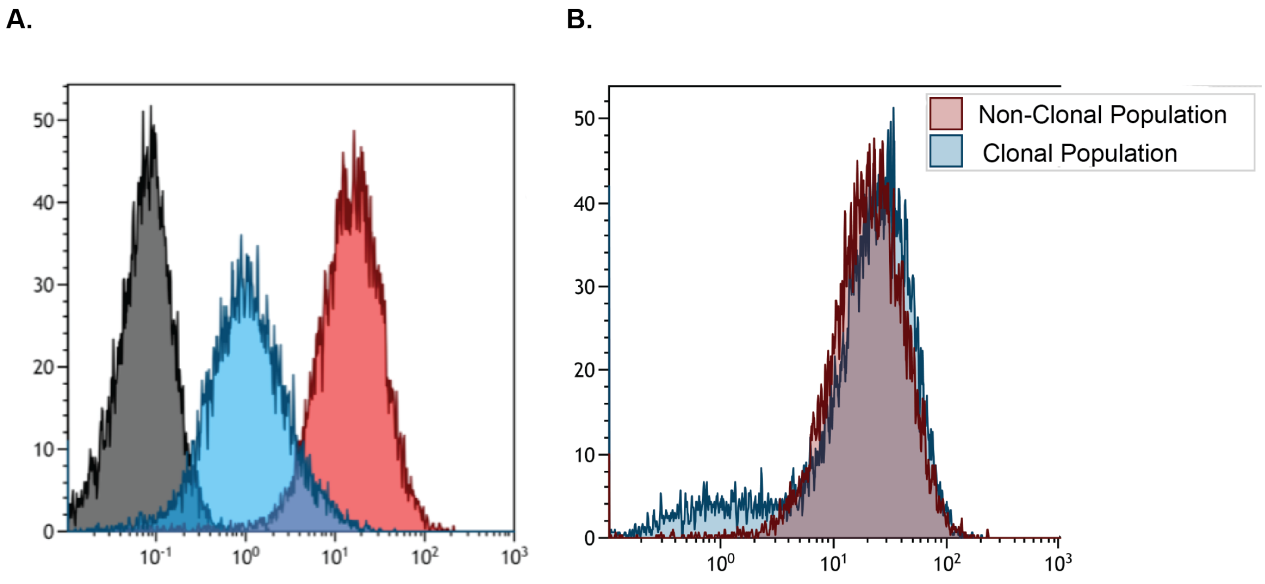


Figure 19 – Human thrombomodulin expression in sorted PAEC

A) Comparison of human thrombomodulin expression in cells before (black) and after recombinase treatment + FACS (red). Expression of hTHBD by human aortic endothelial cells was analyzed for comparison (blue).

B) Comparison of hTHBD expression in a clonal double allelic inserted cell line in the second day after pCMV-PhiC31-NLS transfection (blue) against bulky sorted non-clonal TPAEC (red). This result shows that the protocol using non-clonal cells has the exact same expression as cloned cells.

4.4 Characterization experiments

After sorting of cells expressing hTHBD, characterization experiments were performed to test the new transgenic cell line.

4.4.1 Mono-allelic vs. bi-allelic correct insertion

4.4.1.1 Qualitative measurement

In order to detect remaining pig thrombomodulin copies, PCR amplification capable of amplifying the three possible outcomes after cell treatment was used (Fig. 10, blue primers).

As seen in Figure 20A, correct inserted human thrombomodulin (**) is the most prevalent outcome, followed by deletion of pig thrombomodulin (***). Unmodified pig thrombomodulin (*) could not be detected by this method.

To evaluate further, specific primers for pig thrombomodulin were used (Fig. 10, orange primers). When compared to non-transfected cells, the amount of pig thrombomodulin is much lower (Fig. 20B). This band was cloned for sequencing, and results showed that 5 out 7 clones presented mutation that caused frame shift and knock out of the protein.

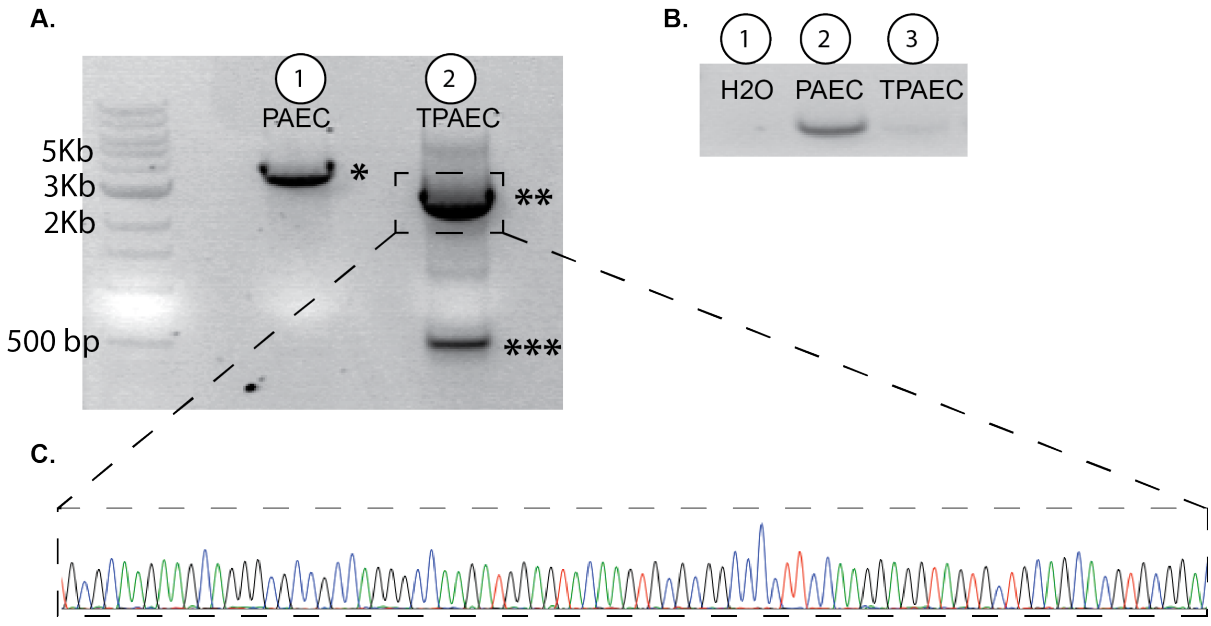


Figure 20 – Genotyping of human thrombomodulin transgenic cell line
A) PCR amplification for thrombomodulin region sequences of wild type (WT) PAEC and sorted TPAEC. In lane 1, untreated PAEC were used as a control for pig thrombomodulin amplification of 3505 bp (*). In lane 2, TPAEC expressing human thrombomodulin enabled amplification of a band of 2525 bp corresponding to the human sequences (**) and a smaller band (455 bp) indicative of complete removal of pig thrombomodulin coding sequence (***) without transgene incorporation. Amplification of full length pig thrombomodulin was not observed. The presence of remaining pig thrombomodulin was more carefully examined in B) by using pig specific PCR primers. Lane 1 represents a no DNA negative control. Lane 2 shows amplification of the pig thrombomodulin gene from untreated pig AEC. Lane 3 shows reduced amplification of the pig gene in human transgene expressing cells. C) Sequencing histogram of band representing human thrombomodulin inserted after pig endogenous promoter. No mix peaks were found in bulky sorted cells, showing that genotype is the same for all cells.

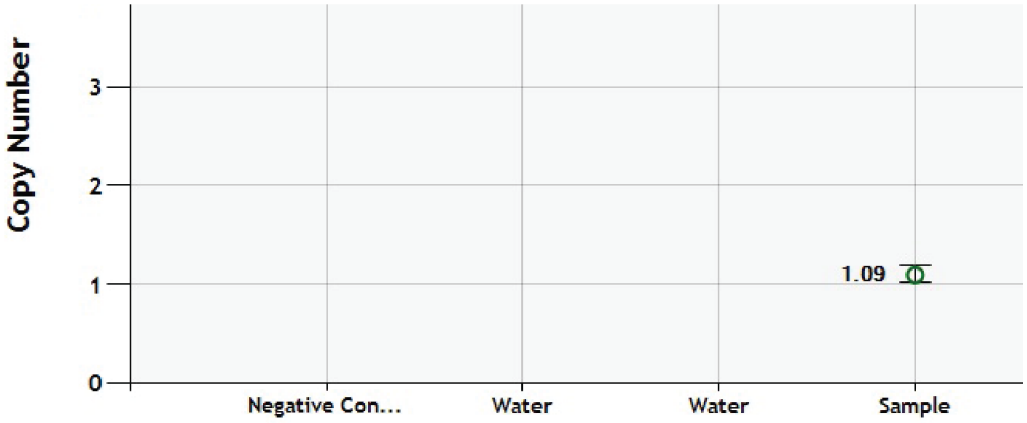
4.4.1.2 Quantitative measurement

In order to determine quantitatively mono-allelic vs. bi-allelic correct insertion, digital droplet PCR was performed (green primers and probes, Fig 11). In sorted experimental pig cells, copy number varied from 1.18 to 1.01 with a mean of

1.09 copies per cell (about 10% of cells with correct biallelic gene replacement) (Fig. 21A).

In regard to total copy number of inserts per cell (yellow primers and probe, fig. 11), including random integration, we found a mean number of 5.28 copies per cell (Fig. 21B).

A.



B.

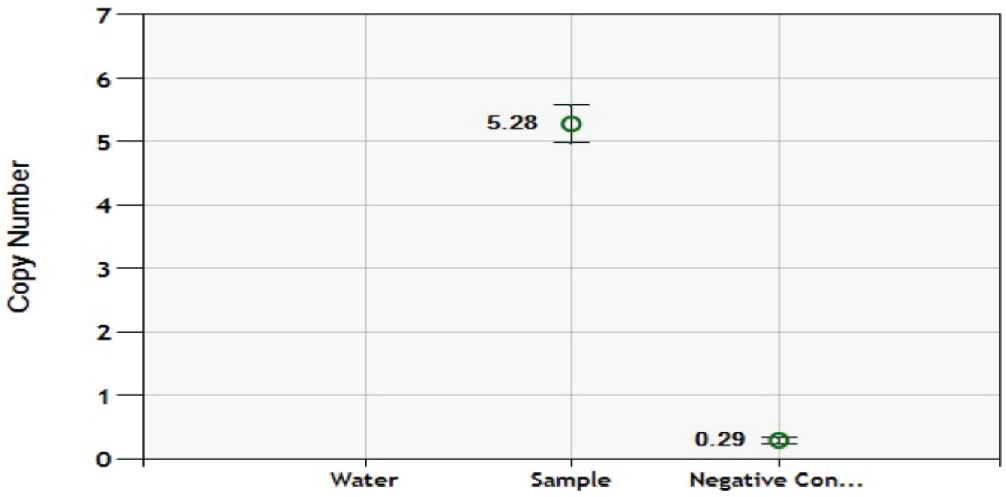


Figure 21 – Human thrombomodulin ddPCR quantification in TPAEC
A) ddPCR of correct integrated construct.
B) ddPCR of total integrated construct.

4.4.2 Sequencing

Sequencing of correct inserted hTHBD band (**) showed the exact predicted sequence, with no mix peaks, confirming that all cells presented the same genotype (Fig. 20C).

4.4.3 Functional assay

The function of human thrombomodulin was tested by its ability to activate protein C in the presence of human thrombin. Figure 22 shows the measured activated protein C in experimental group and positive and negative controls.

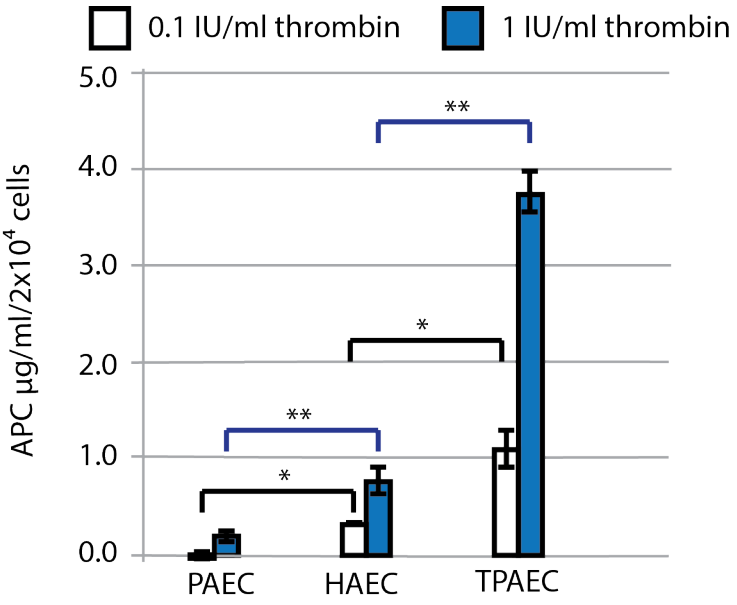


Figure 22 – Activation of protein C in PAEC, HAEC and TPAEC
Functional assay of human thrombomodulin by quantification of activated protein C generated by incubation of protein C and thrombin with the cells. White bars show incubation of protein C with 0.1IU/ml of thrombin and blue bars show incubation of protein C with 1.0 IU/ml of thrombin. TAEC presented more activation of protein C than human controls and WT pig cells in both thrombin concentrations, ANOVA ($p<0.001$).

Mean measured Activated protein C in the experiment with thrombin 0.1 IU/ml was -0.0004µg/ml in PAEC (± 0.02073), 0.3306µg/ml (± 0.011327) in HAEC and 1.0998µg/ml (± 0.18976) in TPAEC ($p<0.001$)

Mean measured Activated protein C in the experiment with thrombin 1.0 IU/ml was 0.2072µg/ml in PAEC (±0.054687), 0.7658 µg/ml (±0.1295) in HAEC and 3.7606µg/ml (±0.21546) in TPAEC ($p<0.001$).

4.4.4 Promoter regulation assay

To evaluate the persistence of promoter regulation, pig transgenic cells for human thrombomodulin were incubated with TNF-Alpha for 3 days (10ng/ml). Cells were compared with untreated controls with qPCR (Fig. 23A) and flow cytometry (Fig. 23B) and showing decreased thrombomodulin expression in the treated group. When compared with human matched controls, the difference in expression was similar (Fig. 23C).

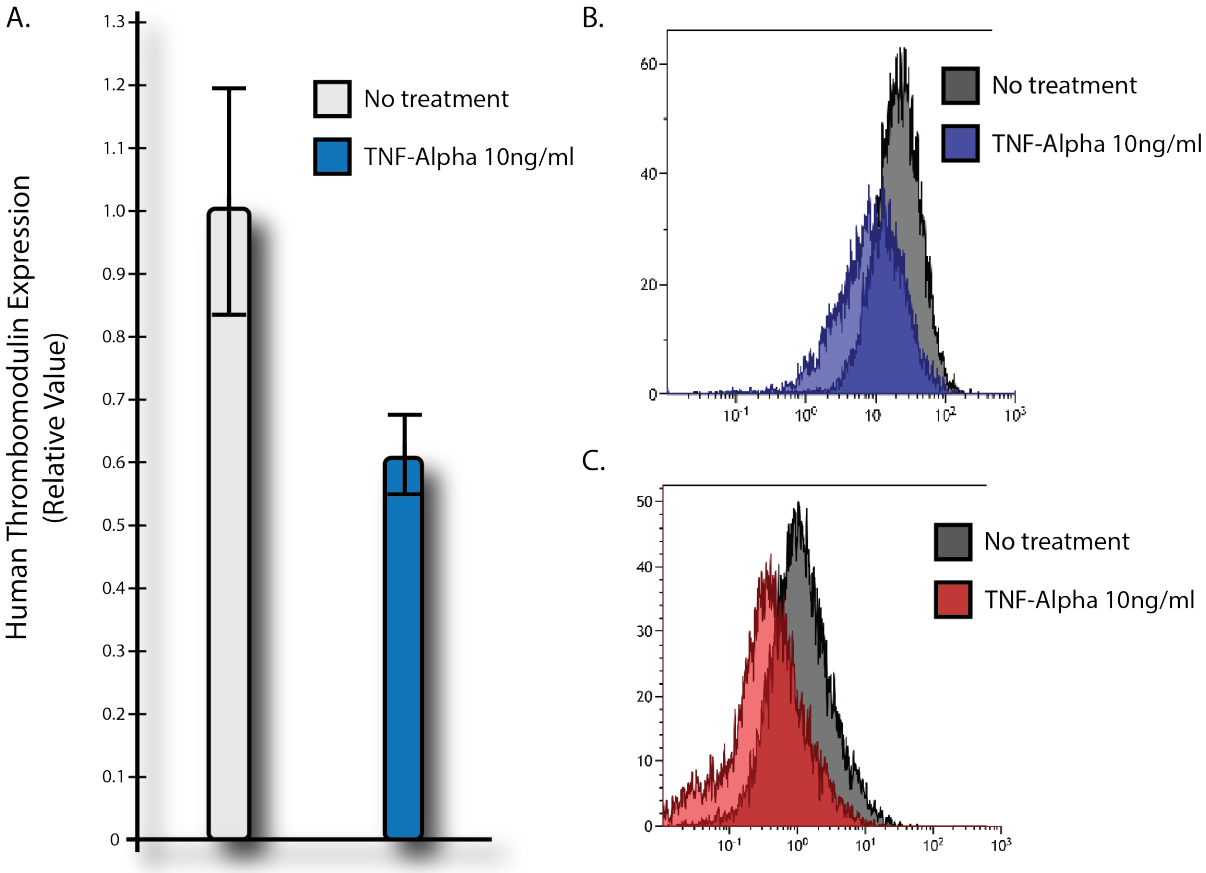


Figure 23 – Promoter regulation assay results
A) qPCR comparing human thrombomodulin transcript levels in transgenic pig aortic endothelial cells (TPAEC) incubated with TNF-alpha for 3 days (blue) versus untreated TPAEC (light gray).

B) Comparison of transgenic pig aortic endothelial cells not treated (black histogram) and treated (blue histogram) with TNF-alpha.

C) Human aortic endothelial cells flow cytometry histogram of non-treated (black) and treated (red) cells with TNF-Alpha.

4.5 B2M experiments

The same experiment was performed with B2M gene. After transfecting dual gRNA for pig B2M removal together with a puromycin insert with hidden human B2M, puromycin positive cells were sorted and negative SLA I cells were separated and grown (Fig. 24A).

This step selected pig cells with complete knockout of pig B2M. After that, recombinase plasmid were transfected to those cells, and positive human B2M cells were sorted with human specific antibody in two populations, as seen in figure 24B.

ddPCR were performed (Fig. 24C) in each of this population, as well as sequencing (Fig. 24D). Digital PCR showed that the population with more expression of human B2M presented more than one copy per cell, while the other population showed small number of correct inserted transgene. Sequencing results showed that there was mixed peaks in the region of homology arm.

This can be explained by the fact that a PAM sequence (removed in the 5' HA arm transition to the ATGG sequence) was reconstituted by accident when cloning this arm into the insert plasmid. This allowed the 5' CRISPR gRNA to target the inserts, allowing removal of the 5' HA and trap from it. In this scenario, random integration could happen.

Even with this experimental design problem, it was possible to obtain a clonal pattern of expression of human B2M with at least one copy per cell in the

correct location based on digital PCR in a subpopulation. This population presented the same expression pattern as human cells, as shown in Fig. 24B.

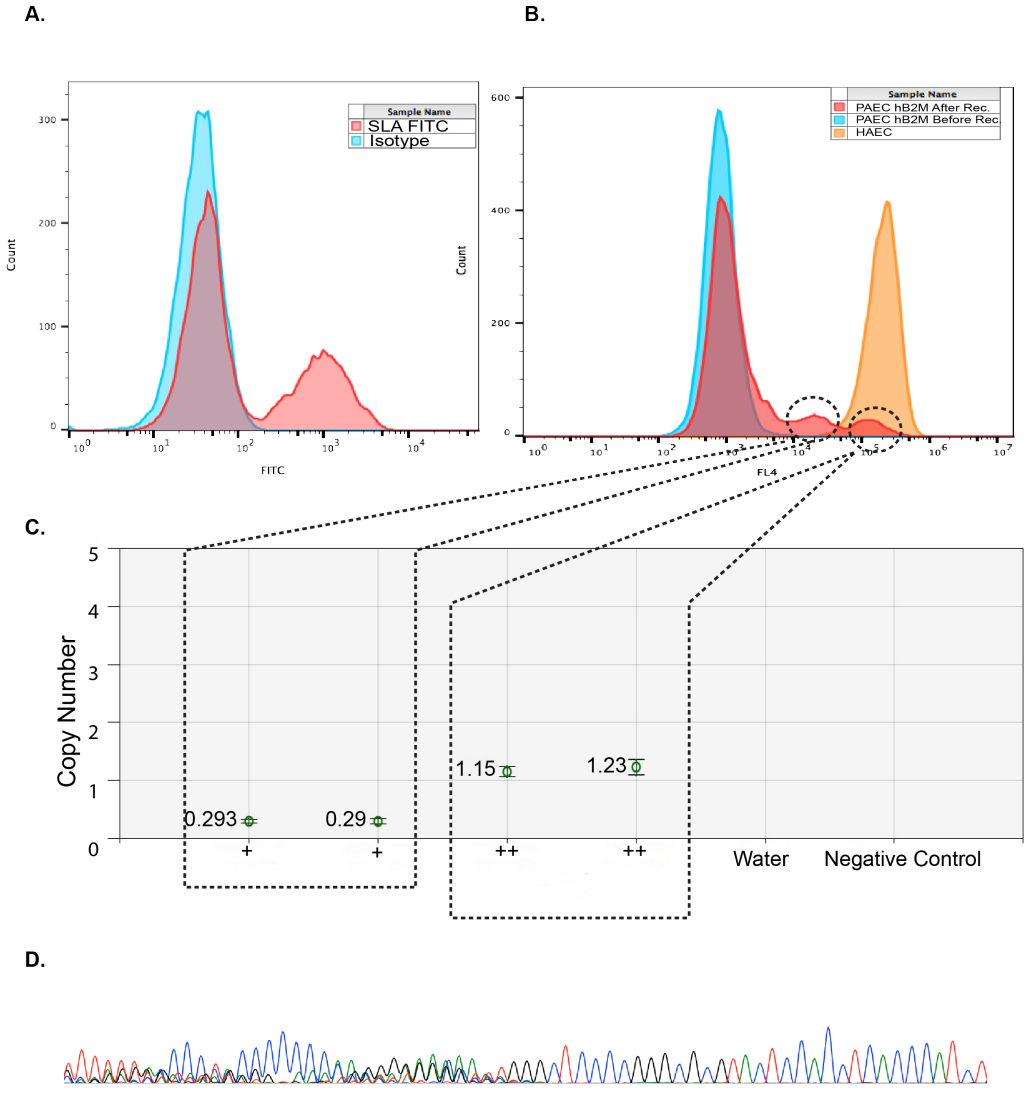


Figure 24 – B2M experiments results

A) SLA I stained puromycin selected PAEC in red and isotype staining in blue.
B) Human specific B2M stained puromycin selected PAEC before (blue) and after (red) recombinase. HAEC stained for human B2M.
C) ddPCR in duplicate of corrected inserted hB2M in weaker (0.293 copies per cell) and stronger peak (1.15-1.23 copies per cell).
D) gDNA sequencing of stronger hB2M peak cell population showing mixed peaks at 5' HA region.

5. DISCUSSION

5. Discussion

Pig is an important model for disease studies and a potential candidate for unlimited organs for xenotransplantation.

After the development of multiple nucleases, knockout animals are easier and faster to be generated, with consistent phenotype in spite of different genotype (any frame-shift mutation will generate the same functional end result).

However, so far, generation of gain of function transgenic pigs were difficult and unpredictable since different genotypes will generate different phenotypes in the adult animal.

Since there is a shortage of targeted mutation for large animals, mainly due to lack of long lasting stem cell lines for the lengthy protocols used for mice, for example, most transgenic animals are generated by random integration.

Random integration generated transgenic pigs will present different expression profiles in each animal, with patchy organ expression of transgene and susceptible to methylation of exogenous promoters, decreasing expression over time (95).

Also, some techniques for generation of transgenic animals with more predictable expression pattern usually require multiple steps and multiple generations of animals, such as recombinase DNA integration. In comparison to smaller animals, pigs have a relatively long gestation time (114 against 20 days)

and take longer to reach sexual maturity (5-6 months against 4-8 weeks in mice) (105-107).

In addition to that, housing large animals is expensive, and producing intermediary transgenic pigs for two-step procedures and screening for the correct expression profile can be time consuming.

Thrombomodulin transgenic pigs have been generated before, however, none of them with a consistent expression in endothelial tissues. The techniques varied from using viral promoters with random integration (104, 108, 109), cloning pig endogenous promoter (110) and endothelial specific promoter surrounded by insulators (111). Expression of human thrombomodulin in aortic endothelial cells in those experiments was as low as 8% in the adult animal (111).

Using the same promoter, Iwase archived 96% positive expression of thrombomodulin in aortic endothelial cells using insulator (111), however, no expression on kidneys for xenotransplant (85).

This inconsistence is very prejudicial for xenotransplant experiments comparing different immunosuppressive regimens, because positive controls can range from no expression to 96%.

In addition to that, none of these previous pigs were generated with knock out of the endogenous pig thrombomodulin. This may be the reason why the function of human thrombomodulin in those cells could not reach the same as human cells, in spite that their expression was higher than in human cells for some clones.

That happened because both pig and human thrombomodulin can bind human protein C, but only the latter complex can activate human protein C (84), generating a competition for substrate, archiving lower results.

For those reasons we developed a new technique, clonal gene transplant (CGT), which replaces the pig gene for the human counterpart in a clonal fashion, allowing bypassing cell screening, being suitable for generation of transgenic animals with an predictable expression pattern.

In this study we have established a protocol for gene swap with clonal expression pattern using promoterless strategy associated with a trap to impair expression if incorrectly inserted after a random promoter. Also, genotyping was identical in all working transgene loci. This technique achieved 100% biallelic gene replacement and 100% single allele pig-to-human gene swap.

Because of the similarity of phenotype and genotype described above, clones could be combined, allowing a very fast procedure, generating cells for somatic cell nuclear transfer in 14-20 days after transfection.

Also, using the endogenous promoter we could maintain complete gene regulation not shown in other pig models (104). This maintained regulation could be important in disease models, where a trigger can start gene expression or stop gene expression, which would be more physiologic than unaltered gene expression.

In addition to that, the most important advantage of preserving the promoter with in locus integration is consistent and tissue specific gene expression after animal generation as shown by Raife in mice models (98).

For that, first we needed to select primary cells with endothelial gene expression that had good life span and high cloning capability. We utilized primary pig aortic endothelial cell lines for the experiments.

These cells were utilized previously in our service to generate pigs (unpublished observations by Estrada, Reyes, Santos). This choice is very important both for a molecular point of view, and with an expression pattern point of view.

The most commonly utilized cell line for somatic cell nuclear transfer is fibroblast, including when generation of endothelial specific transgenic animal is done. Any experiment utilizing this kind of cell line would generate animals with incorrect expression and regulation pattern of any endothelial gene.

Second, we validated the best size of 5' homology arms for the specific correct insertion strategy. We had better results by using small 5'Homology arms (60 base pairs), because bigger homology arms cloned the endogenous promoter, making random integrated inserted cells to survive as well. We hypothesize that the size of homology arm could be bigger, as long as it does not reach the pre-initiation complex region, which could trigger random integrated cells to express the selection markers if inserted after a promoter.

Orlando et al achieved a similar result in homologous derived integration using 50bp homology arms when compared to sizes 15 times larger. However, the overall correct integration was much lower (up to 10%) (112) due to lack of optimized protocols suitable for primary cells for SCNT.

Third, we inverted the usual arrangement of DNA insert. Most protocols for target mutation usually have the gene of interest in the 5' and the selection marker downstream with its own promoter. Using conventional cassettes in addition to dual CRISPR protocol, as low as 13.6% of the colonies have the insert in the right place as reported by Chen (113).

This means that most cells are surviving because of random integration of the expression/selection cassette, making cloning and genotyping necessary. In our experiments for example, there were 5 copies of the insert per cell, but only one in the correct location. This shows that a lot of other cells could have survived if a selection marker with promoter were used, decreasing the number of correct inserted cells in the overall recovered amount.

In addition to that, there is no safe way to guaranty that selection marker will be removed from the cell in the second step, unless other cloning step is performed. In the same manuscript Chen used dual CRISPR for gene replacement, only 42.5% of clones had the selection marker removed after Cre recombinase (113). In combination with the first step, 2 sets of cloning in the same cell line were required, which would be impossible in pig primary cells for somatic cell nuclear transfer.

This inversion allowed 100% biallelic gene swap in integration experiments, and 100% single allele integration in gene replacement experiments. Further protocol optimization is being done to allow 100% biallelic gene insertion in both kinds of experiments.

Since we did not get the same result when adding human thrombomodulin to the final insert, the hypothesis that the high similarity between pig and human thrombomodulin was impairing homologous recombination in the homology arms region was made. As the arm had only 60bp and the thrombomodulin gene has 1.8kb, the homology could be happening inside this gene.

To try to avoid this from happening, we customized the human thrombomodulin by changing the nucleotides but not changing the amino acids from the protein. This was made by codon optimization for pig base usage in a way that the least amount of homology was obtained. After codon modification, there was no homology in BLAST alignment between new human thrombomodulin sequence and pig thrombomodulin.

In spite of this effort, we still did not get any colony after selecting only biallelic inserted cells with different antibiotic selection marker.

Further protocol optimization is being done to allow pig–to-human biallelic gene replacement.

Fourth, by incorporating the in-frame stop/off-frame start codon trap, if the insert was integrated in the coding sequence of a random gene, the stop trap would interrupt selection marker to be translated. Also, if the insert was integrated after a random promoter, before transcription initiation, an off-frame start codon would initiate off-frame RNA translation of the selection marker, again, not allowing antibiotic resistance gene to be expressed by the cell line. In addition to that, the trap is maintained into the insert if NHEJ happens in the targeted location, causing an off-frame expression of selection marker through this endogenous promoter,

generating a truncated protein and cell death by the antibiotic in the media. This allowed the cell population to have the exact same genotype at the target location (HR) and same expression profile, since no random integrated inserts contributed to human thrombomodulin expression.

Fifth, we utilized serine recombinase PhiC31 for removal of the selection marker. With the promoter outside strategy for cell selection, only cells with correct insert and recombination will express the selection markers in the first step and the gene of interest in the second step respectively.

After recombination, this site becomes inactive in the absence of co-factors; so new recombination in successive experiments would not be affected.

This is particular important in the case of manipulating SLA class I, since there are several genes close together, so persistence of an active site could impair to ability to do multiple alterations in this segment, which would not be possible with more commonly used tyrosine recombinases (Cre, FLP). In this family of recombinases, the recombinase site remains active, even after recombination.

This is the first time in our knowledge of a promoter outside strategy using PhiC31 recombinase. This site is usually 250bp long and has an ATG sequence inside of it, so this approach in the full size is impossible.

Some reports states that the full size recombinase site is more efficient than the minimal size (114). So a balance in the size of recombinase site and absence of ATG sequence was important for the high efficiency of our protocol.

When the gene of interest was hidden behind the selection cassette, we could note that the efficiency of removal of the cassette using codon optimized PhiC31 recombinase site and modified sites (size) was very high (limited probably only by transfection efficiency of the cell line). Previous in vitro studies with excision using the most commonly used Cre/ LoxP and FLP/FRT excision showed that Cre would only recombine 70% of substrate against 100% of FLP, however, less quantity of enzyme was necessary (115).

One in vivo study comparing efficiencies of resolution of Cre, FLP, FLPo, PhiC31 and PhiC31o showed that the efficiencies of recombination of PhiC31o were similar to Cre, in about 80% of clones (102). However, this study used integrated expression cassette of the recombinase, while in our study we use transient expression of the recombinase, showing very efficient recombination of a bigger cassette.

More importantly, in this protocol we achieved a clonal expression pattern without cloning cells, showed by at least one copy per cell in the correct location by digital droplet PCR, and the perfect match of expression profile of clone and sorted non-clonal cells.

Copy number variation (CNV) detection using real-time PCR presents procedural difficulties (116).

In this method, quantitative data is acquired from the cycle threshold (CT), a point where the fluorescent signal increases above background. External calibrators or comparison to endogenous controls are necessary to evaluate the

amount of an unknown. Amplification efficiencies affect Ct values, reducing the accuracy of this method for absolute quantitation (103).

Digital PCR is a method based on dilution of template DNA to independent non-interacting partitions (117). Inside each of these partitions, template DNA can be amplified if present or not amplified if not present.

As digital PCR relies on a binary end-point, either positive or negative (one or zero, respectively), amplification efficiencies has little interference in the evaluation of DNA copy number (103).

The utilization of different fluorescent probes permits simultaneous evaluation of a particular DNA region of interest (ROI) in comparison to a reference amplicon in a single reaction (103), allowing single reaction CNV evaluation.

Although ddPCR was used before to estimate copy number, to our knowledge, this is the first time it was utilized to estimate correct integrated copy number in a non-clonal population, because there is a lack of models for this kind of integration.

In addition to thrombomodulin gene replacement, this new technique (CGT) was also tested in B2M gene. Our data show that it inserted the gene in the correct location as predicted, however, because of an experimental design problem, sequencing presented mix peaks.

This problem was caused because the PAM sequence from the target sequence, which was removed from the 5’ Homology arm (ATG sequence) from the insert, was re-integrated once this arm was cloned using AscI restriction enzyme. With this, the CRISPR used to target pig B2M ATG region, could cut this

pig genomic region, in addition to the insert (before and after integration). This resulted in random integration of the insert expressing the selection marker, as well as correct integrated gene to have different genotype in different cells because of non-homologous end-joining correction after gene integration.

Although the results were not perfect, we could show that B2M promoter also works in driving the selection marker to allow cell selection.

By utilizing this approach, cell culture procedure is shortened and made it easy, since no cloning is necessary, and a single cell-sorting round is necessary to select the cells with transgene expression.

The only limitation of this model is the necessity of an active promoter that is strong enough to drive the antibiotic resistance gene. Although tested with some limitations, these requirements were met in replacement of pig to human thrombomodulin, B2M and Swine Leukocyte Antigen (SLA) class I to Human Leukocyte Antigen (HLA) class I (data not shown) in aortic endothelial cell lines.

For all the mentioned reasons, this model would aid in the making of not only animals for xenotransplant, but as well for disease models.

One good example of this would be Huntington's disease. To date, there is not a single pig model with consistent expression of the transgenic protein and correlated symptoms, mainly because random integration and silencing possibility (118).

Since the phenotype can only be analyzed after years, it is difficult and expensive to screen several animals for the expected symptoms in traditional random integration model.

In addition to that, Huntington's disease has several phenotypes, based on the length of CG repeats of human gene.

Our technique could be use to generate pigs with same expression pattern and different CG repeats to evaluate clinical symptoms in older age, since the only variable would be the CG repeats, instead of copy number, positional effect, expression in the target tissue and silencing over time as seen in conventional models.

In summary, this new "clonal gene transplant" model is easy, fast, works with different genes, consistent, and could become the new standard for generation of transgenic animals.

6. CONCLUSIONS

6. Conclusions

- Pig aortic endothelial cells have a high transfection efficiency, long life span, and good cloning capabilities
- Pig aortic endothelial cells are ideal for generation of transgenic pigs that express endothelial transgenes
- This new technique (Clonal Gene Transplant) was feasible, easy to perform and reproducible for replacement of pig to human thrombomodulin using pig endogenous promoter
- The technique was also possible in other gene as shown in replacement of pig B2M to human B2M, in spite of some experiment design problems
- Gene expression was at least the same as the human cell controls, even though most transgenic pig cell lines only presented one copy of the insert in the right location instead of two copies
- This technique will allow generation of transgenic animals with predictable expression pattern not susceptible to loss of expression over time
- It will also save time and money because transgenic animal screening and re-cloning will not be required

7. REFERENCES

7. References

1. Fritscher-Ravens A, Ghanbari A, Cuming T, Kahle E, Niemann H, Koehler P, et al. Comparative study of NOTES alone vs. EUS-guided NOTES procedures. Endoscopy. 2008;40(11):925-30.

2. Kragh PM, Nielsen AL, Li J, Du Y, Lin L, Schmidt M, et al. Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. Transgenic Res. 2009;18(4):545-58.

3. Niemann H, Kues WA. Transgenic farm animals: an update. Reprod Fertil Dev. 2007;19(6):762-70.

4. Petters RM, Alexander CA, Wells KD, Collins EB, Sommer JR, Blanton MR, et al. Genetically engineered large animal model for studying cone photoreceptor survival and degeneration in retinitis pigmentosa. Nat Biotechnol. 1997;15(10):965-70.

5. Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, et al. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science. 2008;321(5897):1837-41.

6. Gun G, Kues WA. Current progress of genetically engineered pig models for biomedical research. Biores Open Access. 2014;3(6):255-64.

7. Stoltz DA, Rokhlina T, Ernst SE, Pezzulo AA, Ostedgaard LS, Karp PH, et al. Intestinal CFTR expression alleviates meconium ileus in cystic fibrosis pigs. J Clin Invest. 2013;123(6):2685-93.

8. Ostedgaard LS, Meyerholz DK, Chen JH, Pezzulo AA, Karp PH, Rokhlina T, et al. The DeltaF508 mutation causes CFTR misprocessing and cystic fibrosis-like disease in pigs. Sci Transl Med. 2011;3(74):74ra24.

9. Umeyama K, Watanabe M, Saito H, Kurome M, Tohi S, Matsunari H, et al. Dominant-negative mutant hepatocyte nuclear factor 1alpha induces diabetes in transgenic-cloned pigs. Transgenic Res. 2009;18(5):697-706.

10. Renner S, Braun-Reichhart C, Blutke A, Herbach N, Emrich D, Streckel E, et al. Permanent neonatal diabetes in INS(C94Y) transgenic pigs. Diabetes. 2013;62(5):1505-11.

11. Ramsoondar J, Mendicino M, Phelps C, Vaught T, Ball S, Monahan J, et al. Targeted disruption of the porcine immunoglobulin kappa light chain locus. Transgenic Res. 2011;20(3):643-53.

12. Mendicino M, Ramsoondar J, Phelps C, Vaught T, Ball S, LeRoith T, et al. Generation of antibody- and B cell-deficient pigs by targeted disruption of the J-region gene segment of the heavy chain locus. Transgenic Res. 2011;20(3):625-41.

13. Bongoni AK, Kiermeir D, Jenni H, Bahr A, Ayares D, Klymiuk N, et al. Complement dependent early immunological responses during ex vivo xenoperfusion of hCD46/HLA-E double transgenic pig forelimbs with human blood. Xenotransplantation. 2014;21(3):230-43.

14. Watanabe M, Nakano K, Matsunari H, Matsuda T, Maehara M, Kanai T, et al. Generation of interleukin-2 receptor gamma gene knockout pigs from somatic cells genetically modified by zinc finger nuclease-encoding mRNA. PLoS One. 2013;8(10):e76478.

15. Huang J, Guo X, Fan N, Song J, Zhao B, Ouyang Z, et al. RAG1/2 knockout pigs with severe combined immunodeficiency. J Immunol. 2014;193(3):1496-503.

16. Yang D, Wang CE, Zhao B, Li W, Ouyang Z, Liu Z, et al. Expression of Huntington's disease protein results in apoptotic neurons in the brains of cloned transgenic pigs. Hum Mol Genet. 2010;19(20):3983-94.

17. Baxa M, Hruska-Plochan M, Juhas S, Vodicka P, Pavlok A, Juhasova J, et al. A transgenic minipig model of Huntington's Disease. J Huntingtons Dis. 2013;2(1):47-68.

18. Jakobsen JE, Johansen MG, Schmidt M, Dagnaes-Hansen F, Dam K, Gunnarsson A, et al. Generation of minipigs with targeted transgene insertion by recombinase-mediated cassette exchange (RMCE) and somatic cell nuclear transfer (SCNT). Transgenic Res. 2013;22(4):709-23.

19. Luo Y, Li J, Liu Y, Lin L, Du Y, Li S, et al. High efficiency of BRCA1 knockout using rAAV-mediated gene targeting: developing a pig model for breast cancer. Transgenic Res. 2011;20(5):975-88.

20. Sieren JC, Meyerholz DK, Wang XJ, Davis BT, Newell JD, Jr., Hammond E, et al. Development and translational imaging of a TP53 porcine tumorigenesis model. J Clin Invest. 2014;124(9):4052-66.

21. Flisikowska T, Merkl C, Landmann M, Eser S, Rezaei N, Cui X, et al. A porcine model of familial adenomatous polyposis. Gastroenterology. 2012;143(5):1173-5 e1-7.

22. Sommer JR, Estrada JL, Collins EB, Bedell M, Alexander CA, Yang Z, et al. Production of ELOVL4 transgenic pigs: a large animal model for Stargardt-like macular degeneration. Br J Ophthalmol. 2011;95(12):1749-54.

23. Scott PA, Fernandez de Castro JP, Kaplan HJ, McCall MA. A Pro23His mutation alters prenatal rod photoreceptor morphology in a transgenic swine model of retinitis pigmentosa. Invest Ophthalmol Vis Sci. 2014;55(4):2452-9.

24. Al-Mashhadi RH, Sorensen CB, Kragh PM, Christoffersen C, Mortensen MB, Tolbod LP, et al. Familial hypercholesterolemia and atherosclerosis in cloned minipigs created by DNA transposition of a human PCSK9 gain-of-function mutant. Sci Transl Med. 2013;5(166):166ra1.

25. Cooper DK, Gollackner B, Sachs DH. Will the pig solve the transplantation backlog? Annu Rev Med. 2002;53:133-47.

26. Reemtsma K, McCracken BH, Schlegel JU, Pearl MA, Pearce CW, Dewitt CW, et al. Renal Heterotransplantation in Man. Ann Surg. 1964;160:384-410.

27. Makowka L, Gordon RD, Todo S, Ohkohchi N, Marsh JW, Tzakis AG, et al. Analysis of donor criteria for the prediction of outcome in clinical liver transplantation. Transplant Proc. 1987;19(1 Pt 3):2378-82.

28. Gundlach M, Broering D, Topp S, Sterneck M, Rogiers X. Split-cava technique: liver splitting for two adult recipients. Liver Transpl. 2000;6(6):703-6.

29. Rogiers X, Malago M, Gawad K, Jauch KW, Olausson M, Knoefel WT, et al. In situ splitting of cadaveric livers. The ultimate expansion of a limited donor pool. Ann Surg. 1996;224(3):331-9; discussion 9-41.

30. DeVita MA, Vukmir R, Snyder JV, Graziano C. Procuring organs from a non-heart-beating cadaver: a case report. Kennedy Inst Ethics J. 1993;3(4):371-85.

31. Casavilla A, Ramirez C, Shapiro R, Nghiem D, Miracle K, Bronsther O, et al. Experience with liver and kidney allografts from non-heart-beating donors. Transplantation. 1995;59(2):197-203.

32. Cooper DK, Ekser B, Ramsoondar J, Phelps C, Ayares D. The role of genetically engineered pigs in xenotransplantation research. J Pathol. 2016;238(2):288-99.

33. Cooper DK. A brief history of cross-species organ transplantation. Proc (Bayl Univ Med Cent). 2012;25(1):49-57.

34. White DJ, Calne RY. [Xenotransplantation]. Chirurg. 1996;67(4):324-30.

35. Cooper DK. Xenoantigens and xenoantibodies. Xenotransplantation. 1998;5(1):6-17.

36. Galvao FH, Pompeu E, de Mello ES, da Costa Lino Costa A, Mory E, Dos Santos RM, et al. Experimental multivisceral xenotransplantation. Xenotransplantation. 2008;15(3):184-90.

37. Carrington CA, Richards AC, van den Bogaerde J, Tucker AW, White DJ. Complement activation, its consequences, and blockade by gene transfer. World J Surg. 1997;21(9):907-12.

38. Loss M, Przemeck M, Schmidtko J, Arends H, Kunz R, Jager K, et al. Long-term survival of cynomolgus monkeys following pig-to-primate kidney xenotransplantation using h-DAF transgenic donor organs. Transplant Proc. 2000;32(5):1095-6.

39. Hecker JM, Lorenz R, Appiah R, Vangerow B, Loss M, Kunz R, et al. C1-inhibitor for prophylaxis of xenograft rejection after pig to cynomolgus monkey kidney transplantation. Transplantation. 2002;73(5):688-94.

40. Cowan PJ, Chen CG, Shinkel TA, Fisicaro N, Salvaris E, Aminian A, et al. Knock out of alpha1,3-galactosyltransferase or expression of alpha1,2-fucosyltransferase further protects CD55- and CD59-expressing mouse hearts in an ex vivo model of xenograft rejection. Transplantation. 1998;65(12):1599-604.

41. van den Bosch M, Lohman PH, Pastink A. DNA double-strand break repair by homologous recombination. Biol Chem. 2002;383(6):873-92.

42. Barnes DE. Non-homologous end joining as a mechanism of DNA repair. Curr Biol. 2001;11(12):R455-7.

43. Wei C, Liu J, Yu Z, Zhang B, Gao G, Jiao R. TALEN or Cas9 - rapid, efficient and specific choices for genome modifications. J Genet Genomics. 2013;40(6):281-9.

44. Whyte JJ, Prather RS. Cell Biology Symposium: Zinc finger nucleases to create custom-designed modifications in the swine (Sus scrofa) genome. J Anim Sci. 2012;90(4):1111-7.

45. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, et al. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature. 2000;407(6800):86-90.

46. Whitelaw CB. Transgenic livestock made easy. Trends Biotechnol. 2004;22(4):157-9; discussion 9-60.

47. Butler JR, Ladowski JM, Martens GR, Tector M, Tector AJ. Recent advances in genome editing and creation of genetically modified pigs. Int J Surg. 2015;23(Pt B):217-22.

48. Liu Q, Segal DJ, Ghiara JB, Barbas CF, 3rd. Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. Proc Natl Acad Sci U S A. 1997;94(11):5525-30.

49. Beerli RR, Segal DJ, Dreier B, Barbas CF, 3rd. Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. Proc Natl Acad Sci U S A. 1998;95(25):14628-33.

50. Beerli RR, Dreier B, Barbas CF, 3rd. Positive and negative regulation of endogenous genes by designed transcription factors. Proc Natl Acad Sci U S A. 2000;97(4):1495-500.

51. Beerli RR, Barbas CF, 3rd. Engineering polydactyl zinc-finger transcription factors. Nat Biotechnol. 2002;20(2):135-41.

52. Bhakta MS, Henry IM, Ousterout DG, Das KT, Lockwood SH, Meckler JF, et al. Highly active zinc-finger nucleases by extended modular assembly. Genome Res. 2013;23(3):530-8.

53. Kim S, Lee MJ, Kim H, Kang M, Kim JS. Preassembled zinc-finger arrays for rapid construction of ZFNs. Nat Methods. 2011;8(1):7.

54. Gonzalez B, Schwimmer LJ, Fuller RP, Ye Y, Asawapornmongkol L, Barbas CF, 3rd. Modular system for the construction of zinc-finger libraries and proteins. Nat Protoc. 2010;5(4):791-810.

55. Bibikova M, Golic M, Golic KG, Carroll D. Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases. Genetics. 2002;161(3):1169-75.

56. Bibikova M, Beumer K, Trautman JK, Carroll D. Enhancing gene targeting with designed zinc finger nucleases. Science. 2003;300(5620):764.

57. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. Nat Rev Genet. 2010;11(9):636-46.

58. Cradick TJ, Ambrosini G, Iseli C, Bucher P, McCaffrey AP. ZFN-site searches genomes for zinc finger nuclease target sites and off-target sites. BMC Bioinformatics. 2011;12:152.

59. Mak AN, Bradley P, Cernadas RA, Bogdanove AJ, Stoddard BL. The crystal struct
ure of TAL effector PthXo1 bound to its DNA target. Science. 2012;335(6069):716-9.

60. Deng D, Yan C, Pan X, Mahfouz M, Wang J, Zhu JK, et al. Structural basis for sequence-specific recognition of DNA by TAL effectors. Science. 2012;335(6069):720-3.

61. Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, et al. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic acids research. 2011;39(12):e82.

62. Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK. FLASH assembly of TALENs for high-throughput genome editing. Nat Biotechnol. 2012;30(5):460-5.

63. Briggs AW, Rios X, Chari R, Yang L, Zhang F, Mali P, et al. Iterative capped assembly: rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. Nucleic acids research. 2012;40(15):e117.

64. Schmid-Burgk JL, Schmidt T, Kaiser V, Honing K, Hornung V. A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes. Nat Biotechnol. 2013;31(1):76-81.

65. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-23.

66. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol. 1987;169(12):5429-33.

67. Wiedenheft B, Sternberg SH, Doudna JA. RNA-guided genetic silencing systems in bacteria and archaea. Nature. 2012;482(7385):331-8.

68. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature. 2011;471(7340):602-7.

69. Li P, Estrada JL, Burlak C, Montgomery J, Butler JR, Santos RM, et al. Efficient generation of genetically distinct pigs in a single pregnancy using multiplexed single-guide RNA and carbohydrate selection. Xenotransplantation. 2015;22(1):20-31.

70. Shimizu A, Hisashi Y, Kuwaki K, Tseng YL, Dor FJ, Houser SL, et al. Thrombotic microangiopathy associated with humoral rejection of cardiac xenografts from alpha1,3-galactosyltransferase gene-knockout pigs in baboons. Am J Pathol. 2008;172(6):1471-81.

71. Cowan PJ, Roussel JC, d'Apice AJ. The vascular and coagulation issues in xenotransplantation. Current opinion in organ transplantation. 2009;14(2):161-7.

72. Galili U. Discovery of the natural anti-Gal antibody and its past and future relevance to medicine. Xenotransplantation. 2013;20(3):138-47.

73. Salama A, Evanno G, Harb J, Soulillou JP. Potential deleterious role of anti-Neu5Gc antibodies in xenotransplantation. Xenotransplantation. 2015;22(2):85-94.

74. Burlak C, Paris LL, Lutz AJ, Sidner RA, Estrada J, Li P, et al. Reduced binding of human antibodies to cells from GGTA1/CMAH KO pigs. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2014;14(8):1895-900.

75. Estrada JL, Martens G, Li P, Adams A, Newell KA, Ford ML, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/beta4GalNT2 genes. Xenotransplantation. 2015;22(3):194-202.

76. Robson SC, Cooper DK, d'Apice AJ. Disordered regulation of coagulation and platelet activation in xenotransplantation. Xenotransplantation. 2000;7(3):166-76.

77. Buhler L, Basker M, Alwayn IP, Goepfert C, Kitamura H, Kawai T, et al. Coagulation and thrombotic disorders associated with pig organ and hematopoietic cell transplantation in nonhuman primates. Transplantation. 2000;70(9):1323-31.

78. Crikis S, Cowan PJ, d'Apice AJ. Intravascular thrombosis in discordant xenotransplantation. Transplantation. 2006;82(9):1119-23.

79. Ierino FL, Kozlowski T, Siegel JB, Shimizu A, Colvin RB, Banerjee PT, et al. Disseminated intravascular coagulation in association with the delayed rejection of pig-to-baboon renal xenografts. Transplantation. 1998;66(11):1439-50.

80. Ramackers W, Klose J, Winkler M. Xeno-kidney transplantation: from idea to reality. Transplant Proc. 2012;44(6):1773-5.

81. Esmon CT, Esmon NL. The link between vascular features and thrombosis. Annu Rev Physiol. 2011;73:503-14.

82. Ito T, Maruyama I. Thrombomodulin: protectorate God of the vasculature in thrombosis and inflammation. J Thromb Haemost. 2011;9 Suppl 1:168-73.

83. Wang L, Jiang R, Sun XL. Recombinant thrombomodulin of different domains for pharmaceutical, biomedical, and cell transplantation applications. Med Res Rev. 2014;34(3):479-502.

84. Roussel JC, Moran CJ, Salvaris EJ, Nandurkar HH, d'Apice AJ, Cowan PJ. Pig thrombomodulin binds human thrombin but is a poor cofactor for activation of human protein C and TAFI. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2008;8(6):1101-12.

85. Iwase H, Liu H, Wijkstrom M, Zhou H, Singh J, Hara H, et al. Pig kidney graft survival in a baboon for 136 days: longest life-supporting organ graft survival to date. Xenotransplantation. 2015;22(4):302-9.

86. Mohiuddin MM, Reichart B, Byrne GW, McGregor CG. Current status of pig heart xenotransplantation. Int J Surg. 2015;23(Pt B):234-9.

87. Cooper DK, Satyananda V, Ekser B, van der Windt DJ, Hara H, Ezzelarab MB, et al. Progress in pig-to-non-human primate transplantation models (1998-2013): a comprehensive review of the literature. Xenotransplantation. 2014;21(5):397-419.

88. Mohiuddin MM, Corcoran PC, Singh AK, Azimzadeh A, Hoyt RF, Jr., Thomas ML, et al. B-cell depletion extends the survival of GTKO.hCD46Tg pig heart xenografts in baboons for up to 8 months. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2012;12(3):763-71.

89. Kuwaki K, Tseng YL, Dor FJ, Shimizu A, Houser SL, Sanderson TM, et al. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. Nat Med. 2005;11(1):29-31.

90. Mohiuddin MM, Singh AK, Corcoran PC, Hoyt RF, Thomas ML, 3rd, Lewis BG, et al. Role of anti-CD40 antibody-mediated costimulation blockade on non-Gal antibody production and heterotopic cardiac xenograft survival in a GTKO.hCD46Tg pig-to-baboon model. Xenotransplantation. 2014;21(1):35-45.

91. Mohiuddin MM, Singh AK, Corcoran PC, Hoyt RF, Thomas ML, 3rd, Ayares D, et al. Genetically engineered pigs and target-specific immunomodulation provide significant graft survival and hope for clinical cardiac xenotransplantation. J Thorac Cardiovasc Surg. 2014;148(3):1106-13; discussion 13-4.

92. Baldan N, Rigotti P, Calabrese F, Cadrobbi R, Dedja A, Iacopetti I, et al. Ureteral stenosis in HDAF pig-to-primate renal xenotransplantation: a phenomenon related to immunological events? American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2004;4(4):475-81.

93. Hall VJ. Early development of the porcine embryo: the importance of cell signalling in development of pluripotent cell lines. Reprod Fertil Dev. 2012;25(1):94-102.

94. Nowak-Imialek M, Niemann H. Pluripotent cells in farm animals: state of the art and future perspectives. Reprod Fertil Dev. 2012;25(1):103-28.

95. Kong Q, Wu M, Huan Y, Zhang L, Liu H, Bou G, et al. Transgene expression is associated with copy number and cytomegalovirus promoter methylation in transgenic pigs. PLoS One. 2009;4(8):e6679.

96. Zhu J, Park CW, Sjeklocha L, Kren BT, Steer CJ. High-level genomic integration, epigenetic changes, and expression of sleeping beauty transgene. Biochemistry. 2010;49(7):1507-21.

97. Garrels W, Mates L, Holler S, Dalda A, Taylor U, Petersen B, et al. Germline transgenic pigs by Sleeping Beauty transposition in porcine zygotes and targeted integration in the pig genome. PLoS One. 2011;6(8):e23573.

98. Raife TJ, Dwyre DM, Stevens JW, Erger RA, Leo L, Wilson KM, et al. Human thrombomodulin knock-in mice reveal differential effects of human thrombomodulin on thrombosis and atherosclerosis. Arterioscler Thromb Vasc Biol. 2011;31(11):2509-17.

99. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell. 2003;115(3):281-92.

100. Telugu BP, Ezashi T, Roberts RM. The promise of stem cell research in pigs and other ungulate species. Stem Cell Rev. 2010;6(1):31-41.

101. Waghmare SK, Estrada J, Reyes L, Li P, Ivary B, Sidner RA, et al. Gene targeting and cloning in pigs using fetal liver derived cells. J Surg Res. 2011;171(2):e223-9.

102. Raymond CS, Soriano P. High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells. PLoS One. 2007;2(1):e162.

103. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem. 2011;83(22):8604-10.

104. Miwa Y, Yamamoto K, Onishi A, Iwamoto M, Yazaki S, Haneda M, et al. Potential value of human thrombomodulin and DAF expression for coagulation control in pig-to-human xenotransplantation. Xenotransplantation. 2010;17(1):26-37.

105. Swindle MM, Makin A, Herron AJ, Clubb FJ, Jr., Frazier KS. Swine as models in biomedical research and toxicology testing. Vet Pathol. 2012;49(2):344-56.

106. Andrews WW, Ojeda SR. A detailed analysis of the serum luteinizing hormone secretory profile in conscious, free-moving female rats during the time of puberty. Endocrinology. 1981;109(6):2032-9.

107. Murray SA, Morgan JL, Kane C, Sharma Y, Heffner CS, Lake J, et al. Mouse gestation length is genetically determined. PLoS One. 2010;5(8):e12418.

108. Petersen B, Ramackers W, Tiede A, Lucas-Hahn A, Herrmann D, Barg-Kues B, et al. Pigs transgenic for human thrombomodulin have elevated production of activated protein C. Xenotransplantation. 2009;16(6):486-95.

109. Yazaki S, Iwamoto M, Onishi A, Miwa Y, Hashimoto M, Oishi T, et al. Production of cloned pigs expressing human thrombomodulin in endothelial cells. Xenotransplantation. 2012;19(2):82-91.

110. Wuensch A, Baehr A, Bongoni AK, Kemter E, Blutke A, Baars W, et al. Regulatory Sequences of the Porcine THBD Gene Facilitate Endothelial-Specific Expression of Bioactive Human Thrombomodulin in Single- and Multitransgenic Pigs. Transplantation. 2014;97(2):138-47.

111. Iwase H, Ekser B, Satyananda V, Bhama J, Hara H, Ezzelarab M, et al. Pig-to-baboon heterotopic heart transplantation--exploratory preliminary experience with pigs transgenic for human thrombomodulin and comparison of three costimulation blockade-based regimens. Xenotransplantation. 2015;22(3):211-20.

112. Orlando SJ, Santiago Y, DeKelver RC, Freyvert Y, Boydston EA, Moehle EA, et al. Zinc-finger nuclease-driven targeted integration into mammalian genomes using donors with limited chromosomal homology. Nucleic acids research. 2010;38(15):e152.

113. Chen Y, Cao J, Xiong M, Petersen AJ, Dong Y, Tao Y, et al. Engineering Human Stem Cell Lines with Inducible Gene Knockout using CRISPR/Cas9. Cell Stem Cell. 2015;17(2):233-44.

114. Groth AC, Olivares EC, Thyagarajan B, Calos MP. A phage integrase directs efficient site-specific integration in human cells. Proc Natl Acad Sci U S A. 2000;97(11):5995-6000.

115. Ringrose L, Lounnas V, Ehrlich L, Buchholz F, Wade R, Stewart AF. Comparative kinetic analysis of FLP and cre recombinases: mathematical models for DNA binding and recombination. Journal of molecular biology. 1998;284(2):363-84.

116. Aldhous MC, Abu Bakar S, Prescott NJ, Palla R, Soo K, Mansfield JC, et al. Measurement methods and accuracy in copy number variation: failure to replicate associations of beta-defensin copy number with Crohn's disease. Hum Mol Genet. 2010;19(24):4930-8.

117. Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA. Quantitation of targets for PCR by use of limiting dilution. Biotechniques. 1992;13(3):444-9.

118. Holm IE, Alstrup AK, Luo Y. Genetically modified pig models for neurodegenerative disorders. J Pathol. 2016;238(2):267-87.

8. APPENDIX

DNA sequences utilized in the study

Human codon optimized human thrombomodulin:

ATGCTGGGTGTGCTCGTCCTGGGCGCCCTGGCATTGGCGGGGCTGGGTTTC
CCTGCCCCCGCTGAGCCCCAACCCGGCGGGTCTCAGTGTGTGGAACACGAT
TGTTTCGCCCTGTATCCAGGACCAGCCACCTTTCTGAACGCCTCTCAGATCTG
TGACGGACTGAGAGGACATCTCATGACAGTCAGGTCATCCGTCGCCGCCGAC
GTGATTAGCCTCCTGCTCAATGGAGACGGAGGCGTGGGCAGGAGAAGGCTG
TGGATTGGACTCCAGCTGCCTCCAGGATGTGGAGACCCAAAAAGACTGGGCC
CACTCAGAGGATTTCAGTGGGTGACCGGCGATAATAACACATCCTATTCCCG
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GCCGTGAGCGCTGCCGAAGCTACCGTGCCCTCCGAACCCATTTGGGAAGAG
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Modified PhiC31 AttP sequence:

AGAAGCGGTTTTCGGGAGTAGTGCCCCAACTGGGGTAACCTTTGAGTTCTCT
CAGTTGGGGGCGTAGGGTCGCCGACATGACAC

Modified PhiC31 AttB sequence:
TCACGGTCTCGAAGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCC
CGGGCGCGTACTCCACCTCACCCATCTGGTCCA

60 bp 5' Homology arm:
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GCTGACACC

2548 bp 5' Homology arm:
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Puromycin-hTHBD insert:

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9. ATTACHMENTS

Faculdade de Medicina da Universidade de São Paulo
Avenida Dr. Arnaldo, 455
Pacaembu – São Paulo – SP

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificamos que o projeto intitulado **"Geração de linha de células primárias de porco para xenotransplante com expressão de trombomodulina humana usando promoter endógeno nos dois alelos"** protocolo nº **166/15** sob a responsabilidade de **Luiz Augusto Carneiro DAlbuquerque** e **Rafael Miyashiro Nunes dos Santos**, apresentado pelo Departamento de Cirurgia - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Faculdade de Medicina da USP em reunião de 02.12.15

Vigência do Projeto	Novembro/2016
Espécie/linhagem	Células endoteliais de aorta de suínos
Nº de animais	Não se aplica
Peso/Idade	Não se aplica
Sexo	Não se aplica
Origem	Não se aplica

CEUA-FMUSP, 02 de Dezembro de 2015

Dr. Eduardo Pompeu
Coordenador
Comissão de Ética no Uso de Animais

Version: 8. 2012

Indiana University – School of Medicine IACUC
Institutional Animal Care and Use Committee (IACUC) Animal Protocol Review Form

For IACUC Office Use Only					
Protocol Number:	10447 MD/R		Old Protocol Number:	10021	
Approval Date:	11/28/2012	3-Year Expiration Date:	11/28/2015		
Amendment #:		Amendment Approval Date			

Instructions

Sections - A through **Section - I** are the base protocol and required for all protocols. **Sections – J** through **Section - W** apply dependent on what procedures any animal may undergo on this protocol. Please click on each box and enter the information requested.

Section A - Principle Investigator and General Protocol Information

Please select one. If you are amending at the time of continuation, you will need to submit 60 days before the continuation date.

☐ New Study ☒ Amendment Complete the **amendment summary below** ☐ Continuing Review Complete the continuing review **Section X** at the end of this form ☐ Pilot Study

Amendment Summary of Changes
In this section please summarize all changes proposed to the protocol in this amendment, and then edit the appropriate sections to reflect those changes. **IMPORTANT:** Study amendments **MAY NOT** be instituted until written approval is received from the School of Medicine Animal Care and Use Committee (IACUC). Remember to update your searches to document the proposed changes are not duplicative and that alternatives are not available.

A1 – withdrawn 03/03/2013

A2 – Approved 03/28/2013
Addition of 70 new pigs, 32 wildtype pigs and 32 ASGR1-/- pigs, and 6 CD55-GGTA1$^{-/-}$/CMAH$^{-/}$. 3Rs and duplication searches updated, statistics updated. These additions will allow the study of ASGR1 function in xenogeneic platelet phagocytosis in the absence of the GGTA1 genetic modification. The addition of CD55 should decrease xenogeneic human antibody binding to pig cells.

A3 – Approved 6/25/2013
Addition of possible paralytic use during non-survival surgeries
Addition of saline drip line during non-survival surgeries

The saline drip will allow us to maintain blood volume during surgery. We need to use Pancuronium for the porcine multiple organ harvests in order to be able to dissect around the vena cava without diaphragmatic contractions. In the absence of the paralytic, dissection around the vena cava results in violent involuntary contractions that precludes fine dissection near the liver.

Addition of possible paralytic use during non-survival surgeries
Addition of saline drip line during non-survival surgeries
Addition of possible catheterization prior to non-survival surgery

The saline drip will allow us to maintain blood volume during surgery. We need to use paralytic for the porcine multiple organ harvests in order to be able to dissect around the vena cava without diaphragmatic contractions. In the absence of the paralytic, dissection around the vena cava results in violent involuntary contractions that precludes fine dissection near the liver. The catheterization is to minimize possibility of bladder damage that could contaminate body cavity, and internal organs, with urine.

A4 – Approved 12/11/2013
Addition of 32 new pigs 26 CMAH$^{-/-}$/GGTA1$^{-/-}$/iGB3$^{-/-}$ pigs and 6 CMAH$^{-/-}$/GGTA1$^{-/-}$/SLAI$^{-/-}$ pigs. CMAH$^{-/-}$/GGTA1$^{-/-}$/SLAI$^{-/-}$ is a new genotype added. This addition will allow the study of platelet phagocytosis in triple knockout out pig with carbohydrate modifications. The additional deletion of SLAI should decrease human antibody binding to porcine cells. 3Rs and duplication searches updated, statistics updated.
We were already approved for 6 CMAH$^{-/-}$/GGTA1$^{-/-}$/iGB3$^{-/-}$ pigs. These initial 6 were being used in the xenoantigen experiments and will now also be used in the phagocytosis experiments in conjunction with the additional 26 requested.

A5 – Approved 03/24/2014
Removal of blood pressure monitoring during neuromuscular blockade section P; pigs do not have tails to monitor blood pressure accurately. Upon speaking with veterinarian, he advised that heart rate monitoring was sufficient.

A6 – Approved 04/22/2014
Addition of 6 $GGTA1^{-/-}/SLA1^{-/-}$ pigs

These pigs will allow for comparison to the $GGTA1^{-/-}$ pig to see if SLA is a xenoantigen. We are asking to add these animals primarily as a means to remain accurate with our documentation. We had originally requested to add $CMAH^{-/-}/GGTA1^{-/-}/SLAI^{-/-}$. We are now asking for $GGTA1^{-/-}/SLA1^{-/-}$ because when the new litter of pigs was born we noticed that while the CMAH gene was indeed modified, it retained at least partial activity. In other words, we did modify GGTA1, SLA 1 and the CMAH genes, however the CMAH gene retained activity (SLA1 and GGTA1 did not). We did phenotype the cells used to make this litter and believed they were deficient n CMAH activity at the time of nuclear transfer. The failure in phenotyping appears to correspond exactly with the time period of our vendor switching reagents that we use in the CMAH phenotyping. The new reagents behave differently from the reagents we used to use. The fact that the CMAH phenotype differs from what we initially requested does not alter our ability to gain information from these animals pertinent to xenotransplantation. The use of these animals will follow the description in A4.

Additional information explaining importance of SLA-1 xenoantigen study. We plan to use these animals to study human immune responses to proteins synthesized by SLA-1 genes. We hypothesize that these will be antigenic to some humans for the following reasons: 1) SLA-1 proteins are >70% identical to HLA-1 proteins (the human homologs of SLA). 2) Many patients needing organ transplants have high levels of antibodies to HLA-1 proteins as a result of blood transfusions or pregnancies. We have access to antibodies obtained from humans that have abundant levels of HLA-1 specific antibodies and will use these reagents to test our hypothesis.

A7 – Approved 05/22/2014
Change of Principal investigator to A. Joseph Tector from Leela L Paris.

A8 – Approved 08/22/2014
Addition of 6 $GGTA1^{-/-}$,$CMAH^{-/-}$, $B4GALNT\text{-}2^{-/-}$ pigs.
A major aim of this protocol is to identify and eliminate xenoantigens in pigs by genetic engineering. A recent report suggests that the β1,4 N-acetylgalactosaminyl transferase 2 (β 4GALNT-2) gene directs the synthesis of pig xenoantigens . This report identified β 4GALNT-2 as a relevant gene in xenoantigenicity by screening pig gene expression libraries in vitro and probing them with antibodies collected from baboons exposed to pig cardiac tissue. We are asking to add 6 animals to the protocol that lack this gene in order to extend the evaluation of this gene and its role in xenoantigenicity. Human beings have been reported to have antibodies to the product of this gene, but these antibodies have not been evaluated in the xenotransplant setting. We are unable to find reports of production or analysis of pigs containing the proposed genotype.

Byrne, Guerard W., Paul G. Stalboerger, Zeji Du, Tessa R. Davis, and Christopher GA McGregor. "Identification of new carbohydrate and membrane protein antigens in cardiac xenotransplantation." *Transplantation* 91, no. 3 (2011): 287-292.

Administrative Changes – Approved 02/27/2015
Updated IBC protocol number

Administrative update-Approved 03/20/2015
changed protocol associates- removed several PAs added E Nus, J Hooker

Ammendment 9:
Addition of ~~64~~ 39 new pigs: $GGTA1^{-/-}$, $B4GALNT\text{-}2^{-/-}$, $ASGR1^{-/-}$ (n=13); $GGTA1^{-/-}$, $B4GALNT\text{-}2^{-/-}$ (n=13); $CD18^{-/-}$, $GGTA1^{-/-}$, $B4GALNT\text{-}2^{-/-}$, $VWF^{-/-}$ (n=13).

One aspect of this protocol is to examine platelet consumption by pig livers. We are asking for 48 novel pigs to address this question by continuing our in vitro perfusion of pig livers with platelets obtained from non-human primates (NHP). These proposed ex vivo perfusions will be performed to guide xenotransplant studies that will be performed by collaborators at the Yerkes primate center under a separate IACUC protocol. Yerkes has made significant strides in the survival of xenotransplantation of pig tissues into NHP. They have the longest-surviving kidney xenotransplant which continues to survive for more than 5 months [1]. Given the successes we have had at minimizing xenoantigenicity of pig tissues for humans and non-human primates [2], it is now a realistic goal to test liver xenotransplantation in a non-human primate model, which will serve as a pre-clinical model to guide efforts in human xenotransplantation. The Yerkes center will provide the NHP blood needed to complete this study.

These ex vivo studies are necessary because pig livers also destroy non-human primate platelets [3]. This amendment requests the additional pig strains to test three novel pig strains to determine if it is possible to minimize NHP-platelet consumption by pig livers by eliminating platelet receptors from the swine. We are proposing to delete three receptors, one-at-a-time (ASGR1, CD18, VWF). We propose to do this in pigs lacking GGTA1$^{-/-}$ and B4GALNT-2$^{-/-}$ genes because these genes produce the only verified xenoantigens recognized by non-human primates [2]. The three platelet receptors are promising targets because 1) We have shown that eliminating ASGR1 reduces pig liver consumption of human platelets [4]. 2) We have shown CD18 down-regulation reduces pig Kuppfer cell binding of human platelets [5]. 3) VWF is well characterized as a platelet-binding molecule, and incompatibilities between pig VWF and human platelets have been reported [6]. These novel strains will also be tested to determine if antigenic compatibility with the primate immne system is altered by the genetic modifications by drawing blood from the pigs and mixing it with NHP lymphocytes and antibodies. Literature searches do not yield alternative methods that represent the multifactorial environment of the intact liver. This research is not duplicative because the strains of pig are completely novel. Pigs lacking either VWF or ASGR1 have been produced but on different backgrounds and have not been used to examine uptake of NHP platelets. Please see attached and updated literature searches to support this requested amendment in agreement with the 3R principles. We have refined our perfusion analyses to eliminate the need for pig platelet perfusion studies. This reduces the number of needed animals by 50% and allows us to use three fewer experimental animals per group to detect meaningful differences.

1. Higginbotham, L., Mathews, D., Breeden, C. A., Song, M., Farris, A. B., Larsen, C. P., ... & Adams, A. B. (2015). Pre□transplant antibody screening and anti□CD154 costimulation blockade promote long□term xenograft survival in a pig□to□primate kidney transplant model. *Xenotransplantation*.
2. Estrada, Jose L., Greg Martens, Ping Li, Andrew Adams, Kenneth A. Newell, Mandy L. Ford, James R. Butler, Richard Sidner, Matt Tector, and Joseph Tector. "Evaluation of human and non□human primate antibody binding to pig cells lacking GGTA1/CMAH/β4GalNT2 genes." *Xenotransplantation* (2015).
3. Yeh, Heidi, Zurab Machaidze, Isaac Wamala, James W. Fraser, Nalu Navarro□Alvarez, Karen Kim, Christian Schuetz et al. "Increased transfusion□free survival following auxiliary pig liver xenotransplantation." *Xenotransplantation* 21, no. 5 (2014): 454-464.
4. Paris, Leela L., Jose L. Estrada, Ping Li, Ross L. Blankenship, Richard A. Sidner, Luz M. Reyes, Jessica B. Montgomery et al. "Reduced human platelet uptake by pig livers deficient in the asialoglycoprotein receptor 1 protein." *Xenotransplantation* (2015).
5. Chihara, Ray K., Leela L. Paris, Luz M. Reyes, Richard A. Sidner, Jose L. Estrada, Susan M. Downey, Zheng-Yu Wang, A. Joseph Tector, and Christopher Burlak. "Primary porcine Kupffer cell phagocytosis of human platelets involves the CD18 receptor." *Transplantation* 92, no. 7 (2011): 739-744.
6. am Esch, Jan Schulte, Miguel A. Cruz, Jonathan B. Siegel, Josef Anrather, and Simon C. Robson. "Activation of human platelets by the membrane-expressed A1 domain of von Willebrand factor." *Blood* 90, no. 11 (1997): 4425-4437.

If this is a **Replacement Study** for an Expiring Application, Please provide the study number of the expiring study this application will replace	10021

[x] **Research** [ ] **Teaching** [ ] **Other (explain):**

NOTE: For teaching protocol, you must disclose that animal(s) will be used and provide an alternative for those who object to using animals.

Title of Project: The study of pig organs for xenotransplantation

Is this Project Funded? [x] Yes [ ] No

Name of Funding Agency? Departmental funds

The Title of the Funding Project?

Name of PI on Grant:

Note: If funded by VA, please complete the Animal Component of the Research Protocol Form.

Principal Investigator:	A Joseph Tector			Degree(s):	MD, PhD
Campus Address:	MS B005			Department/School:	Surgery
Campus Phone:	2743251	Campus Fax:	2747429	IU Network ID:	Jtector

CO- Principal Investigator:	Jose Estrada	Degree(s):	PhD, DVM
Campus Address:	MS 264	Department/School:	Surgery

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Campus Phone:	2740790	Campus Fax:		IU Network ID:	estradaj

AAALAC Procedures Check List

This Checklist is part of your application. Check those that apply to your research project.			
	Hazardous Materials		Survival Surgery
	Multiple Survival Surgery		Restraint
x	Anesthesia		Lab Housing > 12 hours
	Special Diet or Treating Feed/Water		Special Housing

Investigator Assurance

Completion and signing of this form are the responsibility of the principal investigator. Completion of the approval process will fulfill Public Health Service and USDA requirements under the federal Animal Welfare Act, and will serve to remind users and the public of Indiana University School of Medicine's commitment to humane care and use of animals. In signing this form, I assure the following:

I certify that:

- all studies described in this protocol will be conducted in compliance with all applicable policies & guidelines (i.e., PHS policy, the Animal Welfare Act, "Guide to the Care and Use of Laboratory Animals", applicable University policies, Standard Operating Procedures) described by Indiana School of Medicine IACUC.
- all individuals listed on this protocol who are exposed to the physical risks associated with animal contact (AT RISK) are participating in this institution's Occupational Health & Safety Program.
- if applicable, individuals listed on this protocol have or will have received training in aseptic surgical methods and techniques, and if applicable, in the proper use of anesthetics, analgesics and tranquilizers.
- the individuals listed on this protocol have received training in procedures for reporting animal welfare concerns.
- the IACUC will be notified regarding any unexpected study results that impact the welfare of the animals and that any unanticipated pain or distress, morbidity, or mortality will be reported to the appropriate veterinary staff and the IACUC as soon as possible.
- all procedures, treatments, anesthetic and analgesic regiments will be adhered to as outlined in this protocol. Any deviations will be submitted via an amendment form and not employed until approved.
- the proposed work and animals in this protocol match the proposed work and animals in the sponsored programs identified in the funding section of this protocol.

x	**By checking this box,** I acknowledge responsibility for this protocol and assure that the individuals listed on this protocol are qualified (or will be adequately trained) to conduct the studies described in this protocol in a humane manner.	
Joseph A. Tector		05/16/2014
Typed Name of PI		**Date**

Section B – Research Sites

Will any of the research activities in this protocol be conducted completely or partially in Veteran's Administration (VA) facilities, or conducted in approved off-site locations and facilities by VA researchers while on VA time.	
	Yes Approval is required from the Veterans Administration Research & Development Committee.
X	No

Where will animals be housed?	
x	LARC
	Methodist
	Other:
For Other: Please identify those facilities. Have you submitted an Animal Care and Use Form to those campuses?	
	Yes
	No

Will Live Animals be taken outside the Laboratory Animal Research Center?		
	Yes	If Yes, please complete the rest of this section
x	No	If No, go to next section

Where will animal use occur? Complete the table below to identify each location where animals will go if they leave LARC for any purpose.		
Building and Room Number	Procedure	Location for Records Maintenance

If animals used in this protocol will be housed or held outside of LARC for more than twelve (12) consecutive hours, provide the building name, room, and phone number of contact person in the area. Such areas are subject to inspections by the IACUC, AAALAC site visitors, and regulatory officials.			
Building and Room Number	Number of Animals at Any One Time	Length of time animals will be housed	Phone Number

Scientific Justification for housing animals outside the LARC from more than 12 consecutive hours:

Section C – Personnel Associates

Personnel Qualifications:
In the table below provide the following:

- Information on each person who will be responsible for performing any experimental animal procedure
- List every procedure they will be performing and on which species each will be performed.
- Indicate how they were or will be trained to perform the procedure (hands-on assistance, direct supervision, coursework),
- Who trained or will train them(veterinarian, faculty supervisor, laboratory technician, postdoctoral fellow, graduate student), and
- How much experience they have had performing each procedure (for example, three years, 50 animals)

If you have additional personnel, please copy and paste additional rows as needed. For assistance contact the IACUC office.

PI Name:	Tector, A. Joseph
IU Network ID:	atector
List Experience and what training will take place for people with no prior experience:	Over 15 years in swine research and an internationally recognized liver transplant surgeon. He has performed hundreds of successful swine operations including aortic cannulations, laparotomy, and sternotomy , tissue/organ harvests, and blood draws.
Role on Protocol (surgery, care, order animals, etc.,)	Performing non-survival surgeries on swine, tissue/organ harvesting from swine, and blood draws from swine

Name:	Estrada, Jose
IU Network ID:	EstradaJ
List Experience and what training will take place for people with no prior experience:	Trained in veterinary school. 20 years of experience with animal surgeries including swine and is a faculty supervisor. He will be trained by senior member for swine non-survival surgeries and tissue harvests.
Role on Protocol (surgery, care, order animals, etc.,)	Assisting in swine Surgery, protocol, tissue harvest

Name:	Chihara, Ray K.
IU Network ID:	rchihara
List Experience and what training will take place for people with no prior experience:	Surgical training and at least 3 years of experience in porcine surgeries including aortic cannulations, laparotomy, and sternotomy, swine blood draws, and swine tissue/organ harvest- trained with hands on assistance by A. Joseph Tector.
Role on Protocol (surgery, care, order animals, etc.,)	swine surgeries, swine blood draws, swine prep, swine tissue/ organ harvest

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Name:	Lutz, Andrew
IU Network ID:	alutz
List Experience and what training will take place for people with no prior experience:	Surgical training 1 year experience with porcine non-survival surgeries including aortic cannulations, laparotomy, and sternotomy and swine blood draws; trained by faculty supervisor
Role on Protocol (surgery, care, order animals, etc.,)	Porcine non survival surgeries, swine blood draws, swine tissue/organ harvest, pig prep

Name:	Sidner, Richard
IU Network ID:	rsidner
List Experience and what training will take place for people with no prior experience:	More than 25 years experience doing swine surgeries including aortic cannulations, laparotomy, and sternotomy; was trained by faculty surgeon with hands on assistance.
Role on Protocol (surgery, care, order animals, etc.,)	pig non survival surgeries, pig tissue/organ harvest

Name:	Reyes, Luz
IU Network ID:	luzreyes
List Experience and what training will take place for people with no prior experience:	has 3 years experience in swine non survival surgeries, tissue harvest, and animal prep was trained by Leela Paris; will be trained by Lab technician or faculty supervisor to draw blood from swine
Role on Protocol (surgery, care, order animals, etc.,)	swine non-survival surgeries, swine blood draws, swine tissue/organ harvest,

Name:	Wang, Zheng-Yu
IU Network ID:	zywang
List Experience and what training will take place for people with no prior experience:	more than 20 years of experience mouse models hands on training by faculty supervisor. 1 year experience in pig-non survival surgery, aortic cannulations, laparotomy,and sternotomy, tissue/organ harvest trained by faculty supervisor Christopher Burlak.
Role on Protocol (surgery, care, order animals, etc.,)	swine Surgeries, swine tissue/organ harvest,

Name:	Sanders, Carrie
IU Network ID:	csanders
List Experience and what training will take place for people with no prior experience:	At least 3 years of experience in swine non-survival surgeries including aortic cannulations, laparotomy, and sternotomy, was trained by transplant surgeon faculty supervisor A. Joseph Tector at IUSM
Role on Protocol (surgery, care, order animals, etc.,)	non-survival pig surgeries and pig organ/tissue harvest

Name:	Services, LARC
IU Network ID:	
List Experience and what training will take place for people with no prior experience:	Trained
Role on Protocol (surgery, care, order animals, etc.,)	animal monitor, blood draws, euthanasia, animal disposition, procedures with anesthesia, care of swine, etc

Name:	Blankenship, Ross
IU Network ID:	rlblanke
List Experience and what training will take place for people with no prior experience:	Will be trained by faculty supervisor Leela Paris and technician Sue Downey, surgical resident Andrew Lutz, with hands on access to perform pig non-survival surgeries including aortic cannulations, laparotomy, and sternotomy, pig blood draws, pig organ/tissue harvests
Role on Protocol (surgery, care, order animals, etc.,)	swine blood drawing, swine tissue/organ harvest, swine non-survival surgeries

Name:	Tector, Matthew
IU Network ID:	mtector
List Experience and what training will take place for people with no prior experience:	15 years in large animal research and swine surgeries including aortic cannulations, laparotomy, and sternotomy trained by faculty supervisor with hands on assistance. He will be trained by Sue Downey on swine

	blood draws.
Role on Protocol (surgery, care, order animals, etc.,)	Non-survival surgeries on swine, tissue/organ harvesting, and blood draws on swine

Name:	Downey, Susan
IU Network ID:	sudowney
List Experience and what training will take place for people with no prior experience:	Med Tech, phlebotomy, large animal models, 4 years swine surgical experience trained by faculty supervisors through direct surpervision and hands on assistance to perform non survival surgeries including aortic cannulations, laparotomy, and sternotomy. She was trained by reading a book on pig anatomy for swine blood draws. She has been successfully drawing blood from swine for over a year. She will be trained by LARC staff to anesthetize and euthanize swine.
Role on Protocol (surgery, care, order animals, etc.,)	Non-survival surgeries on pigs, tissue/organ harvesting, and blood draws on pigs, swine anesthesia, swine euthanasia.

Name:	William, C. Goggins
IU Network ID:	wgoggins
List Experience and what training will take place for people with no prior experience:	Board certified transplant surgeon, will be trained by A. Joseph Tector with non-survival pig surgeries including aortic cannulations, laparotomy, sternotomy , and blood draws. Dr. Tector and Su Downey will be responsible for direct supervision.
Role on Protocol (surgery, care, order animals, etc.,)	Swine non survival surgeries, swine blood draws, swine tissue/organ harvest

Name:	Elizabeth Diana Liska
IU Network ID:	eliska@IUhealth.org
List Experience and what training will take place for people with no prior experience:	Will be trained by Carrie Sanders and Sue Downey with non-survival pig surgeries including aortic cannulations, laparotomy, sternotomy , and blood draws. No previous swine experience.
Role on Protocol (surgery, care, order animals, etc.,)	Swine non survival surgeries, swine blood draws, swine tissue/organ harvest

Name:	Elizabeth Jordan Pearsall
IU Network ID:	epearsall@IUhealth.org
List Experience and what training will take place for people with no prior experience:	Will be trained by Carrie Sanders and Sue Downey with non-survival pig surgeries including aortic cannulations, laparotomy, sternotomy , and blood draws. No previous swine experience.
Role on Protocol (surgery, care, order animals, etc.,)	Swine non survival surgeries, swine blood draws, swine tissue/organ harvest

Name:	James Butler
IU Network ID:	JRButler
List Experience and what training will take place for people with no prior experience:	Will be trained by Sue Downey and A. Joseph Tector with non-survival pig surgeries including aortic cannulations, laparotomy, sternotomy, and blood draws. He is a MD doing his surgical residency. He has had two years experience on pig surgeries in the surgical resident program under Lisa Fisher at IUSM.
Role on Protocol (surgery, care, order animals, etc.,)	Swine non survival surgeries, swine blood draws, swine tissue/organ harvest

Name:	Shekhar A. Kubal
IU Network ID:	SAKUbal@IUPUI.edu
List Experience and what training will take place for people with no prior experience:	Board certified transplant surgeon, trained by A. Joseph Tector, at least 1 year experience with non-survival pig surgeries including aortic cannulations, laparotomy, sternotomy , and blood draws. Dr. Tector and Su Downey will be responsible for direct supervision.
Role on Protocol (surgery, care, order animals, etc.,)	Swine non survival surgeries, swine blood draws, swine tissue/organ harvest

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Name:	Burcin Ekser
IU Network ID:	BEkser@IUPUI.edu
List Experience and what training will take place for people with no prior experience:	David K C Cooper MD, PHD at the University of Pittsburgh, trained Burcin Ekser, MD. He has performed at least 20 non-survival surgeries on pigs in a similar manner as this protocol. He also has experience in baboon surgeries and xenotransplants. He is a trained transplant surgeon. He will be trained by Sue Downey, Dr. Leela Paris, Dr. Kubal, and/or Dr. AJ Tector on the particulars of this protocol.
Role on Protocol (surgery, care, order animals, etc.,)	Swine non survival surgeries, swine blood draws, swine tissue/organ harvest

Name:	Mouhamad Alloosh
IU Network ID:	malloosh@iupui.edu
List Experience and what training will take place for people with no prior experience:	Overall summary – Mouhamad Alloosh has 14 years of experience in swine research. Anesthesia via telazol and xylazine, isoflurane and mask drop = >1000 pigs, multiple anesthesia sessions in some pigs; trained by M. Sturek (IUSM) via hands-on assistance and direct supervision. Telemetry surgery (including vascular access port placement) = ~20 pigs; trained by M. Sturek (IUSM); B Carter (U of Missouri) and J Tune (IUSM) via hands-on assistance and direct supervision. Post-operative pain management = >100 pigs; trained by M. Sturek (IUSM); B. Carter (U of Missouri) and A Blickman (IUSM) via hands-on assistance and direct supervision. Urine collection using metabolic cage; self-trained in removing a pan from the metabolic cage; pigs are not even touched to accomplish this; training to be verified by LARC technical staff. Central line placement and ear vein catheterization = ~>500 pigs; trained by J Carroll (U of Missouri) via hands-on assistance, direct supervision – One session of direct instruction was provided; "Coursework" – Powerpoint presentation provided more detailed diagrams, etc. Blood sampling with caval puncture (jugular vein and vena cava) = ~400 pigs; trained by B. Carter (U of Missouri) via hands-on assistance; direct supervision Metabolic assessment (including sling restraint, body weight, tail-cuff blood pressure, and intravenous glucose tolerance test (IVGTT) = ~1000 assessments; trained by M. Sturek (IUSM); via hands-on assistance and direct supervision. Swine husbandry (diet/oral medication dosing) = ~1000 pigs; trained by M. Sturek (IUSM); via hands-on assistance and direct supervision. Angiography and intravascular ultrasound (IVUS) = ~950 assessments; trained by M. Sturek (IUSM); E. Mokelke (U of Missouri) via hands-on assistance and direct supervision. Euthanasia (conduct and verify) = ~800 pigs by isoflurane overdose and excision of the heart; trained by M. Sturek (IUSM); E Mokelke (U of MissourI) via direct supervision
Role on Protocol (surgery, care, order animals, etc.,)	Swine non survival surgeries, swine blood draws, swine tissue/organ harvest

Name:	Rafael Santos
IU Network ID:	rasantos@iupui.edu
List Experience and what training will take place for people with no prior experience:	Training for non-survival pig surgeries for organ procurement and exsanguination. PA is a MD and Surgeon in Brazil. He will be trained by A. Joseph Tector MD transplant surgeon.
Role on Protocol (surgery, care, order animals, etc.,)	They will assist perform non-survival pig surgeries, pig tissue/organ procurement and exsanguination

Name:	Joseph Ladowski
IU Network ID:	jmladows
List Experience and what training will take	Training for assisting on non-survival pig surgeries for organ procurement

place for people with no prior experience:	and exsanguination. PA is a 2nd year medical student at IUPUI School of Medicine. He will be trained by A. Joseph Tector MD transplant surgeon or Rafael Sanchez MD
Role on Protocol (surgery, care, order animals, etc.,)	They will assist non-survival pig surgeries, pig tissue/organ procurement and exsanguination

Name:	Greg Martens
IU Network ID:	gmartens
List Experience and what training will take place for people with no prior experience:	Greg is a surgical resident. He had previously swine experience working with Sue Downey and other associates within the surgery department 2 years ago. He is to be further trained by A. Joseph Tector, and Susan Downey to perform the non-survival pig surgeries and pig organ/tissue harvests.
Role on Protocol (surgery, care, order animals, etc.,)	He will assist with non-survival pig surgeries, pig tissue/organ harvest

Name:	Shunji Nagai
IU Network ID:	snagai
List Experience and what training will take place for people with no prior experience:	They are to be trained by A. Joseph Tector, Rafael Sanchez, Andy Lutz and Carrie Sanders to perform the non-survival pig surgeries and pig organ harvest.
Role on Protocol (surgery, care, order animals, etc.,)	They will assist with non-survival pig surgeries, pig tissue/organ harvest

Name:	Jane Hooker
IU Network ID:	jahooker
List Experience and what training will take place for people with no prior experience:	She has been trained by Sue Downey at IUPUI to handle pigs as part of the “pig watching” efforts aimed at maintaining the health and safety of newborn animals. She has performed this role for over 1 year.
Role on Protocol (surgery, care, order animals, etc.,)	Jane will be trained by Sue Downey and/or LARC staff to assist with handling pigs during blood draw procedures. Jane will serve in an assistant handling role and will not perform the blood draw.

Name:	Elisabeth Nus
IU Network ID:	enus
List Experience and what training will take place for people with no prior experience:	Will be trained by Carrie Sanders and Sue Downey with non-survival pig surgeries including aortic cannulations, laparotomy, sternotomy , and blood draws. No previous swine experience.
Role on Protocol (surgery, care, order animals, etc.,)	Swine non survival surgeries, swine blood draws, swine tissue/organ harvest

Name:	
IU Network ID:	
List Experience and what training will take place for people with no prior experience:	
Role on Protocol (surgery, care, order animals, etc.,)	

Section D – Purpose, Goals and Hypothesesiacuc

Definitions. Please list all abbreviations that will be used.
HTK- Histadine-Tryptophan-Ketoglutarate ACD- citrate dextrose solution ASGR1- Asialogycoprotein1 GGTA1- glycoprotein galactosyltransferase alpha 1, 3 gene iGB3s-isoglobotriaosylceramide synthase SIRPA- signal regulatory protein alpha

CMAH- cytidine monophosphate-N-acetylneuraminic acid hydroxylase
Xenotransplant- transplantation of living cells, tissues or organs from one species to another
SLA - swine histocompatibility antigen
B4GALNT-2: beta-1,4-N-acetyl-galactosaminyl transferase 2

Lay Summary (Do not exceed 250 words)

Briefly summarize **(In LAYMAN'S TERMS)** the background, general hypothesis, experimental plan, and potential relevance of the proposed research/instruction to human or animal health and/or to the advancement of scientific knowledge (limited to 250 words). Use clear, concise, non-technical, **layman's terms (i.e., language level of a newspaper article for the general public).** As some members of the IACUC are nonscientists, **copying and pasting from a grant application, journal article or abstract is not appropriate, as this would not be considered as layman's terms**. Wherever possible, you should avoid the use of terminology specific to the discipline. Otherwise, **all medical and/or technical terms must be defined in lay language**.

Thousands of people die annually waiting for transplantable organs. One potential solution is using pig organs, but two problems prevent this. First, the human immune system efficiently destroys pig cells. Second, pig livers rapidly eat human platelets leading to uncontrollable bleeding. The overall goal of this project is to determine if new pig strains can be engineered to overcome these problems.

A separate protocol will manage the genetic manipulations needed to create novel pig strains. This protocol will determine if it is possible to identify and eliminate the molecules in pig cells that trigger rejection by the human immune system. Collecting cells and tissues from pigs and mixing them with components of the human immune system in the lab will achieve this. The aim is to determine if destruction of genetically modified pig tissues might be reduced in genetically modified pig cells. This protocol will also perfuse organs, isolated from genetically modified pigs, with human platelets to determine if platelet consumption can be minimized. Strains of pigs will be compared to analyze the effect of each genetic modification.

Success in this project will lead to a new source of organs that may be useful as for transplantation to prolong life and minimize suffering of humans with organ failure. Alternative therapies for these patients currently do not exist.

Please provide a brief hypothesis of your study. Do not cut and paste from your grant (Do not exceed 1 pages).

In the proposed study we hope to identify pig carbohydrates and proteins, which make them to be physiologically and immunologically incompatible for use as a donor transplant to humans. ASGR1 and SIRPα have been identified to be involved in xenogeneic phagocytosis of human platelets by the porcine liver. GGTA1, CMAH, iGB3, and B4GALNT-2 have been identified to modify carbohydrates on the surface of pig cells causing them to be bound by human antibodies. Genetic modification of pigs can lead to more suitable and compatible organs for transplantation into humans.

Thrombocytopenia occurs upon pig liver transplant into baboons and an extreme platelet loss that occurs upon human platelet perfusion into porcine livers. ASGR1 has been shown to mediate human platelet phagocytosis in vitro. To confirm this we seek to use ASGR1 knockout pigs. Pigs will be exsanguinated and perfused with saline and or preservation solution to minimize on ischemia/reperfusion injuries. We will perfuse their livers with either human platelets or porcine platelets and examine uptake. Also, different pig liver cells will be isolated, incubated with pig and human platelets and uptake measured. Expression will also be screened using flow cytometry, confocal microscopy, and western blot. It is hypothesized that liver cells in vitro as well as whole livers ex vivo from ASGR1-knockout, GGTA1-ASGR1 knockout pigs and from GGTA1-/-/CMAH-/-/hSIRPα transgenic pigs will have decreased human platelet uptake compared to wild type, GGTA1-/- or GGTA1-/-/CMAH-/- pigs.

If pig organs are transplanted into baboons or perfused with human blood/antibodies, then human antibodies bind and cause the graft to reject and die. GGTA1, CMAH, IgB3, and B4GALNT-2 have been implicated in the modification of carbohydrates on the pig cell surface. These proteins are non functional in all humans. They are functional in pigs. Therefore the carbohydrate modifications made by GGTA1, CMAH, and IgB3 in pig cells are foreign to humans. B4GALNT-2, is a carbohydrate modifying gene that is inactive in ~3% of humans. People lacking B4GALNT-2 also develop antibodies to carbohydrate modifications produced by this gene. We will test human antibody binding to cells from pigs with GGTA1, CMAH, and IgB3 knocked-out in various combinations. We will also test for complement dependent cytotoxicity to these cells and compare them to human cells. In addition, we will perform mixed lymphocyte reactions to test the human cellular responses to these porcine cells. It is hypothesized that pigs with GGTA1, CMAH,

and IgB3 knocked out will have less human antibody binding and cell cytotoxicity than wt pigs and organs will not reject as quickly. In addition, knockout of these GGTA1, CMAH, IgB3, and B4GALNT-2 genes in the same pig will produce cells that have less antibody binding than the single knockouts cells. It is expected that there will still be some human antibody binding and that other xenoantigens need to be identified. Blood samples from these knockout pigs will also be used to identify other non-Gal, non-HD human antigens. CD55 is a protein on the surface of cells that prevents antibody binding from recruiting other proteins that kill cells. Expression of CD55 in the pigs with modified carbohydrates is predicted to reduce complement dependent cytotoxicity. SLAI, swine leukocyte antigen I is a protein that is reported to be a xenoantigen. There is increased human antibody binding to pig cells after it had been absorbed to SLA I. Knockout of this protein in a GGTA1-/-CMAH-/- pig should reduce human antibody binding even further.
Recent evidence has suggested that modifications to proteins that alter surface carbohydrates may affect thrombocytopenia in pig to human xenotransplantation, therefore any modification to carbohydrate expression made to reduce antigenicity will have to be tested for its effect on platelet phagocytosis.

Success at reducing antigenicity and platelet uptake will open up the possibility to perform xenotransplants in a non-human primate model to provide data that will help to guide initial investigations into pig-to-human xenotransplantation. Therefore, we will test the reactivity of primate immune components (antibodies and lymphocytes) with pig strains. We will also determine if eliminating platelet receptors (ASGR1, CD18, and VWF) from pigs can reduce consumption of NHP by isolated porcine livers. These in vitro studies will provide valuable information to guide NHP pre-clinical studies.

Section E – Experimental Groups and Timelines

Provide a description of all the procedures that the experimental animals will undergo.

- Include a breakdown of experimental groups and for each group, provide a timeline for the experiments proposed. The timeline must be clear and begin with animal procurement, denote the approximate timing of all important manipulations including length of recovery time between surgery(ies) and anesthesia (details on surgeries and anesthesia will be requested in other sections), and conclude with final disposition of the animals. The number of animals designated for each experimental group should be listed and the total for all experiments should be consistent with the total number of animals requested for the study.
- When basing the number of animals needed for *in vitro* experiments, details of *in vitro* experiments are not necessary. Provide a brief summary of the *in vitro* work and describes how the number of animals was determined.
- For studies that necessitate breeding to produce the experimental mice, the total number should include the breeders and the offspring that cannot be used because of lack of the required genotype. (Example: If you need 10 experimental mice and only half of each litter will carry the correct genotype, you may need to generate 24 mice, 4 litters of 6 pups per litter, which added to the number of breeders could mean a total of ~ 30 mice). Animals generated solely for colony maintenance should be justified. **If you wish you could use the Breeding excel sheet, which you need to send in your e-mail with your submission.**
- Timelines may be presented in a narrative, listing step-by-step time events in the experimental protocol or they may be presented in graphic form.

Platelet Phagocytosis Experiments

Once weaned, pigs will be transferred to protocol. Pigs will be bled not more than every 2 weeks to determine expression and harvest platelets for analysis. Once this is determined then pigs may undergo non-survival surgery and tissues, blood and organs will be harvested. Non-survival surgery will occur when pigs are between 3 kg and 130kg. To do in vitro testing human platelet uptake will be compared with porcine platelet uptake as control. To do this pigs will be bled not more than every 2 weeks under general anesthesia. Experiments will be performed so that pig blood draws are finished before animals undergo non-survival surgery. Groups 1 and 3 will also be used as controls for groups 2 and 4. The in vitro work will be performed at the same time for group 1 and group 2 and groups 3 and 4 will occur on same day. The perfusion experiments use the whole liver and cells of the liver are not healthy by the end of experiment. In vitro experiments require perfusion of an entire liver with a cell disruption reagent to harvest cells. There are typically only enough cells to perform the in vitro experiments one time. In addition the timing of the experiments does not allow for repeats in one day.

Groups

1. $GGTA1^{-/-}/CMAH^{-/-}$ 16 livers perfused with human platelets, 16 perfused with pig platelets
2. $GGTA1^{-/-}/CMAH^{-/-}$/hSIRPA 16 livers perfused with human platelets, 16 perfused with pig platelets
3. $GGTA1^{-/-}$ 16 livers perfused with human platelets, 16 perfused with pig platelets
4. $GGTA1^{-/-}/ASGR1^{-/-}$ 16 livers perfused with human platelets, 16 perfused with pig platelets
5. wild type 16 livers perfused with human platelets, 16 perfused with pig platelets

6. ASGR1$^{-/-}$ 16 livers perfused with human platelets, 16 perfused with pig platelets
7. GGTA1$^{-/-}$/CMAH$^{-/-}$/iGB3$^{-/-}$ 16 livers perfused with human platelets, 16 perfused with pig platelets
8. GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, VWF$^{-/-}$ 13 livers perfused with NHP
9. GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, ASGR1$^{-/-}$ 13 livers perfused with NHP
10. GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, CD18$^{-/-}$ 13 livers perfused with NHP

Timeline

Day 1- Acquisition of weaned pigs via protocol transfer, no acclimation necessary

Day 1- Month 12: Pigs will be bled not more than once every two weeks (for up to 20 bleeds) to confirm phenotype and test reagents on cells

Day 30-Month 12: Pigs will undergo non-survival surgery once phenotype is confirmed and reagents are tested; some pigs may be transferred to Estrada protocol for breeding depending on results of phenotypic analysis

- Non-survival surgery for perfusion followed by organ harvest
  - Anesthesia
  - Catherization of bladder
  - Paralytic Pancuronium or Vecuronium given
  - Mid-line abdominal incision to access aorta for perfusion
  - Sternotomy to access vena cava for transection
  - Blood draw
  - Bolus of Heparin administered
  - Perfusion to preserve organ quality
  - Euthanasia as blood is replaced
  - Organ harvest

Summary of pig numbers for Platelet Phagocytosis Experiments

Group 1: GGTA1$^{-/-}$/CMAH$^{-/-}$
16 livers perfused with human platelets, 16 perfused with pig platelets **32** total
Group 2: GGTA1$^{-/-}$/CMAH$^{-/-}$/hSIRPA
16 livers perfused with human platelets, 16 perfused with pig platelets, 32 total
Group 3: GGTA1$^{-/-}$
16 livers perfused with human platelets, 16 perfused with pig platelets, 32 total
Group 4: GGTA1$^{-/-}$/ASGR1$^{-/-}$
16 livers perfused with human platelets, 16 perfused with pig platelets, 32 total
Group 5: wild type
16 livers perfused with human platelets, 16 perfused with pig platelets, 32 total
Group 6: ASGR1$^{-/-}$
16 livers perfused with human platelets, 16 perfused with pig platelets, 32 total
Group 7: GGTA1$^{-/-}$/CMAH$^{-/-}$/iGB3$^{-/-}$
16 livers perfused with human platelets, 16 perfused with pig platelets, 32 total
Group 8: GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, VWF$^{-/-}$
13 livers perfused with NHP
Group 9: GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, ASGR1$^{-/-}$
13 livers perfused with NHP
Group 10: GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, CD18$^{-/-}$
13 livers perfused with NHP

Xenoantigen Experiments

Weaned pigs will be bled not more than once every two weeks under anesthesia. Blood cells will be incubated with sera from 3 different age groups, both genders, each of four ABO blood types. Twenty-four different serum types will be used. As per the power analysis performed by biostatistician Barry Katz, 9 different individual human sera for each group will be tested for IgM and IgG binding. That will give 216 assays/group. Each of these sera will be tested on blood cells from 4 different groups of pigs. We will need 8 mls pig blood per assay of 1 human serum sample to give IgM and IgG binding per strain of pig. Blood is good for 1.5 days in this time we can perform 3 tests so 24 mls pig blood. We are asking for 6 pigs so that we can perform multiple assays each week without down time, considering pigs will not be bled more than every 2 weeks. Therefore 6 pigs will be needed for blood to accomplish the n=9 human sera IgG and IgM binding replicates of 24 types of human sera.

Six pigs of each group are requested for blood draws. Blood from animals in the platelet phagocytosis experiment

(groups 1 and 2) will be used in this experiment so no unique animals will be needed. Six animals per genotype will be needed to generate blood for this experiment in groups 5 and 6.

Groups and Pig numbers

1. GGTA1$^{-/-}$/CMAH$^{-/-}$ 0 additional, 6 from Platelet Phagocytosis Study
3. GGTA1$^{-/-}$ 0 additional, 6 from Platelet Phagocytosis Study
7. GB3$^{-/-}$/GGTA1$^{-/-}$ (iGb3s/Gal KO) 6 additional pigs
8. CMAH$^{-/-}$/GGTA1$^{-/-}$/iGB3$^{-/-}$ (iGb3s/GT/CMAH KO) 0 additional, 6 from Platelet Phagocytosis Study
9 CD55-GGTA1$^{-/-}$/CMAH$^{-/-}$ 6 additional pigs
10. CMAH$^{-/-}$/GGTA1$^{-/-}$/SLAI$^{-/-}$ 6 additional pigs
11. GGTA1$^{-/-}$/SLA1$^{-/-}$ 6 additional pigs
12. GGTA1$^{-/-}$,CMAH$^{-/-}$, B4GALNT-2$^{-/-}$ 6 additional pigs

Timeline:

Day 1-Weaned pigs will be transferred to protocol

Day 1-Month 12: Pigs will be bled not more than every 2 weeks for up to 20 bleeds

By month 12: Pigs will be transferred back to Estrada protocol for breeding or will be used in platelet phagocytosis study and undergo non-survival surgery

Pig Totals for all studies

1. GGTA1$^{-/-}$/CMAH$^{-/-}$ (CMAH/GAL) 32
2. GGTA1$^{-/-}$/CMAH$^{-/-}$/hSIRPA (hSIRPA-CMAH/GTKO) 32
3. GGTA1$^{-/-}$ (GTKO) 32
4. GGTA1$^{-/-}$/ASGR1$^{-/-}$ (ASGR1/GAL KO) 32
5. Wild type 32
6. ASGR1$^{-/-}$ 32
7. GB3$^{-/-}$/GGTA1$^{-/-}$ (iGb3s/Gal KO) 6
8. CMAH$^{-/-}$/GGTA1$^{-/-}$/iGB3$^{-/-}$ (iGb3s/GT/CMAH KO) 32
9. CD55-GGTA1$^{-/-}$/CMAH$^{-/-}$ 6
10. CMAH$^{-/-}$/GGTA1$^{-/-}$/SLAI$^{-/-}$ 6
11. GGTA1$^{-/-}$/SLA1$^{-/-}$ 6
12. GGTA1$^{-/-}$,CMAH$^{-/-}$, B4GALNT-2$^{-/-}$ 6
13. GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, VWF$^{-/-}$ 13
14. GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, ASGR1$^{-/-}$ 13
15. GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, CD18$^{-/-}$ 13

Section F - Unnecessary Duplication and the Three R's (Replacement, Reduction, and Refinement)

To comply with federal regulations, please perform a comprehensive search of available literature using key words or search strings to assure the activities involving the use of animals is not unnecessarily duplicative and that alternative non-animals models and alternatives to painful procedures are not available.

For USDA Covered Species: The investigator is required to seek less painful and less stressful alternatives to procedures in order to promote animal welfare and comply with the USDA Policy 12. Please include the ***painful procedure(s) to be performed*** in your Keywords for USDA covered species.

Keywords: Include keywords pertinent to the proposed research topic that would provide a knowledge of the range of methods used to study the research topic proposed, including the common animal models and the typical animal numbers needed for statistical significance. In addition, searches should include specific terms to identify alternative animal or non-animal models. Specific terms that might help to identify alternative or non-animal models include: *alternative*, assay, technique, method, culture (as in cell, tissue, organ), invertebrate, in vitro (method, model, technique), simulation, model,*

isolated (as in cell, tissue, organ), virtual. * "alternative" is not a particularly productive term unless the search is focused on the topic of pharmacology and toxicology. Do not cite your own work.

Example databases to search: PubMed, Biological Abstracts, or contact the Animal Welfare Information Center (AWIC). Link to website to use to conduct the search: http://libguides.library.umkc.edu/alternatives

Database Searched:	PubMed
Key Words/Search Strategy:	pig liver thrombocytopenia;
Range of Years Searched (Example: January 1966-Present):	January 1960-present
Date of Search:	10-15-12

Database Searched:	Pubmed
Key Words/Search Strategy:	CMAH pig antigen
Range of Years Searched (Example: January 1966-Present):	January 1960-present
Date of Search:	10-15-12

Database Searched:	Pubmed
Key Words/Search Strategy:	Xenotransplant pig cell line
Range of Years Searched (Example: January 1966-Present):	January 1960-present
Date of Search:	10-15-12

Database Searched:	Pubmed
Key Words/Search Strategy:	Pig blood draws
Range of Years Searched (Example: January 1966-Present):	January 1960-present
Date of Search:	10-16-12

Database Searched:	Ovid-medline (with consultation of medical librarian)
Key Words/Search Strategy:	See attached appendix 1 for 3R keywords and subject headings
Range of Years Searched (Example: January 1966-Present):	1946-November 6, 2012
Date of Search:	10-19-12

Database Searched:	Ovid-medline (with consultation of medical librarian)
Key Words/Search Strategy:	See attached appendix 2 for duplication keywords and subject headings
Range of Years Searched (Example: January 1966-Present):	1946-November 6, 2012
Date of Search:	11-7-12

Database Searched:	Ovid-medline (with consultation of medical librarian)
Key Words/Search Strategy:	See attached appendix F2 for duplication keywords and subject headings and F1 for 3R keywords and subject headings
Range of Years Searched (Example: January 1966-Present):	1946-present
Date of Search:	02-23-2013

Database Searched:	Ovid-medline (with consultation of medical librarian)
Key Words/Search Strategy:	See attached appendix F2 for duplication keywords and subject headings and F1 for 3R keywords and subject headings
Range of Years Searched (Example: January 1966-Present):	1946-present
Date of Search:	11-15-2013
Database Searched:	Ovid-medline
Key Words/Search Strategy:	See attached appendices for three Rs and duplication evaluation
Range of Years Searched (Example: January 1966-Present):	1946-present
Date of Search:	04-30-2015

Please briefly describe how the above search documents that there are no alternatives to the use of animals, and if

painful procedures are proposed, that less invasive alternatives to these procedures are not available or cannot be used.

There is suggestion of using a pig cell line, we use pig cell lines whenever possible to minimize animal use, however the porcine cell lines available are phenotypically different than primary cells. The pig cell lines available do not phagocytose human platelets and there are no cell lines available with the modifications we suggest to use here. In addition it is important to use primary cells when doing cross match studies because there is incorporation of xenoantigens from the sera and other media components. We are currently working on a way to get the pig cell lines to grow without these components to minimize the use of animals.

Pig blood draws search lead to an article with a different technique listed to insert catheter for daily blood draws, but given that we will not be drawing more than once every two weeks this is more stressful to the pigs. We will consider it though and talk to veterinarian if blood-sampling times need to be increased.
There were 68 articles found when searching for alternatives to the surgical procedures in pigs including organ harvesting thoracotomy, laparotomy, hepatectamy, and catheterization. None were appropriate for the study.
There was not an alternative to the non-survival surgery. There is not a less invasive method for the exsanguination and perfusion of the pig with preservation solution as well as the removal of the liver.

We reran the attached “replace” search to determine if a replacement system could be identified. Of 21 publications we found no alternative methods of study. Cell culture models have been used to study the uptake of platelets by pig-derived cells (Kupffer and endothelial cells). No cell types retain the ability to reproducibly consume platelets long term forcing the collection of livers from animals every time an assay is attempted.

Please briefly describe how the above search documents that the proposed activities are not unnecessarily duplicative of previous activities.

There were over 33 articles retrieved and reviewed with the help of a medical librarian to ensure we are not duplicating research unnecessarily.
The genetic background of the pigs used is different than the ones proposed here. Others have tried to solve xenogeneic thrombocytopenia with the use of drugs, but without much success. No studies of immunological incompetence in CMAH GGTA1 and IGB3 knockout pigs; such pigs do not exist. There are no studies using CD55 in combination with the genetic modifications proposed here. There is a study with the absorption of SLAI antibodies from the human serum before perfusion. This is supportive evidence for our study and not a duplicate.

The ultimate goal of our research is to develop a swine strain that can be used in liver xenotransplantation for humans. To accomplish this, platelet consumption by the organ must be reduced. We found 22 reports based on the attached search “duplication”. There are no reports of stable knockout animals being evaluated for platelet incompatibility that match the proposed strains deletions of potential platelet receptors.

Principle of Replacement.
Provide a brief paragraph describing how the design of your studies reflects due attention to the principle of replacement. Justify the need for involving vertebrate animals and why the species/strain proposed is a good model for this work. Explain why you cannot use non-animal alternatives such as mechanical models, computer simulations, bacteria, yeast; or systems other than live animals, such as cell/tissue culture, isolated organ preparations, etc., or invertebrate animals, are not appropriate.

We are searching for an alternative for human organ transplant; the pig has been discussed in the literature as the most appropriate source. To study the human immunological and physiological response to pig organs and cells it is not appropriate to use an invertebrate. The porcine cell lines available have different antigens on their surface some due to the culture conditions and some due to the immortalization process or they do not have the same phenotype such as that of xenogenic phagocytosis. When possible this study uses cell lines available. We performed a search to look for alternatives with the consultation of a medical librarian. There were 63 results using an alternative animal search, none were appropriate. There were 29 articles with non-animal alternatives search; none were appropriate. Some of the articles were the ones published by this lab. The others were not appropriate because we are specifically looking at pig organs for xenotransplantation. The cell lines available do not exhibit the phagocytic phenotype of primary cells. In addition the conditions of cell culture incorporate non-endogenous antigens on their surface that interfere with xenoantigen analysis. Swine is the most commonly used large animal model for xenotransplantation.

We repeated literature searches to search for replacement alternatives. No models have been described that allow evaluation of the role of an intact liver in creating thrombocytopenia.

Version: 8. 2012

Principle of Reduction
Describe the statistical methods (e.g., power analyses, related literature citation) and/or other rationales (e.g., tissue collection needs, breeding efficiency) that you used to determine the number of animals required. Please be specific and justify group size. **If this is a pilot study**, this should be clearly stated and although power analysis may not be possible, some rationale for the number of animals used must be given. When selecting the method below, strongly consider inserting a chart or table (or submitting as an attachment) to illustrate animal numbers required for each study component.

Method Chosen (Select only One)
Please describe how you determined how many animals you need for each experiment proposed in this application. Enter literature citations, with a brief narrative explaining how they related to the determination of group sizes, **OR** results of your power analysis, i.e., calculations).

	Literature Citation
Provide the citation.	

	Power Analysis (Please provide the following	
Provide Calculations:	Xenoantigen Experiment - Calculated by Barry Katz of IUPUI Biostats department. Based on past similar experiments, genetic modifications produce large mean differences averaging 1.7 standard deviations for IGG and IGM and ranging from 0.7 to 3 standard deviations. To be conservative we performed the sample size calculations for a difference of 1.0 standard deviation. Based on a 5% significance level and a two tailed paired t-test, we estimate that 9 human sera on cell pairs will yield 85% power.	
Major outcome variable being measured:	**IgM and IgG binding (human Sera) (Mean Fluorescence Intensities) (sample of 2 types of cells)**	
Expected variability (e.g., standard deviation):		**1.7 Standard deviations**
Minimum scientifically meaningful treatment effect:		SD difference greater than 6694 for IgG and 9457 for IgM

	Power Analysis (Please provide the following	
Provide Calculations:	Platelet Phagocytosis experiments - Calculated by Barry Katz of IUPUI Biostats department and based on preliminary data with a small number of animals, the observed difference between the human platelet and pig platelet perfusion was 79%. To be conservative we calculated the sample size for a two-tailed t-test with a 5% significance level and a standard deviation of 30%, (pooled SD was 23%). We estimate that 16 animals per group will yield 80% power to detect a difference of 30% between groups.	
Major outcome variable being measured:	Human and pig platelet uptake in liver ex vivo perfusion (percent platelet number in perfusate)	
Expected variability (e.g., standard deviation):		Standard deviation = 23%
Minimum scientifically meaningful treatment effect:		Difference of 30% or more in percent platelet uptake

	Power Analysis (Please provide the following	
Provide Calculations:	Non human platelet phagocytosis experiments: - Calculated by George Eckert of IUPUI Biostats department and based on preliminary data with a small number of animals. The sample size calculations assume a within-group standard deviation of 13% based on prior data. With a sample size of 13 livers per genotype the study will have 80% power to detect a difference between genotypes of 15%, assuming two-sided two-sample t-tests conducted at a 5% significance level. With this sample size, the study will also have 80% power to detect a within-genotype reduction from input to output of 11% using paired t-tests.	
Major outcome variable being measured:	Non human platelet uptake in liver ex vivo perfusion (percent platelet number in perfusate)	
Expected variability (e.g., standard deviation):		Standard deviation = 13%
Minimum scientifically meaningful treatment effect:		Difference of 15% or more in percent platelet uptake

Principles of Refinement.

Please describe any refinements to your experimental design that have incorporated the tenets of the Three R's. Refinement includes, but is not limited to, the following:

- Proper assessment of potential pain and distress, including both behavioral signs (based on the animal's normal behavior patterns) and to physiological parameters (e.g. plasma cortisol and catecholamine levels, white blood cell counts, cardiovascular parameters). When in doubt, investigators should assume that the animal is in pain and take steps to alleviate it.
- Responsible use of anesthetics, analgesics, and sedatives when appropriate;
- Setting appropriate endpoints for the experiment or stages of the experiment;
- Using general techniques to reduce stress and minimize complications (e.g. proper handling and restraint methods, good surgical technique /post-operative care); and
- Seeking additional training for unfamiliar procedures or techniques to be used;

Senior members of the team will train junior members of the study in swine specific surgery. In addition, some members of the team will be trained by LARC staff to perform certain procedures such as anesthesia and euthanasia. All pigs are anesthetized for blood draws and surgical procedures. Blood from pigs and organs will be shared with others to reduce the number of animals. Pigs used for blood draws will also be used in non-survival surgeries to reduce the number of animals.

Section G – Animal Numbers/Experimental Groups

Provide the total number, strain, and species of animal in the table below.

The total number should include animals that are born but not used as well as all the animals required for the maintenance of the colonies for the period requested. Refer to section 15 to provide breeding information.

- **Rodents:** Categorize rodents as noted below; however, breeding animals may be included in Classification C.
- **Category B:** Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery but not yet used for such purposes.
- **Category C:** Teaching, research, experiments, or tests conducted involving no or only very brief pain or distress, with no need for or use of pain relieving drugs.
- **Category D:** Experiments, teaching, research, experiments, or tests conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.
- **Category E:** Teaching, experiments, research, surgery, or tests conducted involving accompanying pain or distress to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will would have adversely affected the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.

Species and Number of Animals

Species	Strain/ Nomenclature/genotype	tg/KO/KI (Check all that apply)			Weight/ Age	Source ("LARC Vendor" is acceptable)	Total Number of Animals per USDA Category			
		tg	KO	KI			B	C	D	E
Swine/sus scrofa	GGTA1$^{-/-}$,CMAH$^{-/-}$, B4GALNT-2$^{-/-}$		X		400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol			6	
Swine/sus scrofa	GTKO		x		400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol			32	
Swine/sus scrofa	GGTA1$^{-/-}$/SLA-/-		x		400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol			6	
Swine/sus scrofa	CMAH-/-/GGTA1-/-/SLA I-/-		x		400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol			6	

Swine/sus scrofa	CMAH-/-/GGTA1-/-		x		400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol			32	
Swine/sus scrofa	ASGR1/GAL KO		x		400 g-400 lbs/ 0 - 3 years	Transfer from Estrada protocol,			32	
Swine/sus scrofa	hSIRPA-CMAHKO/GTKO	x	xx		400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol,			32	
Swine/sus scrofa	iGb3s/GT/CMAH KO		x		400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol			32	
Swine/sus scrofa	iGb3s/GAL KO		x		400 g-400 lbs/ 0 - 3 years	Transfer from Estrada protocol			6	
Swine/sus scrofa	CD55-CMAH-/-/GAL-/-		x		400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol			6	
Swine/sus scrofa	ASGR1 KO		x		400 g-400 lbs/ 0 - 3 years	Transfer from Estrada protocol,			32	
Swine/sus scrofa	wildtype				400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol, purchase from Larc Vendor			32	
Swine/sus scrofa	GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, VWF$^{-/-}$				400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol, purchase from Larc Vendor			~~16~~13	
Swine/sus scrofa	GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, CD18$^{-/-}$				400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol, purchase from Larc Vendor			~~16~~13	
Swine/sus scrofa	GGTA1$^{-/-}$, B4GALNT-2$^{-/-}$, ASGR1$^{-/-}$				400 g-400 lbs/ 0-3 years	Transfer from Estrada protocol, purchase from Larc Vendor			~~16~~13	
				Total Numbers of Animals					~~28~~62	

				93	

For Category E Protocol – complete the following

Provide a scientific justification to explain why the use of anesthetics, analgesics, sedatives or tranquilizers during and/or following painful or distressing procedures is contraindicated:

For USDA Covered Species – complete the following

Please explain why this species is being used.
Swine are being used for the direct study of the interactions of human blood, blood components, tissue, organs, etc, interact with that of porcine organs, blood, tissue, and cells. This study is provide data for the xenotransplant of porcine organs therefore the use of the swine is needed.

Section H – Potential- Experimental Complications and Emergency Management Plan

Can the animals be euthanized for health reasons before completion of the experiment?	
x	**Yes, please answer the following questions.**
Please identify the criteria that will be used to determine when animals must be euthanized before completion of the experiment	
There are no anticipated complications on this study. LARC veterinary staff will be consulted if there are complications or symptoms. As recommended by LARC veterinarians.	
Describe any complications/symptoms that may occur:	
Failure to eat and drink for > 48 hours; clinical symptoms of systemic illness or physiologic impairments that are not responsive to veterinary medical intervention and treatment; loss of 20% of pre-study body weight (animals weighed weekly); signs of pain that are not relieved by analgesic drugs (guarding, vocalization, depressed attitude, increased aggression, grinding teeth); dehiscence of wounds with evidence of infection not responsive to veterinary treatment.	
At what point will these complication/symptoms will be managed?	
At the recommendations of LARC veterinarians	
How will the complications/symptoms be managed/treated?	
As per the recommendations of LARC veterinarians.	
Who will be responsible for managing the complications?	
Name:	LARC veterinarians
Campus Phone:	Emergency Phone:
In the case of an animal emergency requiring euthanasia by the LARC veterinary staff in the absence of PI staff, please describe what samples should be collected post-mortem.	
Please notify prior to euthanasia for sample collection or keep whole piglets at 4°C (refrigerated). If pigs are too large to keep at 4°C, please take blood samples in ACD tubes (yellow top blood collection tubes with citrate dextrose anticoagulation solution (Tector drawer at Conrad Farms) prior to euthanasia and remove liver, kidney, aorta, spleen, ear, and keep in Saline at 4°C (refrigerator).	
In the case of emergency veterinary care, are there classes of drugs that cannot be used?	
no	
x	**No, Complete the questions about the pilot study.**
Has a pilot study been performed to determine if pre-death endpoints can be identified?	
	Yes
	No

In the face of distinct signs that a process is causing irreversible pain or distress, alternative endpoints should be sought to satisfy both the requirements of the study and the needs of the animals. Humane endpoints support the concept of refinement, which is a means to achieve a more humane use of animals

To determine what clinical signs can be observed prior to death, and therefore represent significant indicators of pain, suffering, and subsequently death, and to determine the time course of events as well as determine the needed frequency of monitoring, a pilot study to identify pre-death endpoints or verify that pre-death endpoints cannot be used must be performed. Once parameters have been established and the correlation with death validated these indicators can be used as the defined endpoint of the experiment. This ultimately results in a reduction of animal suffering.

If you performed a pilot study, provide the experimental outline for a pilot study Explain how you determined the appropriate number for the pilot.

Section I - Euthanasia

The IUSM IACUC and LARC adhere to the approved methods of euthanasia as recommended by the American Veterinary Medical Association Panel on Euthanasia. If animals are to be euthanized under this protocol, even if different procedures within the protocol require separate methods, agents, dosages or routes of administration to accomplish euthanasia, this section should be completed for each euthanasia procedure. Note that a secondary method of euthanasia is required to ensure death.

Final Disposition of Animal		
x	Euthanasia	
	Return to Colony	
X	Transfer to a Different Protocol (following IACUC and LARC procedures to transfer animals)	
	Other:	

Select a primary and a secondary method of euthanasia
Anesthetic overdose (this includes carbon dioxide delivered by a gas cylinder, flow meter, and regulator. The use of dry ice is unacceptable)

Species	Agents	Method, Dosages (mg/kg) or Routes (IM, IV, IP) of Administration
swine	Exsanguination/ perfusion under anesthesia	Under anesthesia as per non-survival surgery protocol, Swine will be perfused with Saline and or HTK. Check cessation of heartbeat
Swine	Barituric acid derivative 100-150 mg/kg	IV injection 100-150 mg/kg. Check for cessation of heartbeat and/or a bilateral pneumothorax will be performed.

Physical methods to assure death following the use of agents listed above: check all that apply		
Method	Yes	Explanation
Pneumothorax		
Exsanguination	x	On the anesthetized animal, exsanguinations and perfusion will be performed. Animals will be bled from the vena cava and perfused with saline or HTK via the aorta. For death verification cessation of heartbeat will be checked.
Cervical Dislocation*		
Perfusion	x	
Decapitation		
Other		
*NOTE: Cervical Dislocation cannot be used in rats > 200 g BW		

Other Methods of Euthanasia:
If a physical method of euthanasia is to be used without prior anesthetic, please provide a detailed description of the proposed methods and the reasons requiring that this method be used. Cervical dislocation or decapitation without sedation or anesthesia requires a scientific justification.

Species	Name of Person(s) Responsible for Performing **Physical Method of Euthanasia**

If LARC Staff are requested to perform euthanasia, a written request specifying the animal(s) and date to perform such euthanasia must be signed by the principal investigator, co-investigator, faculty sponsor or responsible technician.

In the event that euthanasia becomes necessary for clinical reasons, standard methods will be used.

Experimental Protocol Summary

This Checklist is part of your application. Check all those that apply to your research project. Where you have checked, complete the **appropriate sections**.

x	Agent Administration [Section J]
	Behavior Testing [Section K]
	Food/Water Deprivation/Restriction [Section L]
	Irradiation [Section M]
	Special Caging/Husbandry [Section N]
x	Surgical Procedures (Non-Survival/Terminal, Survival, Multiple Survival) [Section O]
x	Anesthesia/Sedation/Analgesia [Section P]
x	Blood Sampling [Section Q]
	Creating a Disease Condition [Section R]
	Immunization [Section S]
	Hybridoma [Section T]
	Prolonged Restraint/Aversive Stimuli [Section U]
	Breeding, Weaning, and Genotyping [Section V]
	Annual Continuing Review

Section J – Agent Administration

This protocol will not receive IACUC approval until the Principal Investigator provides evidence of approval from the appropriate regulatory committee (e.g. IBC, Recombinant DNA, Radiation Safety). **If you have multiple agents, you are more than welcome to submit a separate sheet as an attachment to the IACUC office with this form.**

Do you have an IBC approved protocol for the work described in this study?		
X	Yes, if yes, please provide the IBC #:	1744
	No	

Non-Hazardous Agents (provide the following information):

Agent Name(s):	Heparin pharmaceutical grade
Route of administration:	IV
Dosage Range (Mg/kg or gm & mL):	Approximately 100 IU/kg .5mL- 20 mL of 1000 U/mL
Frequency of Administration:	One time dose during non-survival surgery
Possible Complications	Uncontrolled bleeding
How complications will be resolved:	It is non survival surgery.

Is the compound prepared in compliance with the IACUC NPD policy? NPD IACUC Policy. If no, please provide justification for the use of Non-Pharmaceutical grade chemical compounds	x	Yes		No

Hazardous Chemicals (provide the following information):

Chemical Name(s):						
Nature of chemical, e.g. carcinogen, toxin, teratogen:						
Route of administration:						
Dosage Range (PFU, CFU, Log number vrions, etc.):						
Route of excretion:		Is the bedding hazardous?		Yes		No
Will humans be exposed?				Yes		No
Will the carcass be hazardous?				Yes		No
Disposal method for wastes and carcasses?						

Is the compound prepared in compliance with the IACUC NPD policy? NPD IACUC Policy. If no, please provide justification for the use of Non-Pharmaceutical grade chemical compounds		Yes		No

Infectious Agents:

Name of Agent(s):								
Indicate Animal Biosafety Level:		ABSL1		ABSL2		ABSL2+ 3 Precautions		ABSL3

Route of Administration:
Dosage (PFU, CFU, Log number vironsvrions, etc.):
If the agent is infectious to humans or animals indicate: ☐ Humans ☐ Animals
If the agent is shed in feces, urine or body secretions indicate: ☐ Feces ☐ Urine ☐ Body secretions
Will the carcass be treated as infectious? Follow up with Blood, Body Fluids and rDNA sections below. ☐ Yes ☐ No
Disposal method for wastes and carcasses?

Blood, Body Fluids, Normal or Neoplastic Tissue:

Name of Agent(s):
Indicate Animal Biosafety Level: ☐ ABSL1 ☐ ABSL2 ☐ ABSL2+ 3 Precautions ☐ ABSL3
Route of Administration:
Will Universal Precautions be followed under the OSHA Bloodborne Pathogen Standard? ☐ Yes ☐ No
Are all staff current in their annual BBP training requirement? ☐ Yes ☐ No
Will the carcass be infectious? ☐ Yes ☐ No
Disposal method for wastes and carcasses?

Recombinant DNA *(including transgenic animal constructs)*:

Describe the host/vector system: hSIRPA vector- pEGFP N1 with human SIRPA insert originally in Top10 ecoli prior to transfection by electroporation and Somatic Cell Nuclear Transfer or Breeding
Does the modified gene translate into a potentially harmful product? no
Route of Administration: Already in pig upon receiving from Estrada protocol
Dosage (PFU, CFU, Log number vrions, etc.): N/A
If the agent is infectious to humans or animals indicate: ☐ Humans ☐ Animals
If the agent is shed in feces, urine or body secretions indicate: ☐ Feces ☐ Urine ☐ Body secretions
Will the carcass be infectious? ☐ Yes ☒ No
Disposal method for wastes and carcasses? Normal Disposal Method

Describe the host/vector system:
Does the modified gene translate into a potentially harmful product?
Route of Administration:
Dosage (PFU, CFU, Log number vrions, etc.): N/A
If the agent is infectious to humans or animals indicate: ☐ Humans ☐ Animals
If the agent is shed in feces, urine or body secretions indicate: ☐ Feces ☐ Urine ☐ Body secretions
Will the carcass be infectious? ☐ Yes ☒ No
Disposal method for wastes and carcasses? Normal Disposal Method

Safety Procedures for Animal Handlers - (LARC Personnel and Laboratory Staff)

Who has the potential to be exposed to this material? ☐ **LARC staff** ☐ **Laboratory staff**

Personal Protection *(indicate personal protective apparel/procedures to be used)*:

☐ Hair cover ☐ Gown ☐ Lab coat ☐ Booties
☐ Waterproof boots ☐ Safety glasses ☐ Goggles ☐ Face shield
☐ Respiratory mask (type) Gloves (type):
Personnel Disease Surveillance Procedures and frequency

Materials Handling:

☐ In cabinet *(indicate type)*:
☐ Chemical Fume Hood ☐ Biosafety cabinet
Decontamination of area after use:
Procedures:
Location of each hood used:

Waste Disposal:

☐ Bedding/feces/urine is hazardous: Duration: (days)
Indicate disposal method:
☐ Incinerate ☐ Autoclave
☐ Hazardous chemical waste disposal face shield ☐ Radioactive waste disposal face shield
☐ Decontaminate cage before washing. Procedures:

Other safety/preventative procedures *(describe including items such as vaccinations, post-exposure treatment)*:

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Section K – Behavioral Testing

Describe all behavioral tests used in this protocol and provide scientific justification for the need for conducting these tests, especially for those tests that cause the animal distress, such as the physical or acoustic shock tests.

State the number of animals for each behavioral test. How long the animals will be subjected to the testing for a single episode? Total number of times animal will be tested in this study? How long will an animal be allowed to recover between test episodes?

List the special behavioral testing equipment that will be used
How will the equipment be cleaned or sanitized in between uses?

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Section L – Food and/or Water Deprivation/Restriction

Withholding food overnight in preparation for surgery would not be considered food deprivation/restriction unless the species is rodents. **Provide justification and complete the table below.**

Species	Number of Animals	Duration, Frequency and Extent	Monitoring Distress	Building and Room # where monitoring records will be kept

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Section M - Irradiation

Irradiation of Animals
Please complete the table below to identify the dose and schedule (if more than one dose) for irradiation and cell transplant. Please contact the In Vivo Therapeutics Core Manager at 274-8811 for procedures and to obtain a copy of the SOP for the Irradiators. Irradiation projects must be approved by the Radiation Safety Office (RSO) prior to approval (submitted to the Core Manager). Please attach the protocol to the e-mail when you submit this document.

Dose per Fraction/#Fractions	Type of Animal	Project Description

Radioactive Materials (provide the following information): Must be approved by the Radiation Safety Office

Where will the radiation be used: *(bldg. and room no.)*					
Name of Permit Holder project completed under:					
Radioisotope or radiation source:					
Route of Administration:					
Doses administered (mCi)":					
Chemical Forms:		Is radioactivity excreted?		Yes	No
Will there be an exposure rate off the animal?				Yes	No

Are carcasses radioactive?		Yes		No
Disposal method for wastes and carcasses?		RSO		LARC
How long is the half-life of this isotope?				

Radiation-producing Machines
Contact the Radiation Safety Office if radiation-producing machines will be used with animals. The RSO may give specific training for these.

- ☐ Fluoro, Project Description:
- ☐ X-Ray, Project Description:
- ☐ CT, Project Description:
- ☐ Other, Project Description:

Safety Procedures for Animal Handlers - (LARC Personnel and Laboratory Staff)

Who has the potential to be exposed to this material? ☐ **LARC staff** ☐ **Laboratory staff**

Personal Protection *(indicate personal protective apparel/procedures to be used)*:

☐ Hair cover	☐ Gown	☐ Lab coat	☐ Booties
☐ Waterproof boots	☐ Safety glasses	☐ Goggles	☐ Face shield
☐ Film Badges			

☐ Respiratory mask (type) Gloves (type):

Materials Handling:

☐ In cabinet *(indicate type)*:
☐ Chemical Fume Hood ☐ Biosafety cabinet
Decontamination of area after use:
Surveys performed required? ☐ Yes, if "yes," all areas less than 200 cpm/100 cm^2 (must be to be released) ☐ No
Procedures:
Location of each hood used:

Waste Disposal:

☐ Bedding/excreta radioactive: Duration: (days)
Indicate disposal method:

☐ Incinerate	☐ Autoclave
☐ Bedding/excreta disposed as normal	☐ Bedding/excreta disposed through RSO

☐ Decontaminate cage before washing:

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Section N - Special Caging/Husbandry

Will the use of wire bottom caging for rodents be required? Please note that *The Guide for the Care and Use of Laboratory Animals* recommends against using wire bottom caging.

☐ Yes
Provide a scientific justification for doing so. This justification should acknowledge the existing literature on this topic.

☐ No

Does this study involve housing in non-standard room environments?

☐ Yes
Provide the temperature, humidity, light cycle and duration. Note: Normal environmental controls in LARC are a 12:12 light dark cycle (with light during the day), 70F +/- 2 F, humidity is between 30-70 relative humidity.

x No

Please select any special caging or environment requirements from the list below.

- ☐ Temperature
- ☐ Humidity
- ☐ Light Cycle
- ☐ Caging/Pen (including wire-bottom & open-bottom caging)
- ☐ Bedding Changes
- ☐ Bedding Type
- ☐ Autoclaved

	Other
Please provide information needed to describe special caging or environment requirements checked.	

Are special husbandry or handling practices required? (NB: all rodents are socially housed in BSL1 on contact bedding as standard practice in LARC)	
	Yes
Describe all deviations from standard procedures and practices	
x	No

Will this protocol require any other special animal husbandry requirements for any species? (Variations in caging size, housing density, cage change frequency, dietary manipulations, etc.)	
x	No, only standard housing conditions will be required
	Caging & Environment Special Requirements (please answer the next question below)
	Diets – Food and/or Water Special Requirements
	Care – Special Requirements
Please provide information needed to describe the special husbandry requirement checked.	

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Section O – Surgical Procedures

Please select from the table below what type of surgery will be performed.

Definitions:

Survival surgery is any surgical procedure from which the animal subject recovers consciousness after anesthesia.

- **Major surgery** penetrates and exposes a body cavity or produces substantial impairment of physical or physiological functions (e.g., laparotomy, craniotomy, thoracotomy, bone fracture).
- **Minor surgery** does not penetrate and expose a body cavity and does not produce substantive physical or physiological impairment (e.g., wound suturing, vessel cannulation, endoscopic procedures).

Non-Survival/Terminal surgery is any surgical procedure in which the animal is terminated before recovering consciousness after anesthesia.

	Survival Surgery - Major
	Survival Surgery - Minor
	Multiple Major Survival Surgery(ies)
x	Non-Survival/Terminal Surgery

Within the description of the surgical procedure(s), you must include the following:

- Pre-surgical preparation of the animals (example: Fasting, pre-anesthetic medication & surgical prep of the surgical site)
- Indicate if the Pre-Op Preparation descriptions found in Appendix 2 will be used. See Appendix 2 for more information.

Detailed description of Every Surgical Procedure
Preparation After anesthesia is administered the pig is prepped and draped in sterile fashion. Paralytic given by IV to reduce pig movement during surgery. During the surgery nerves are touched and the pigs start to kick making it dangerous for both the surgeon and the pig. Possible Bladder Catheterization to minimize possibility of bladder damage that could contaminate body cavity, and internal organs, with urine. The perineal area is clean as the surgical region is done. With the help of a lubricated speculum, the female external urethral opening is located in the floor of the vagina (in the upper part because the pig is on her back). A Foley catheter 6-12 French size with stylet is inserted in the urethral opening. The catheter is passed through the urethra until it

reaches the bladder (urine will be seen flowing). Then, the balloon is inflated and the catheter attached to the closed-system urine drainage bag.

Midline-abdominal incision.
A midline incision is made from the suprasternal notch to the pubis. Bilateral subcostal incisions may be made to enhance exposure.

Sternotomy. A median sternotomy may be performed to enter the pericardium and the chest. This enables us to have access to transect the supra-hepatic vena cava.

Exposure of abdominal aorta
The retroperitoneum may be dissected to expose the abdominal aorta. This is for blood collection, cannulation, and subsequent perfusion with either saline and or HTK. Saline may also be perfused into the pig through an IV drip line to preserve volume during exsanguination.

Organs procured after euthanasia.

No surgery will last more than 4 hours with pig under anesthesia.

If any animals are to be subjected to **more than one survival surgery (Major or Minor) in this protocol**, explain and justify multiple survival major surgery procedures in individual animals. Please list how much time will lapse between each procedure.

Will more than **one survival surgery (Major or Minor) occur?**	
	Yes – Provide Justification
x	No

Please list all symptoms that are expected experimental outcomes as a result of procedures implemented in this protocol.				
Change in general appearance	Change in normal appetite	Changes in other physical characteristics	Change in behavior	Other

Describe when the above symptoms occur:

Describe any post-op complications that may occur:

At what point will this be managed?

How will the complications be managed/treated?

Who will be responsible for managing the post-op complications (list all that may apply)			
Name:			
Campus Phone:		Emergency Phone:	

Post-Operative Care: (see IACUC Guidelines for additional information) Describe details of post-operative care procedures including the name of person(s) responsible, medications (including dosage, route, and frequency), treatments, procedures or observations, and schedule for each survival procedure. Describe the clinical signs of pain indicating the need for analgesics if analgesic drugs are to be administered. **For USDA Species** postoperative recovery records must be maintained in the animal room accessible to veterinary and LARC staff. Animal Welfare Act Regulations require submission to the LARC and retention in veterinary care files of postoperative care records for each mammal having major survival surgery (except laboratory rats and mice) at the conclusion of postoperative care. If LARC personnel will be involved in providing postoperative care, arrangements should be made when scheduling the surgical procedure with the

LARC Manager. **For Mice and Rats**, records can be kept in the laboratory room, but the records need to be available for LARC, IACUC and regulatory agencies, if requested.

Explanation of Post-Operative Care – if the Post-Operative Care listed in Appendix 3 will be followed, see (Appendix 3) to help with the explanation.

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Section P – Anesthesia/Sedation/Analgesia

Pharmaceutical-grade medications

Investigators are expected to use pharmaceutical-grade medications and anesthetics whenever they are available, even in acute procedures. Non-pharmaceutical grade chemical compounds should only be used in animals after specific review and approval by the IACUC for reasons such as scientific necessity or non-availability of an acceptable veterinary or human pharmaceutical-grade product. Cost savings alone are not an adequate justification for using non-pharmaceutical grade compounds in animals. Please provide justification for the use of Non-Pharmaceutical grade chemical compounds. See the Non-Pharmaceutical IACUC Policy to help with providing justification or Non-pharmaceutical grade substance recipes. If you want to use your own recipes, please explain how you are adhering to the policy (i.e., explain their sterility, etc.).

	Are Non-Pharmaceutical grade chemical compounds being used?
x	No
	Yes. If yes, please provide justification for the use of Non-Pharmaceutical grade chemical compounds

For USDA covered species, you must consult with an LARC Veterinarian on the pain management plan:

Provide the date(s) you consulted with LARC Veterinarian:	9-8-12, 9-10-12
Name of LARC Veterinarian:	Blickman, Andrew R

Note:

- Pain management drugs are to be used pre-emptively as well as post-operatively. You must also provide dosing intervals.
- Based on the approved or typical intervals for pain management drugs, **the PI is responsible** for monitoring the animal at appropriate intervals to determine appropriate analgesic management even after hours and the middle of the night. The PI may contract with LARC technical staff to provide these services in advance.

Pain Relief - Analgesic drugs

Unless scientifically or otherwise justified to the IACUC's satisfaction, you are obligated to routinely provide postoperative pain relief for all vertebrate animals undergoing survival surgery.

If analgesic drugs cannot be given, provide scientific reason why they can't be used:
All Surgery is non-survival and is done under general anesthesia. Percutaneous blood collection is also done under general anesthesia.

Pre-Anesthetic Regimen - Select one

x	**Pre-Anesthetic Regimen listed below will be applied**		
	Species	Time food withheld	Time water withheld
	Mouse, Rat, Rodent	none	none
	Rabbit	none	none
	Dog	Overnight (12 hours)	none
	Pig	Overnight (12 hours)	none
	Sheep	48 hours	12 –24 hours
	Fish, Amphibian	none	none

	Other criteria will be followed as specified. List the length of time for withholding food and/or water:

Anesthetic/Sedation Regimen - Induction drug(s) - Select one

	Anesthetic/Sedation Regimen listed in Appendix 1 will be applied (See Appendix 1 for list)

x	**Other criteria will be followed as specified**. Provide the sedative/tranquilizers (name, dose, & route of administration). The maintenance anesthetic agents(s) (name, dose, & route of administration).

• TKX: Telazol (500 mg) + Ketamine (250 mg) + Xylazine (250mg); 1 cc per 50 lbs; IM) +/- Pentothal (10-20 mg/kg) IV if needed for intubation + Isoflurane by inhalation through ET tube using precision vaporizer, to effect with waste gas scavaging
· KX: Telazol (500 mg) + Ketamine (250 mg) + Xylazine (250mg); 1 cc per 50 – 100 lbs; IM) +/- Isoflurane via mask using precision vaporizer, to effect with waste gas scavaging for sedation for minor procedures such as blood collection
• TDT: Telazol (5mg/kg of 100mg/ml bottle) + Dexdomitor (.001mg/kg) + Torbugesic (butorphanol 0.1 mg/kg) IM (draw up individually and mix into 1 syringe).
• Ketamine-Xylazine (1g bottle ketamine + 142 mg Xylazine, 0.2 cc per kg, IM; split into two sites if >5ml total dose.)
• Propofol 2-6mg/kg IV slow to effect.

Anesthetic/Sedation Monitoring

Check all that apply			
x	Visual Examination		EKG
x	Heart Rate		Blood Pressure
x	Respiration	x	Temperature
x	Reflexes (please refer to next question	x	O_2
			EEG

Anesthesia Monitoring - Select one

x	**Anesthesia Monitoring listed in Appendix 4 will be applied (See Appendix 4)**

	Other monitoring criteria will be used as specified.

Describe: How you determine that the plane of anesthesia is adequate. How frequently you will monitor the animal. What you would observe that would indicate that additional anesthetic needed to be administered.

Anesthetic Recovery Monitoring - Select one

	Not applicable. These criteria do not apply to this study.

x	**Anesthetic Recovery Monitoring listed in Appendix 5 will be used (Appendix 5)**

	Other procedures will be applied

Describe: How often you observe the animal during the immediate recovery period. Where records of anesthesia procedures are kept (building and room #).

Analgesia Management/Analgesic drugs – Select One (Appendix 6)

x	**Not applicable. These criteria do not apply to this study**

	Analgesic used – please complete table below

Species	Procedure	Analgesic Name	Dose	Route	Minimum Duration

	If analgesic drugs cannot be given, provide scientific reason why they can't be used.
	• Include a statement that this section should include the use of pain management drugs pre-emptively as well as post-operatively, as well as providing dosing intervals. • Based on the approved or typical intervals for pain management drugs, the PI is responsible for monitoring the animal at appropriate intervals to determine appropriate analgesic management even after hours and the middle of the night. The PI may contract with LARC technical staff to provide these services in advance.

Neuromuscular blocking agents (pancuronium, succinylcholine, paralytic drugs) – Select one

	These agents will not be used.

x	These agents will be used.
	List the drug(s), dosage(s) and route of administration. Describe in detail how you will determine that an adequate level of anesthesia is being maintained while the animal is paralyzed. Paralytic agents cannot be used without anesthetics and assisted ventilation.
	Pancuronium in pigs (0.02- 0.15 mg/kg IV) or Vecuronium in pigs (0.08 to 0.10 mg/kg) Additional care is taken when the pigs are given paralytic. The pigs are under deep surgical anesthesia and put on positive pressure ventilation prior the administration of the paralytic. In addition to the methods mentioned above to determine depth of anesthesia, the pigs must be monitored by ECG, oxygen saturation and during the time that the paralytic is in effect. If pig's heart rate increases during the paralysis, we will increase isofluorane anesthesia. If pig starts to over breathe ventilator, then isofluorane will be increased and an additional dose of paralytic will be administered. If necessary, the paralysis can be reversed by iv atropine (0.6-1.2 mg) followed immediatedly by a slow iv injection of pyridostigmine (10-20 mg).

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Section Q – Blood Sampling

If blood sampling will be done in multiple species, insert a line for each species and each method.				
Species	Method of withdrawl	Max volume to be withdrawn	Number of samples	Intervals between sampling
swine	Ear vein, Jugular, Cephalic vein, subcutaneous abdominal vein, tail vein, visible subcutaneous vein	200 mL or 10% of animal blood volume (7 mL/kg x weight (kg), whichever is lower	No more than 20	At least 2 weeks

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Section R – Creating a Disease Condition

	Implanted Device/catheter

Device	Monitoring Procedures Parameters monitored and frequency of monitoing	Names of people who will provide Chronic Care	Office Phone Number	After Hours Phone Number	Building and Room # where monitoring records will be kept

	Naturally Occuring or Experimentally Induced Pathological Condition or Disease

Disease or Condition	Monitoring Procedures Parameters monitored and frequency of monitoing	Names of people who will provide Chronic Care	Office Phone Number	After Hours Phone Number	Building and Room # where monitoring records will be kept

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Section S – Immunization

List the antigens and adjuvants that will be used:

Immunization - Select one

	Procedures listed below will be applied			
Species	Immunization Route & Injection Volumn/Site	Number of Sites per Animal & Location	Blood Collection Schedule	Terminal blood collection procedure - Anesthesia, volume, route of blood collection
Mouse	Subcutaneous, 0.05 ml or IP	Up to 5; dorsal neck & back; or 0.10 ml by IP injection	0.1-0.2 ml can be withdrawn every 2 weeks via facial or saphenous or tail clip or lateral metatarsal vein	General anesthesia is induced and 1.0-1.5 ml is collected by cardiac puncture or orbital sinus puncture
Rabbit	Subcutaneous 0.05-0.10 ml	Up to 10; dorsal skin along sides of back; hair clipped & skin disinfected prior to injections	Up to 35 ml can be withdrawn by ear artery or vein puncture every 2 weeks.	General anesthesia is induced then collect 100-150 ml by cardiac puncture
Note: Anesthetic/Sedative given before injections and survival blood collections. Please remember to fill out the Anesthesia Section.				

	Other procededures will be applied
Provide: Provide the injection site, volume/site, and frequency of the immunization. List the method, frequency and volume of blood to be withdrawn.	

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Section T – Hybridoma

Hybridoma - Select One

	General Critera listed below will be applied		
Priming Agent, volume, injection route	Number of abdominal taps performed to collect ascites	Schedule for collection of ascites fluid
Pristane, up to 0.5 ml given by IP injection	Two survival taps followed by terminal non-survival tap. Fluid is collected using a 22 (or smaller) gauge needle.	Mice are observed twice weekly. Ascites is collected when the abdomen is as large as a near-term pregnant female

	Other Critera will be applied
Provide: The Priming Agent and volume. Cells Injected. Schedule for collection of ascites. Number of abdominal taps. Size of needle used. How progression of ascites will be monitored. Frequency of observations. What criteria will be used to perform abdominal taps.	

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Section U – Prolonged Restraint/Aversive Stimuli

If using a physical restraint: Complete the table below if performing experiments involving animals in which they are restricted from full ambulation and free range of motion (does not include restraint while under anesthesia).

Method/Type and Intensity	Duration/Frequency /Adaptation of Animal	Monitoring Procedures Parameters monitored and frequency of monitoring	Building and Room # where monitoring records will be kept	Husbandry provided to animal (wipe urine to prevent urine scald, express bladder, etc)

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Section V – Breeding, Weaning, and Genotyping

Breeding and Weaning

Complete the information for each strain you plan to breed. **If you wish you can complete the breeding form instead and submit it as an attachment to the IACUC office with this form.**

Strain Designation	Mutation/Transgene/Genotypic Manipulation	Source of Breeders

Describe the characteristics that results from the genotype. Include any detrimental impact on the immune system or other clinical problems or anomalies.

Describe the breeding scheme for each strain to be bred. Indicate how many females per male will be housed and the planned mating system (brother-sister, back-cross, cross-breeding of transgenics, etc)

Complete the table using your best estimates of the number of breeders that will yield the required number of experimental animals i.e. the number justified under number of experimental animals required.

Strain	Average Litter Size	# Experimental Animals Needed*	# Breeders Needed*	# Litters Needed	Sex Used	% Used (genetics)	# Euthanized Weanlings*	Total # Animals Needed (sum of * columns)

Provide an explanation of the percentage to be used (genetics; gender) of the pups produced

How will non-experimental animals or retired breeders be euthanized?

At what age will mice be weaned?	
	21 days old (or younger)
	Other:

Genotyping						
Describe	Age of	Anesthesia to	Instrument(s)	Technique(s)	Size of	Method(s) used to

the method to be used and site of the biopsy	animal	be used (for older animals)	to be used	to be employed	sample	monitor for & minimize complication(s) such as bleeding

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Annual Continuing Review
(Complete when submitting your annual continuing review.)

Have you encountered any problems while conducting this study?	
	Yes – did you consult with a veterinarian regarding these problems?
	No

If yes, Did you consult with a veterinarian regarding these problems?	
	Yes
	No, please explain

Provide the following information for each species approved in this protocol. Note that the number used represents the "Total number" used to date on the protocol.

Species	Total Number Approved	Total Number Received	Total Numbers Born In House

For Pain **Level "E"** studies, provide the following:

How many animals went under pain level "E" procedures	Where there any problems encountered?	Any refinements added to the study?	Was an amendment filed to update the protocol?

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Appendix 1 - Anesthetic/Sedation General Criteria

Species	Recommended Drugs + Protocols for Induction & Maintenance of Anesthesia
Mouse	• Xylazine (5-10mg/kg) + ketamine (90-150mg/kg) IP, IM • Xylazine (2.5-5mg/kg) + acepromazine (1.0-2.5mg/kg) + ketamine (90-100mg/kg) IP, IM • Medetomidine (1-2mg/kg) + ketamine (75mg/kg) IP; minor short procedures only • Sodium pentobarbital [Nembutal®] (50-90mg/kg) IP • Sodium pentobarbital (IACUC recipe) IP • Thiopental (IACUC recipe) IP, IV • Urethane (IACUC recipe) IP • Isoflurane inhaled to effect, with waste gas scavenging • Tribromoethanol [Avertin®] [for single survival anesthesia only] (125-250mg/kg IP [0.2 ml of a 1.25 % solution/10 g body weight ~160mg/kg]); made fresh w. sterile saline, sterilely filtered, stored refrigerated and protected from light. Discard if pH < 5.0.

Rat	• Xylazine (5-10mg/kg) + ketamine (40-95mg/kg) IP, IM • Xylazine (5-10mg/kg) + acepromazine (2.5mg/kg) + ketamine (90-100mg/kg) IP, IM • Medetomidine (0.4mg/kg) + ketamine (60-75mg/kg) IP; minor short procedures only • Tiletamine/zolazepam (Telazol®) 20-40mg/kg) IP) +/- xylazine (5-10mg/kg) IP • Sodium pentobarbital [Nembutal®] (30-60mg/kg) IP • Sodium pentobarbital (IACUC recipe) IP • Thiopental (IACUC recipe) IP, IV • Urethane (IACUC recipe) IP • Isoflurane inhaled to effect, with waste gas scavenging • Tribromoethanol [Avertin®] [for single survival anesthesia only] (300mg/kg IP; made fresh w. sterile saline, sterilely filtered, stored refrigerated and protected from light. Discard if pH < 5.0.
Swine	• TKX: Telazol (500mg) + Ketamine (250mg) + Xylazine (250mg); 1 cc per 50 lbs; IM) +/- Pentothal (10-20mg/kg) IV if needed for intubation + Isoflurane by inhalation through ET tube using precision vaporizer, to effect with waste gas scavenging; 50-75% of this dose can be used in minor, quick procedures • TDT: Telazol (5mg/kg of 100mg/ml bottle) + Dexdomitor (.001mg/kg) + Torbugesic (butorphanol) 0.1mg/kg) IM (draw up individually and mix into 1 syringe). • Ketamine-Xylazine (1g bottle ketamine + 142mg Xylazine, 0.2 cc per kg, IM; split into two sites if >5ml total dose.) Especially for renal studies. • Propofol 2-6mg/kg IV slow to effect.
Sheep	• Diazepam (0.11mg/kg) IV + Ketamine (4.4mg/kg) IV for induction + Isoflurane by inhalation through ET tube, to effect, with waste gas scavenging • Ketamine (20-30mg/kg, IV) then 1-3% Isoflurane by inhalation through ET tube, to effect, with waste gas scavenging • Add: xylazine 0.03-0.05mg/kg IM, wait 10-15 minutes then give ketamine 5mg/kg + diazepam 0.3-0.5mg/kg IV to effect. Ketamine/midazolam may also be used. • Propofol 4-6mg/kg IV slow to effect for induction prior to Isoflurane.
Fish	• Immersion in buffered tricaine methanesulfonate (MS 222): 100mg/L for anesthesia and 50mg/l for sedation; buffered to pH 7.0-7.5 with sodium bicarbonate. Use dechlorinated water. • Ketamine 30mg/kg IV (anesthesia less than 3 minutes). • Ketamine 10-80mg/kg IM + Medetomidine 0.06-4mg/kg IM • Analgesia: Butorphanol 0.4mg/kg pre-op; Morphine 5mg/kg; Ketoprofen 2mg/kg IM
Rabbit	• Isoflurane inhaled from a precision vaporizer, to effect, with waste gas scavenging • Acepromazine + Butorphanol + Isoflurane. A 50:50 mixture of 10mg/ml Acepromazine + Butorphanol dosed at 1mg/kg; given IM or SQ. Given as pre-medication ½ hour prior to Isoflurane for surgical procedures. Or acepromazine 0.3mg/kg + butorphanol 0.50 - 0.75mg/kg given 15-20 minutes prior to procedure/surgery. • Ketamine (35mg-50mg/kg) + Xylazine (5-10mg/kg) IM +/- Glycopyrrolate 0.1mg/kg given IM or SQ; followed by Isoflurane, inhaled to effect, with waste gas scavenging. • Acepromazine (0.3mg/kg) + Butorphanol (0.75mg/kg) given 15-30 minutes prior to surgery for sedation. • Dexdomitor 0.05mg/kg + buprenorphine (0.05mg/kg) + ketamine 5-10mg/kg IM
Dog	• Sedation: Dexdomitor (dexmedetomidine) 0.005- 0.01mg/kg + Butorphanol 0.2mg/kg IV • Pentothal induction (22mg/kg) given IV +/- Atropine (0.04mg/kg) IM or SC + Isoflurane by inhalation from precision vaporizer through endotracheal tube (ET) to effect, with waste gas scavenging. • Propofol 2-8mg/kg slow IV to effect for induction, followed by Isoflurane. Isoflurane inhaled from precision vaporizer to effect by endotracheal tube, with waste gas scavenging • Ketamine/Diazepam or Ketamine/Midazolam (ketamine 5-10mg/kg + diazepam or midazolam at 0.1-0.2mg/kg); IV for induction followed by Isoflurane to effect. Isoflurane inhaled from precision vaporizer using endotracheal tube, with waste gas scavenging • Acepromazine (0.025-0.1mg/kg) + Butorphanol (0.2-0.4mg/kg) IM or SC given 30 minutes prior to induction with Propofol 2-8mg/kg slow IV, followed by Isoflurane to effect by ET, with waste gas scavenging. • Dexmedetomidine hydrochloride (Dexdomitor®) 375 mcg per meter squared IV or 125-500 mcg per meter squared IM given as pre-medication 15-30 minutes prior to Isoflurane induction; reverse the Dexmedetomidine HCL with equal volume of Atipamezole hydrochloride (Antisedan®) IM. • Sodium Pentobarbital (20-30mg/kg) given IV • Ketamine 5-10mg/kg + midazolam 0.1- 0.2mg/kg IV or IM

Frog	• MS-222 immersion; buffered; use dechlorinated water • Wide variation in doses of MS-222 • Larval amphibians and totally aquatic gilled species: 200-500mg/Liter • Salamanders and Frogs: 500mg/L to 2 grams/L • Toads: 1-3 grams/Liter • Analgesia: much variability; examples: Morphine 114mg/kg in the dorsal lymph sac (5 hrs analgesia); Buprenorphine 14mg/kg in the dorsal lymph sac; Butophanol 25mg/kg intracoelomic (12 hrs analgesia).

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Appendix 2 – Pre-Op Preparation description

Species	Pre-surgical preparation description
Rodent (mice of genus Mus, rats of genus Rattus)	Surgery Area: The laboratory area is disinfected before use. Hands are washed prior to donning surgical gloves. Surgeon Prep: The surgeon wears sterile surgical gloves, cap, mask and clean lab coat or gown. Patient Prep: If hair is present over the incision site, it is removed with clippers to include a wide margin around the incision area. Skin is disinfected using several alternating rounds of surgical disinfectant scrub/solution (betadine, hibiclens, chlorhexadine, etc.) with 70% isopropyl alcohol rinses. Care is taken to avoid over-wetting fur outside of the surgical area as this will increase hypothermia. (Consider application of disinfectant/alcohol using cotton-tip applicators rather than larger gauze squares which may be harder to control application in small areas). Instrument Prep: Instruments, suture, wound clips, and implanted devices are sterilized prior to surgery and a sterile field is maintained during surgery. Instruments are re-sterilized between rodents using an autoclave or bead sterilizer, or they are rinsed with sterile saline between animals and separated by function (skin, internal organs, etc.). Instruments separated by function and rinsed with sterile saline between uses will not be used on more than 4-5 mice prior to re-autoclaving. Sterile drapes are used if the abdominal or thoracic cavities are opened. Aseptic technique is used.
USDA-covered species (including hamsters, gerbils and other covered rodents)	Surgery Area: Surgery is done in a dedicated operating room. Surgeon Prep: The surgeon wears a mask, cap and sterile surgical gloves and sterile gown. The surgeon performs a thorough hand scrubbing before donning gloves. Patient Prep: The hair over incision areas is removed leaving a wide skin margin. A sterile skin prep is done using multiple alternating rounds of surgical disinfectant scrub (betadine, hibiclens, chlorhexadine, etc.) with 70% isopropyl alcohol rinses. Instrument Prep: Instruments, suture, wound clips, biopsy devices, and implanted devices are sterile and a sterile field is maintained during the procedure. Instruments are re-sterilized between animals. Incision sites are sterilely draped. Aseptic technique is used.

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Appendix 3 – Post-Operative Care

Species	Prost-surgical preparation description
Rodent	Incisions are checked daily until they are healed. Appetite, water consumption, general body condition, attitude, and mobility are checked daily. Sutures and wound clips are removed 7-10 days after surgery. Additional analgesic drugs are given for pain control as needed. Records are kept by the P.I. Records should be available for the IACUC during semi-annual inspections and veterinary staff when requested. Records must be maintained for three years past protocol expiration.
USDA-covered species	Incisions are checked at least once daily until sutures are removed on days 10-14 post-op. Appetite, water consumption, general body condition, attitude, and mobility are checked daily. Additional analgesic drugs are given for pain control as needed. Records are kept with the individual animal's record in LARC for three years after the animal is euthanized.

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Appendix 4 – Anesthesia Monitoring

Species	Anesthesia Monitoring Procedures

Rodent	Signs of adequate anesthesia: Rodents will be unresponsive to surgical or procedural stimulation. They will not have withdrawal reflexes when their tails or legs are pinched. Incisions will not be made until loss of reflex responses has occurred. Anesthetized animals have regular respiration rates. Their ears and feet remain pink, indicating that peripheral perfusion is adequate. A source of supplemental heat (recirculating hot water pad, microwaveable gel pack, hot water bottle, or heating pad) will be used to prevent hypothermia. Criteria for administration of additional anesthetic: Respiration rate increases in response to surgical or procedural stimulation, withdrawal reflexes return. Monitoring frequency during procedure: Monitor every 2-3 minutes
Cat, Farret, Hamster, Guinea Pig	Signs of adequate anesthesia: The animal is unconscious with good muscle relaxation and absent jaw tone. There is no limb withdrawal reflex when the toes are pinched. The palpebral reflex is absent. The respiration and heart rates are stable, and these do not increase in response to surgical or procedural stimulation. The mucous membrane color is pink, and the capillary refill time is 1-3 seconds. A source of supplemental heat (recirculating hot water pad, microwaveable gel pack, hot water bottle, or heating pad) will be used to prevent hypothermia. Criteria for administration of additional anesthetic: Respiration and/or heart rates increase in response to surgical or procedural stimulation, jaw tone returns, withdrawal reflexes return. Monitoring frequency during procedure: Monitor every 5 minutes
Dog, Pig, Sheep, Rabbit	Signs of adequate anesthesia: The animal is unconscious with good muscle relaxation and absent jaw tone. There is no limb withdrawal reflex when the toes are pinched. The palpebral reflex is absent. The respiration and heart rates are stable, and these do not increase in response to surgical or procedural stimulation. The mucous membrane color is pink, and the capillary refill time is 1-3 seconds. Criteria for administration of additional anesthetic: Heart rate and/or respiration rate increases in response to surgical stimulation; the palpebral reflex returns, jaw tone returns, limb withdrawal reflexes return. Monitoring frequency during the procedure: Monitor every 10 minutes and record observations on a permanent anesthesia record form. This record form must be turned into the LARC and be kept with the animal's chart.
Amphibians & Fish	Signs of adequate anesthesia: Loss of righting ability, decreased abdominal respiratory movement (frogs); lack of response to surgical or procedural stimulation Criteria for administration of additional anesthetic: Return of responses during procedural stimulation; increased abdominal respiratory movement (frogs) Monitoring frequency: Monitor every 2-3 minutes during the procedure

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Appendix 5 – Recovery Monitoring Procedures

Species	Pre-surgical preparation description
Rodents	Monitoring frequency during recovery period: Rodents will be observed at least once every 15 minutes until they are awake enough to walk with a wobbly gait then at least every 30 minutes until they can walk with a normal gait. They will be kept in a warmed recovery cage that contains no bedding. They will not be returned to the animal room until they can walk with a normal gait. Anesthetic Monitoring records: Records are kept by the P.I. (Records should be available for review by the IACUC during semi-annual inspections.)
Cat, Ferret, Hamster, Guinea Pig	Monitoring frequency during recovery period: Monitor every 10 minutes until they are awake enough to walk with a wobbly gait then at least every 30 minutes until they can walk with a wobbly gait. They will be turned over at least once every 30 minutes until they can maintain themselves in sternal recumbence. They will be kept in a warmed recovery cage that contains no bedding. Anesthetic Monitoring records: Records are kept with the individual animal's record in LARC for three years after the animal is euthanized. Then the records are returned to the P.I.
Dog, Pig, Sheep, Rabbit	Monitoring procedures during the recovery period: Animals will be monitored at least once every 15 minutes and vital signs (temperature, heart rate, respiration rate, and capillary refill time) will be assessed and recorded on the permanent anesthesia record form or in the chart. Animals recover under direct observation of trained animal care technicians or veterinarians. Monitoring continues until animals can maintain themselves in voluntary sternal recumbence. Animals are returned to regular housing areas when approved to do so by the attending DVM. Anesthetic Monitoring records: Records are kept with the individual animal's record in LARC for three years after the animal is euthanized. Then the records are returned to the P.I.

Amphibians & Fish	Monitoring procedures during the recovery period: Transfer to a clean tank of conditioned water, monitor until swimming and righting ability are regained. Anesthetic Monitoring records: Records are kept by the P.I. (Records should be available for review by the IACUC during semi-annual inspections.)

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Appendix 6 – Analgesics

Mice	• Buprenorphine 0.05-2.0mg/kg SQ every 6-12 hours SQ, IP, or IM • Buprenorphine 0.05-2.0mg/kg every 6-12 hours SQ or IM + Carprofen 5mg/kg once daily (Blue book says Bup is effective for 3-5hours in mice. Dr Boehm said she is fine with the current protocol) • Butorphanol 2-5mg/kg SQ or IM every 4 hours (Blue book says TorbTord is effective for 1-2 hours in mice. Carprofen 5mg/kg Change to 5-10mg/kg orally or SQ daily; can be combined with opioids • Meloxicam 1.0-10.0mg/kg SQ, IP daily; can be combined with opioids • Morphine 5-10mg/kg SQ every 2-4 hours
Rat	• Buprenorphine 0.01-0.05mg/kg SQ or IM every 8-12 hours • Buprenorphine 0.01-0.05mg/kg SQ or IM every 8-12 hours + Carprofen 5mg/kg once daily (Blue book says Bup is effective for 6-8hhours in rats. Dr Boehm said she is fine with the current protocol) • Butorphanol 2mg/kg SQ or IM every 2-4 hours (Blue book says Torb is effective for 1-2hours in rats. Carprofen 5mg/kg Change to 5-10mg/kg orally or SQ daily; can be combined with opioids • Morphine 2-10mg/kg IM or SQ • Meloxicam, 1.0-3.0mg/kg PO, SQ, IP daily; can be combined with opioids • Ketoprofen, 5-20mg/kg SC or orally daily
Rabbit	• Buprenorphine 0.01-0.05 change to 0.015 – 0.1mg/kg SC or IM every 6-12 hours • Carprofen, 4mg/kg SC daily • Meloxicam, 0.2mg/kg change to 0.2 to 0.3mg/kg SC daily up to 3 days • Ketoprofen, 3mg/kg SC daily • Morphine 2-5mg/kg SC every 2-3 hours. Change to every 3-4 hours • Butophanol 0.01-0.05mg/kg SC or IM every 4 hours. Change to Butorphanol 0.1-0.5mg/kg • Hydromorphone-- for rescue analgesia 0.05-0.1mg/kg SC or IM every 4 hours
Dog	• Buprenorphine 0.005-0.02mg/kg SQ, IM or IV every 8-12 hours • Butorphanol 0.1-0.4mg/kg SC, IM every 4-6 hrs • Carprofen, 4mg/kg SC single dose change to 4mg/kg once daily or 2mg/kg BID; 4mg/kg orally (long term is tolerated) • Ketoprofen, 2mg/kg daily for up to 3 days; 1mg/kg daily orally up to 5 days • Meloxicam, 0.2mg/kg SC single dose; 0.2mg/kg orally on day 1, then 0.1mg/kg orally daily (well tolerated long term) • Morphine 0.25-0.5mg/kg SC or IM every 4 hours. • Fentanyl patch: may require approximately 24 hours to take effect so other opioids such as hydromorphone or morphine will need to be given before blood levels reach a therapeutic range. 50 mcg patch for 10-20 kg dog; • 50-75 mcg patch for 21-30 kg dog. Over 30kg may need 75-100mcg patch. Lasts up to 3 days. • Deramaxx 1-3mg/kg per os every 24 hours. • Hydromorphone: usually used for rescue analgesia and pre-emptively: 0.1mg/kg SC, IM; every 4 hours • Local: lidocaine; lidocaine/bupivacaine; lidocaine patch
Pig	• Buprenorphine 0.01-0.05mg/kg IM every 8-12 hours • Carpofen, 2-4mg/kg SC daily • Flunixin 1-4mg/kg SC or IM every12-24 hours • Fentanyl patch placed on skin of back; takes 24 hours to take effect; must use other opioids such as hydromorphone or morphine before blood levels reach a therapeutic range; may last 3 days. Note that titration of the dose in swine may be difficult so effects are highly variable. • Butorphanol 0.1-0.3mg/kg SC or IM every 4-6 hours. • Hydromorphone: usually used for rescue analgesia and pre-emptively: 0.1mg/kg SC, IM; every 4 hours • Local: lidocaine; lidocaine/bupivacaine; lidocaine patch

Sheep	• Buprenorphine 0.005-0.01mg/kg given IM or SQ every 4 hours • Carprofen, 1.5-2.0mg/kg SC daily up to 3 days. • Flunixin, 2mg/kg SC daily • Phenylbutazone 5-10mg/kg orally • Fentanyl patch adult sheep about 100mcg patch • Hydromorphone: usually used for rescue analgesia and pre-emptively: 0.05mg/kg SC, IM; every 4 hours • Local: lidocaine; lidocaine/bupivacaine; lidocaine patch

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